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ORIGINAL INVESTIGATION

Leptin-deficient mice retain normal appetitive spatial learning yet exhibit marked increases in anxiety-related behaviours

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Abstract
Rationale The individual's emotional state influences food intake in both humans and rodents. Moreover, specific cognitive processes regulating the salient aspects of food reward are also critical for ingestive behaviour. However, the molecular mechanisms underlying such influence remain unclear. Genetic mouse models thus are important tools in dissecting the molecular and pathophysiological processes which cause complex human diseases. Leptin, encoded by the *ob* gene, plays an important part in the energy homeostasis and is critical for the development of obesity.
Objectives In these studies, we assess the impact of leptin on behaviours relevant to anxiety and appetitive learning.
Methods Anxiety-related behaviour was assessed in the light dark box and two tests of hyponeophagia. Spatial learning and behavioural flexibility by re-learning was assessed in an appetitive Y-maze task.
Results Leptin-deficient (*ob/ob*) mice displayed higher levels of anxiety-related behaviour in both anxiety tests. In the appetitive Y-maze task, leptin deficiency caused no deficit in learning or re-learning and acute restrained stress had no influence on the learning process.
Conclusions These results emphasise that whilst leptin has previously been shown to modulate aversively motivated learning we found no difference between leptin-deficient mice and their controls in an appetitive learning task. Moreover, both groups showed behavioural flexibility under stressful conditions. On the other hand, leptin deficiency resulted in marked alterations in behaviours relevant to anxiety.

Keywords Stress · Satiety · Memory · Food intake · Mouse model · Obesity · Leptin · Learning and memory · Anxiolytic · Anxiety · Reward · Maze · Working memory

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Introduction

Leptin, encoded by the *ob* gene, is an adipocytic and brain-derived hormone which plays an important role in energy homeostasis, feeding and body weight regulation (Zhang et al. 1994). Leptin binds to its receptor in the hypothalamus to orchestrate its effects on metabolic homeostasis. The arcuate nucleus of the hypothalamus contains two types of leptin-sensitive neurons; orexigenic neurons (co-localising the peptides neuropeptide Y and agouti-related peptide) and anorexigenic neurons (expressing the peptides cocaine amphetamine-related transcript and pro-opiomelanocortin (POMC)). Through its negative feedback action, leptin suppresses food intake by inhibition of the orexigenic

and stimulation of the anorexigenic neurons (Friedman 2000).

In human and animal studies, leptin has been shown to play an important role in the development of obesity (Ahima and Flier 2000; Friedman 2000; Friedman and Halaas 1998). In rare cases, partial leptin deficiency due to mutations of the *ob* gene can be the cause of obesity in humans (Farooqi et al. 2001; Montague et al. 1997) similar to the model of *ob/ob* mice. Most obesity patients, however, have high circulating serum levels of leptin that are positively correlated to the amount of body fat mass (Maffei et al. 1995). These case studies showed several possible factors as causes for obesity, all part of the leptin signalling pathway, such as mutations in the genes encoding leptin and the leptin receptor (Clément et al. 1998), POMC (Krude et al. 1998) or the melanocortin 4 receptor (Vaisse et al. 1998; Yeo et al. 1998).

One animal model of obesity specifically exploits the role of leptin namely the *ob/ob* mouse which was first discovered over 60 years ago (Ingalls et al. 1950). This strain has an autosomal recessive mutation of the *ob* gene on chromosome 6 leading to a complete leptin protein deficiency (Zhang et al. 1994). *Ob/ob* mice are characterised by several metabolic and neuroendocrine abnormalities such as obesity, hyperphagia, hyperinsulinemia, hyperlipidemia, hyperglycemia and insulin resistance coupled with decreased metabolic rate and body temperature and thus are among the most widely studied mouse models of obesity and the metabolic syndrome (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). In these animals, leptin deficiency leads to a lack of suppression of orexigenic neurons and decreased activation of anorexigenic pathways in the arcuate nucleus, resulting in extremely increased food intake and obesity.

There is an increasing awareness that food intake, mood regulation and cognitive function are closely intertwined (Dallman 2010; Morrison 2009). Thus, leptin is poised to have a direct regulatory role in the mediating of behaviours at the interface of emotionality, learning and appetite control. Although there is much more data linking alterations in food intake and metabolic syndrome with depression (Goldbacher and Matthews 2007; McElroy et al. 2004; Vieweg et al. 2008), it is clear that there is a strong relationship between food intake and anxiety (Pallister and Waller 2008; Swinbourne and Touyz 2007). Indeed, obesity and anxiety share many of the same therapeutic targets (Zheng et al. 2006).

