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Mu-opioid receptor knockout mice show diminished food-anticipatory activity

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Abstract

We have previously suggested that during or prior to activation of anticipatory behaviour to a coming reward, μ-opioid receptors are activated. To test this hypothesis schedule induced food-anticipatory activity in μ-opioid receptor knockout mice was measured using running wheels. We hypothesized that μ-knockout mice show little food-anticipatory activity. In wildtype mice we observed that food-anticipatory activity increased proportional to reduced food intake levels during daily scheduled food access, and thus reflects the animal's physiological need for food. μ-Knockout mice do not adjust their schedule induced running wheel behaviour prior to and during feeding time in the same way as wildtype mice; rather than showing more running wheel activity before than during feeding, they showed an equal amount of activity before and during feeding. As food-anticipatory activity is dependent on the mesolimbic dopamine system and μ-opioid receptors regulate dopaminergic activity, these data suggest a change in the dopamine system's activity in μ-knockout mice. As we observed that μ-knockout mice tended to show a stronger locomotor activity response than wildtype mice to the indirect dopamine agonist *d*-amphetamine, it appears that the dopaminergic system *per se* is intact and sensitive to activation. We found no differences in the expression of pro-opiomelanocortin, a precursor of endogenous endorphin, in the arcuate nucleus between μ-knockout mice and wildtype mice during restricted feeding, showing that the μ-opioid receptor does not regulate endogenous endorphin levels. These data overall suggest a role for μ-opioid receptors in adapting reward related behaviour to the requirements of the environment.

Introduction

Free-living animals seem to face an almost impossible task to perform 'when