On the other hand, leptin has been shown to have cognitive enhancing properties and increased memory and learning performance in aversively motivated cognitive tasks in rats and mice (Farr et al. 2006; Oomura et al. 2006) and caused modification of the hippocampal dendritic morphology and neurogenesis (Garza et al. 2008; O'Malley et al. 2007; Oomura et al. 2006). Leptin receptor deficiency in *db/db* mice and *fa/fa* Zucker rats on the other hand caused impaired learning in various behavioural cognitive tasks (Li et al. 2002; Ohta et al. 2003; Oomura et al. 2010; Winocur et al. 2005).

Despite the link between obesity and stress-related disorders such as anxiety (Barry et al. 2008; Scott et al. 2008), there are very few studies behaviourally characterising the *ob/ob* mouse in animal paradigms relevant to anxiety. Moreover, given the link between reward-related cognitive domains and anxiety, it is also surprising that the effects of leptin deficiency on appetitive learning-based tasks have been neglected in this field. One study has shown that leptin-deficient *ob/ob* mice exhibit high levels of anxiety-related behaviour on the elevated plus maze (Asakawa et al. 2003). The aim of this study, therefore, was to further assess the influence of leptin on anxiety-like behaviours and determine if leptin deficiency affects appetitive learning.

Materials and methods

Animals

Male *ob/ob* mice (n=11) and their lean littermate controls (n=12) (Harlan, UK) were used. Animals were generated on a C57BL/6 background and were shipped to the facility at 5 weeks of age. Animals were housed in standard holding cages in groups of four, with a separation of genotypes. The holding room was temperature (21+1°C) and humidity (55±10%) controlled and under a 12-h light/dark cycle (lights on 7.45 A.M.). Water was available ad libitum throughout the whole study. All experiments were in full accordance with the European Community Council directive (86/609/EEC) and the local animal ethical committee.

Behavioural testing

All mice were tested in all experimental procedures described below over a period of 34 days (see timeline Fig. 1). Test sequences were chosen carefully to avoid interaction as far as possible: anxiety tests were performed first as their outcome is highly sensitive to previous experiences. Influence of the anxiety tasks on the following Y-maze testing is highly unlikely, as a 5-day period of gentle habituation to the procedure was performed before actual testing.

Light dark box

The light dark box test is a widely used and well-validated assay for assessing anxiety-like states of an animal (Cryan et al. 2003; Holmes et al. 2002; Jacobson et al. 2007; Mombereau et al. 2004). Animals with reduced anxiety-like

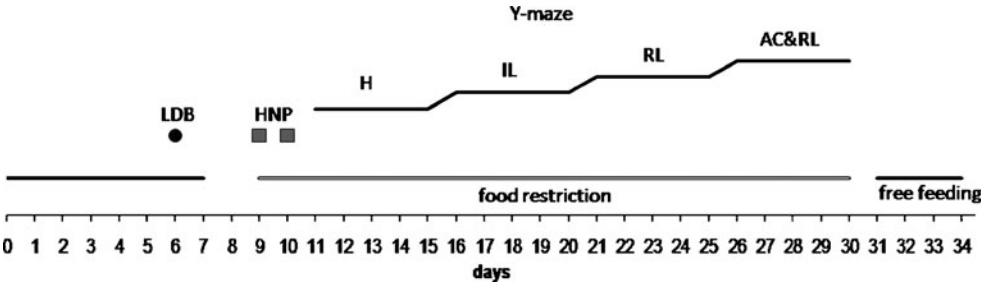

Fig. 1 Timeline of experimental procedures: light dark box (*LDB*), test of hyponeophagia (*HNP*), Y-maze: habituation (*H*), initial learning (*IL*), reversal learning (*RL*), acute stress and reversal learning (*AC&RL*)

behaviour spend more time in the light part of the box and have a longer latency to cross the first time from the light to the dark side, whereas animals with enhanced anxiety-like behaviour spend most of the time in the dark compartment of the box and make fewer transitions between each side.

Apparatus The light dark box test was carried out as previously described (Cryan et al. 2003). Briefly, the box (44×21×21 cm) consisted of an open white compartment (30×22×22 cm) and a black closed compartment (14×21×21 cm). Access from one part of the box to the other was possible through a 12×5-cm door. To increase the anxiogenic effect of the white compartment, side walls were made out of clear plastic, and a neon lamp 50 cm above the light compartment assured additional illumination (380–420 lx).

Procedures Mice were brought into the testing room in their home cages 10 min prior to testing to allow adaptation. Each animal received a single trial, 10 min in duration. The mouse was placed into the light compartment of the box facing away from the access door to the dark part. Latency until the first crossing from the light into the dark area (all four paws) was measured, as well as the number of total transitions and the time spent in each side of the box. After 10 min, the mouse was removed and placed back into the home cage. After each mouse, the apparatus was cleaned with a disinfectant spray (Trigene®) and dried with absorbent paper.

Test of hyponeophagia

Hyponeophagia, the suppression of eating due to anxiety-related states caused by novelty, was assessed by measuring the latency to begin eating in a variety of potentially anxiogenic situations. The level of anxiety-related stimuli was manipulated by using novel food and by conducting the experiment in novel, potentially anxiogenic environments. Tests were carried out essentially as described in the literature (Bannerman et al. 2002; Bannerman et al. 2003; Deacon and Rawlins 2005). Importantly, as described in Deacon and Rawlins (2005), repetitive testing of hyponeophagia does not affect the latency to eat the food reward in control animals.

In this study, hyponeophagia was assessed in two different environments, each with a different level of illumination.

Test 1: bright light conditions (380–400 lx)

The first test of hyponeophagia was carried out in a clear plastic box, similar to the light box used in the light dark box test, and light conditions (neon light 50 cm above box, illumination of 380–460 lx) were identical to the previous setup. This concordance in environmental setup allowed the parallel comparison of anxiety-like behaviour that is unrelated (light dark box) or dependent on food consummation (hyponeophagia). Eight pieces of sweet corn were placed into the centre of the box, and four pieces into each of the corners.

Test 2: low light conditions (100 lx)

In the second task, the floor of a glass cylinder (diameter 21 cm; height 45 cm) was covered to approx. 50% with sunflower seeds. Illumination of the apparatus was 100 lx (room light) and no additional light source was used.

Procedures Both the sweet corn and the sunflower seeds were novel to the mice at the start of these experiments. For each test, mice were brought into the testing room 10 min prior to the start of the experiment. After testing, each mouse was placed into an individual temporary holding cage to prevent social transmission of food preferences between tested and untested mice and returned to its home cage once all animals of the cage had completed the assay.

In both variants of the test, the latency to begin eating was recorded by an experimenter sitting in the testing room next to the apparatus. If a mouse had not eaten (criteria for eating: holding food in hands for 2–3 s) within 2 min, it was removed from the apparatus, placed back into the individual holding cage and retested after a further 2 min. This procedure was carried out until eating occurred but with a maximum number of five trials (total of 600 s). Each mouse received one test session in each task on two subsequent days.

Y-maze training and testing

Spatial reference memory was assessed in a rewarded version of the Y-maze task essentially as previously described in the literature (Deacon et al. 2002, 2007; Reisel et al. 2002). All testing was carried out in the afternoon. Three stages of testing were performed: (1) the initial learning phase, where the first association between the location of the food reward and the spatial reference cues is formed; 2) the stage of reversal learning, where the location of the goal is changed in reference to outside spatial cues and the ability of re-learning of a context can be measured; and (3) the stage of re-learning under the influence of acute restrained stress, where the possibility of stress-induced cognitive interference with the capability of re-learning of a context was assessed.

Apparatus The Y-maze was constructed out of three arms (50 cm×9.5 cm), which were attached to a central triangular area (9.5 cm per side) and arranged at an angle of 120º to each other. The arms were made of black plastic, surrounded by a 0.5-cm-high rim. A small plastic food well was positioned 5 cm from the distal end of each arm. The maze was elevated 80 cm above the floor and could be rotated throughout testing.

Habituation Mice were habituated to the maze in home cage groups to increase exploration and reduce anxiety-related behaviours. Sweetened condensed milk (Carnation®, Nestle) was diluted 50% with water and filled into each of the food wells. Mice were allowed to freely explore the maze and drink the food reward. Once familiar with the maze, mice were placed onto the apparatus individually until they were running freely on the maze and collecting the food reward readily.

Spatial reference memory testing For spatial reference memory testing, the Y-maze was moved to a different testing room and prominent external cues were pinned to the walls in visual height of a mouse on the apparatus.

Initial learning Each mouse was assigned to a goal arm according to their position within the room. Goal arms were counterbalanced between obese and control animals. The maze was rotated by 120º randomly in clock or anticlockwise direction between each trial to prevent potential associations of the correct goal arm with texture or smell of an individual arm of the maze. The starting position for each trial was determined by a pseudorandomised computer sequence, which was different for each mouse; in starting position 0, the mouse was placed on the maze so that the target arm was to its left, in position 1 the target arm was to its right. The pseudorandom sequence contained no more than three consecutive starts from the same position to avoid temporary position preferences in the mice. Animals were tested in groups of eight, with four animals of each experimental group. In each testing group, the distribution of goal positions between animals of the experimental groups was identical. Each mouse of the group received ten trials per day with an intertrial interval of approximately 10 min. The time of testing within an afternoon session was counterbalanced between *ob/ob* and control mice but remained the same for each mouse over successive days of testing. Mice received five consecutive days of initial learning with a total number of 50 trials.

For each trial, the food well on the goal arm was filled with 0.1 ml of sweetened condensed milk, the animal was placed on the end of the start arm and was then allowed to freely run on the maze and the number of entries (all four paws) into the arms was counted until the correct arm was chosen and the reward collected. A trial was scored as successful only if the first choice was correct. Once the task was completed, the mouse was removed from the maze and placed back into its home cage. Between mice, the food wells were not cleaned, so a slight odour of the milk reward remained, to ensure that mice found the correct goal arm not due to olfactory but due to spatial cues.

Reversal learning In the second stage of Y-maze testing, mice were re-assigned to a different goal arm, and starting positions for each mouse were determined by pseudorandomised computer sequence, which was different to the one used in the initial learning phase. New goal positions were assigned counterbalanced between the experimental groups. Testing groups from the initial learning stage remained, with identical assignments of goal position sequences between animals from the experimental groups. Again, animals were tested in ten daily trials for five consecutive days.

Reversal learning under the influence of acute restrained stress In the third stage of Y-maze testing, animals were exposed to 1 h of restrained stress which started 2 h prior to testing. All eight animals of each testing group were placed into mouse restrainer tubes (50 ml tube with an opening for air on each side) for 60 min and were then returned to their home cage for 60 min followed by Y-maze testing. Again, goal positions on the Y-maze were re-assigned for each animal to the last of the three options. Starting positions for each mouse were re-determined by pseudorandomised computer sequences, which were different to those in the phase of initial learning and reversal learning. Again, all mice received a total number of 50 trials, with ten trials per day on five consecutive days.

Food and body weight

Upon arrival, animals were kept on a free-feeding schedule for 8 days, where standard lab chow (2018S Teklad Global

18% Protein Rodent Diet) was given to the animals in pre-weighed portions of 10 g per mouse. Between 9 and 10 A.M., animals were removed from their home cage and weighed. The amount of food consumed was also calculated. For the tests of hyponeophagia and for the period of habituation and testing on the Y-maze, animals were kept on a food restriction schedule for a total of 22 days. To maintain a body weight of 85–90% of the free feeding body weight, animals received pre-weighed portions of standard lab chow inside their home cage after testing, and the amount of fed chow was adjusted to the variations in body weight. At the end of Y-maze testing, animals were kept on a free-feeding schedule for 4 days receiving 15 g of standard lab chow per animal per day. Consumed food per animal and body weights were measured twice per day.

Statistical analysis

Statistical analysis was performed using independent sample *t* tests. Y-maze data were analysed with a repeated measures two-way ANOVA and subsequent estimation of parameters. If data were not spherical, Huynh–Feldt corrections were applied. All tests were carried out at a significance level of $p<0.05$. All analysis was carried out using SPSS 15.0 for Windows (SPPSS Inc., Chicago, USA). All graphs show mean values±SEM. Animals were excluded by two standard deviations from the mean value or when they failed to successfully complete the task.

Results

Anxiety-related measures

Possible differences in anxiety-like behaviour between *ob/ob* mice and lean controls were assessed in two different experiments, the light dark box and the test of hyponeophagia.

Light dark box

Three animals of the *ob/ob* group had to be excluded due to a lack of locomotor activity in the first (and second) half of the test (latencies for the first entry into the dark compartment between 330 and 600 s). Student's *t* test revealed a significant difference between groups in the state of anxiety-like behaviour measured as the percentage of time spent in the light compartment ($t=4.297$; $df=18$; $p<0.001$), with mean values for *ob/ob* mice of 24.00±4.99% and for lean controls of 49.13±3.43% (Fig. 2a).

The total number of transitions between the compartments was significantly lower ($t=6.020$; $df=18$; $p<0.001$) in *ob/ob* mice (18.88±3.34) compared to their controls (50.92±3.72) (Fig. 2b).

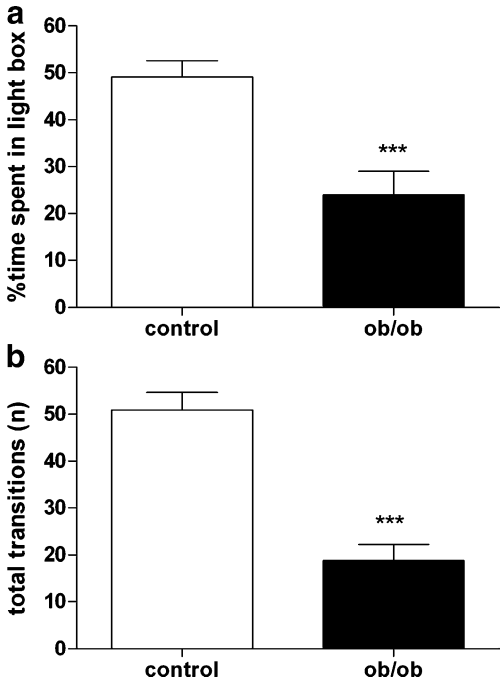

Fig. 2 Leptin-deficient mice have increased anxiety-like behaviour in the light dark box; **a** % time in the light; **b** transitions between both compartments. $n=8$ (ob), 12 (control); ***$p<0.001$, *t* test

Tests of hyponeophagia

As an anxiety-related measurement independent of locomotion and to verify the results obtained in the light dark box (LDB) test, the test of hyponeophagia was carried out. In this setup, animals are not required to move to obtain the food reward. In the first test of hyponeophagia (bright light condition), there was no significant difference between groups in the latency to begin eating (Fig. 3a).

Student's *t* test showed a significant difference between groups in the latency to begin eating ($t=4.019$; $df=18$; $p<0.001$) in the second test of hyponeophagia (low light conditions), with mean values for animals of the *ob/ob* group of 459.55±47.53 s and for animals of the control group of 201.89±40.42 s (Fig. 3b). Animals of the control group (test 1: one mouse; test 2: three mice) had to be excluded due to repetitive jumping out of the apparatus (or attempts to) and thus inability to successfully complete the task.

Spatial reference memory on the Y-maze

Spatial reference memory was assessed in three stages in a rewarded Y-maze task. Results of ten trials were pooled into one block, corresponding to one daily session. Each of the three learning phases lasted 5 days and consisted of 50 trials; thus, mice performed a total volume of 150 Y-maze trials over the entire experimental period. In all three

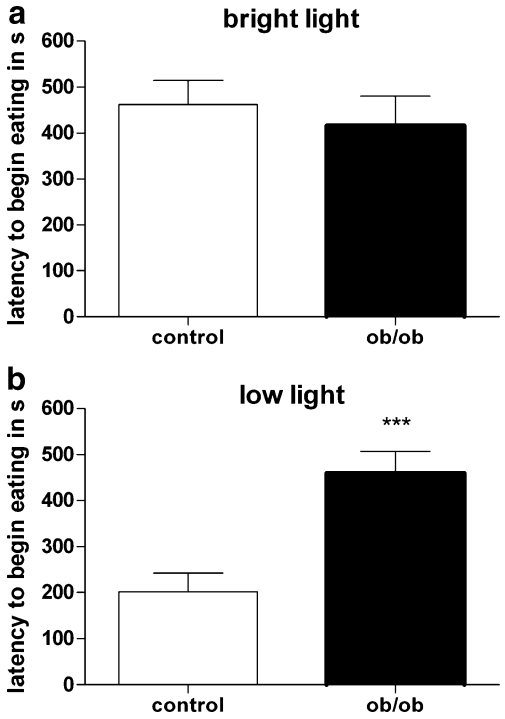

Fig. 3 Anxiety-like behaviour of leptin-deficient mice in two different tests of hyponeophagia; **a** under bright and **b** under low light conditions; n=9–11; ***p<0.001, t test

phases, the significant effect of block shows the learning progress of the animals. Mice of both groups showed equal learning in the initial learning phase and re-learning capabilities with or without the additional influence of acute restrained stress in the phases of reversal learning.

To reveal possible effects of learning stages, the percentage of correct choices in all three phases was analysed with 'phase' and 'block' as the within- and 'group' as the between-subject factor.

There was a significant effect of block [F(4, 84)=209.155; p<0.001] and a significant interaction of phase × block [F(8, 168)=2.836; p=0.006]. No statistical difference was found for group, phase and for the interaction of phase × group, block × group and phase × block × group. Pairwise comparison of phases was not significant for any learning stage. An effect of stress on reversal learning was not detected, as the percentage of choices in two phases was not significantly different (p=0.065) (Fig. 4).

Analysis of the percentage of correct choices in the individual stages revealed in the initial learning phase a significant effect of block [F(4, 84)=63.507; p<0.001], but no significant effect of group or group × block interaction. Parameter estimation showed no significant difference between groups in the individual blocks (Fig. 4).

Analysis of the percentage of correct choices in the phase of the first reversal learning revealed a significant effect of block [F(4, 84)=66.453; p<0.001], but no significant effect of group or group × block interaction. Subsequent estimation of parameters showed no significant difference between the two groups in the individual blocks (Fig. 4).

The percentage of correct choices in the phase of reversal learning 1 h after restraint stress (60 min) was analysed using a two-way repeated measures ANOVA. Analysis revealed a significant effect of block [F(4, 84)= 92.839; p<0.001], but no significant effect of group or interaction between groups and block. Estimation of parameters for each time-point revealed no significant difference between groups (Fig. 4).

The number of entries into the arms was analysed across all stages and for each of the learning phases using statistical tests as described above.

Comparing the number of entries over all phases, analysis showed a significant effect of phase [F(2, 42)= 4.542; p=0.016], of block [F(1.684; 35.357)=218.912; p< 0.001] and a significant interaction of phase × block [F (4.738; 99.500)=6.707; p<0.001]. There was no statistical significance for group, for phase × group, for block × group and for phase × block × group. Pairwise comparison of phases was significant for the initial learning compared to the first reversal (p=0.018), and compared to the second reversal (p=0.021). There was no effect of stress, as the two phases of reversal learning were not significantly different (p=0.922). Animals had significantly less average entries in the initial learning phase (1.256±0.026) compared to the first (1.347±0.025) and the second (1.344±0.024) stage of reversal learning (Fig. 5).

In the initial learning phase, two-way repeated measures ANOVA revealed a significant effect of block [F(1.899;

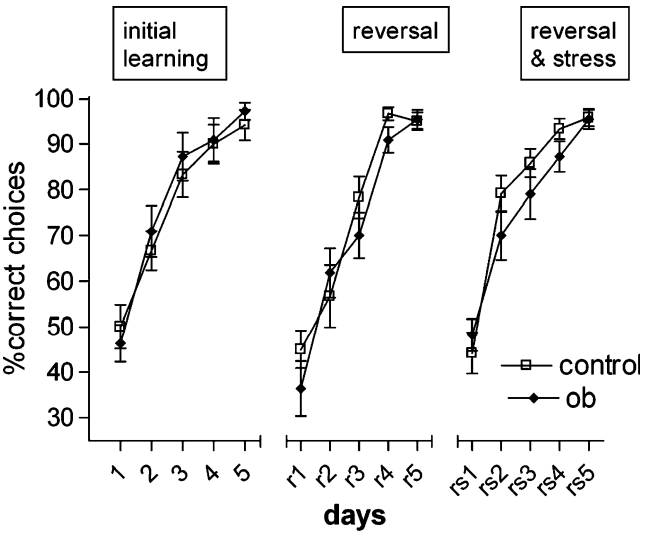

Fig. 4 Appetitive learning in leptin-deficient mice. Percentage of correct Y-maze choices in each of the three phases, initial learning, reversal and reversal and stress, is shown. *Graph* shows average result of performance from ten trials per day (=block). n=11–12

39.879)=62.397; $p<0.001$], but no significant effect of group and interaction of block × group. Estimation of parameters at the individual time-points showed no significant difference between groups in the number of entries (Fig. 5).

At the stage of the first reversal learning, analysis of the number of entries showed a significant effect of block [F(2.372, 49.812)=67.781; $p<0.001$], but no significant effect of group and no significant interaction of block × group. Estimation of parameters showed a significant difference between groups in block 4 ($p=0.035$) with mean values for the number of choices in the control group of 1.033±0.023 and in the obese group of 1.109±0.024. There was no statistical difference in the estimated parameters for the remaining time-points (Fig. 5).

Analysis of the number of entries in the phase of the second reversal learning revealed a significant effect of block [F(4, 84)=118.576; $p<0.001$], but no significant effect of group and no significant interaction of block × group. Estimation of parameters for the individual blocks revealed no significant difference between groups (Fig. 5).

Body weight

After arrival, mice were kept on a free-feeding schedule for 8 days followed by 2 days of restricted feeding for hyponeophagia testing and 20 days of food restriction for Y-maze training and testing. After Y-maze testing, a re-feeding period of 4 days was carried out. Body weight measurements on the 2 days of food restriction for the hyponeophagia testing were excluded from the analysis. Differences in body weight between groups were analysed using repeated measures two-way ANOVA. For the first 8 days, analysis revealed a significant effect of group [F(1, 21)=107.007; $p<0.001$] and of day [F(2.982, 61.487)=359.487; $p<0.001$], and a significant interaction of group × day [F(2.928, 61.487)=149.168; $p<0.001$]. Estimation of parameters showed significance at $p<0.001$ for all days between groups. Body weight averages for obese mice were 26.899±0.446 g and 20.507±0.427 g for controls (Fig. 6).

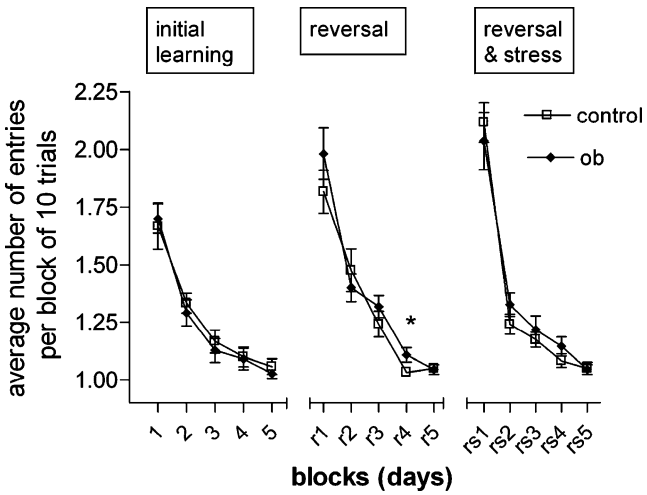

Fig. 5 Appetitive learning in leptin-deficient mice. Average number of entries per block (day) of ten trials in each of the three testing phases. n=11–12. *$p<0.05$ repeated measures ANOVA followed by estimation of parameters

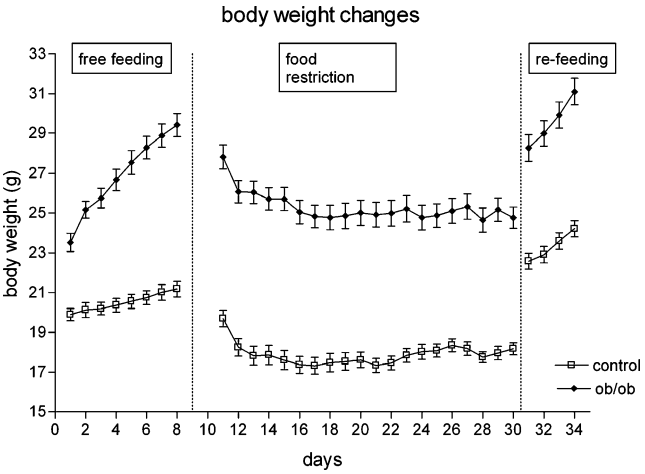

Fig. 6 Body weight changes during the stages of free-feeding, food restriction and re-feeding of leptin-deficient mice. Body weights were significantly different ($p<0.001$) (repeated measures ANOVA followed by estimation of parameters) between groups on all experimental days. n=11–12

During the 20 days of food restriction for Y-maze testing, analysis showed a significant effect of group [F(1, 21)=114.363; $p<0.001$] and of day [F(3.316, 69.642)=48.217; $p<0.001$], and a significant interaction of group × day [F(3.316, 69.642)=8.061; $p<0.001$]. Estimation of parameters showed significant difference at each time-point between groups at a significance level of $p<0.001$. *Ob/ob* animals had average weights of 25.274±0.499 g and lean controls weighed in average 17.880±0.478 g (Fig. 6).

Analysis of differences in body weight in the 4-day period of re-feeding revealed a significant effect of group [F(1, 21)=69.568; $p<0.001$] and of day [F(3, 63)=299.241; $p<0.001$], and a significant interaction of group × day [F(3, 63)=20.572; $p<0.001$]. Estimation of parameters showed significance ($p<0.001$) for all days. Mean body weight values of animals from the obese group were 29.564±0.541 g and from the control group 23.317±0.518 g (Fig. 6).

Discussion

The present study demonstrates that leptin deficiency in *ob/ob* mice leads to an increase in anxiety-like behaviours but does not influence learning and behavioural flexibility, even under acute stressful conditions. These results

emphasise the importance of leptin in emotionality and demonstrate that appetitive learning occurs independent of leptin.

The light dark box creates a naturalistic conflict situation between the natural tendency of rodents to explore novel surroundings and their innate fear of exposure to open, brightly lit environments (Crawley and Goodwin 1980; Cryan and Holmes 2005). Animals with reduced anxiety-like behaviour spend more time in the light part of the box and have a higher number of transitions between compartments, whereas animals with enhanced anxiety-like behaviour spend most of the time in the dark area. Leptin-deficient mice displayed higher levels of anxiety-like behaviour, with significantly less time spent in the light compartment of the box and a reduced number of transitions compared to control animals. These results were confirmed in the test of hyponeophagia. Here, anxiety-like behaviour is measured as suppression of eating behaviour due to anxiety-like states caused by novelty (Bodnoff et al. 1989; Deacon and Rawlins 2005; Dulawa and Hen 2005; Merali et al. 2003). Two different levels of illumination created the potentially anxiogenic source. The high illuminated setup was chosen in reference to the light dark box, to allow comparison of anxiety-like states between tests. Animals of both groups showed strong inhibition in eating behaviour and no difference was thus found between groups due to this floor effect. However, under low illumination conditions in a different environment, obese mice displayed increased inhibition in eating compared to controls. This clearly shows elevated levels of anxiety-like behaviour in the leptin-deficient mice, and that stimulation by food reward could not alter this deficit. These results support the anxiolytic properties of leptin (Liu et al. 2010). Moreover, our data are in agreement with previous findings that described increased levels of anxiety-like behaviour in *ob/ob* mice in the elevated plus maze (Asakawa et al. 2003). The hyponeophagia phenotype is not due to any baseline lack of motivation to eat in *ob/ob* mice as they show normal motivation for food in the acquisition phase of the Y-maze task (this paper) and in the acquisition of an operant-based food reward task (Finger and Cryan, unpublished).

Because in many studies leptin and its receptor have been shown to influence cognitive performance in a variety of aversive learning tasks, we further investigated the effect of leptin deficiency on cognition in an appetitive learning task. To this end, we employed the elevated Y-maze test, a frequently used model to study reference memory in rodents (Deacon et al. 2002, 2007; Reisel et al. 2002). In the initial learning phase, mice were trained to find a rewarded location on the Y-shaped maze according to outside spatial cues. Both groups, *ob/ob* mice and controls, showed equal learning as evidenced by the number of entries and the percentage of correct choices. In order to test possible differences in behavioural flexibility and re-learning capabilities, mice were then re-trained to find a new target position. In this phase of reversal learning, both groups again showed equal performance, with no overall differences.

Given that stress has been shown to alter performance in a non-appetitive Y-maze task (Conrad et al. 2004), we further investigated the effect of acute stress on the animals' ability to re-learn the paradigm. Exposure to 1 h of restraint stress did not alter the behavioural flexibility, and animals of both groups re-learned the test equally. Comparing the performance of animals in all three phases of the experimental procedure revealed no difference in the percentage of correct choices, whereas the process of re-learning was clearly shown in the number of entries. In this parameter, animals had significantly higher values in the stages of reversal learning compared to the initial learning, showing the re-assessing and re-learning over time. Acute stress on the other hand had no influence on this process at any stage. Previous studies by Conrad et al. showed that acute and chronic stress influence performance in a non-rewarded Y-maze task (Conrad et al. 2004; Wright and Conrad 2008). In an appetitive version of the Y-maze task, repeated stress has been shown to disrupt the acquisition of spatial learning, whereas performance in the task was not influenced by stress if learned prior to stress exposure (Ghiglieri et al. 1997). Our studies have a caveat in that all animals received the stressor and we cannot rule out that future studies employing non-stress controls or stressors of longer duration may unmask differential effects of genotype in reversal learning.

These results suggest for the first time, that leptin deficiency has no influence on spatial reference memory. One study previously described similar findings in a rewarded learning and memory task in rats (Paulus et al. 2005). In this study, intra-hippocampal infusion of leptin did not alter memory performance on the radial arm maze. Previous studies in leptin receptor-deficient rodents on the other hand have shown impaired learning in various unrewarded cognitive tasks (Li et al. 2002; Winocur et al. 2005). In the Morris water maze, leptin facilitated spatial learning after administration of low doses whereas an inhibition of learning was observed following application of high concentrations of leptin (Oomura et al. 2010). Most of the studies described the role of leptin on spatial memory performance in non-appetitive tasks, where the motivation for learning was to escape an aversive situation. Our data clearly show that learning performance based on the motivation to obtain a reward, however, is not impaired by leptin deficiency. This somewhat paradoxical finding might be due to the nature of the tasks employed and suggests a more complex role of leptin in learning and memory. Moreover, they

highlight the fact that the neuronal substrates underlying appetitive versus aversive learning are different (Everitt et al. 2003). Leptin is known to play an important part in the motivation for food intake with its anorexigenic properties promoting a negative energy balance by reducing the motivation to eat (Fulton et al. 2000). Considering this, the unaltered learning performance described in this study might underlie a complex interaction between the motivational and cognitive aspects of food intake, leading to a compensation of cognitive deficits due to leptin deficiency and therefore unaltered performance in a rewarded spatial reference memory task. Moreover, we cannot completely rule out that the lack of cognitive deficits in the *ob/ob* animals may be actually due to a higher drive to eat masking a spatial learning deficit.

One of the additional goals of these experiments was to challenge the behavioural repertoire of leptin-deficient mice. It is clear that their obese phenotype and subsequent hypolocomotion may cause a major confounding aspect to their utility in behavioural tasks. Here, we confirm that the *ob/ob* mouse is an appropriate tool for behavioural experiments and is not severely hampered by excessive weight gain, especially if maintained on an appropriate food-restricted schedule as in our appetitive learning task. It is well known that *ob/ob* mice display reduced baseline locomotion when fed ad libitum (Fulton et al. 2006; Laposky et al. 2006). This was seen in the LDB test were three animals of the obese group had to be excluded due to this fact. Thus, it was important to also utilise an anxiety measurement independent of locomotion and to verify the results obtained in the LDB test; hence, the test of hyponeophagia was carried out. As both tests showed higher levels of anxiety-like behaviour in the *ob/ob* mice compared to their controls, this phenotype can be seen as locomotor independent. Interestingly, when on food restriction obese mice displayed baseline movement similar to that in control mice. In the Y-maze task, mice of both groups reached the food reward in equal time intervals, once learning was established.

Taken together, our data further bolster the premise that the actions of leptin go beyond its influence on food intake. Moreover, these data highlight a key role of leptin in anxiety but not in appetitive spatial learning. Future studies should focus on understanding the molecular basis and the neural circuits involved in these effects.

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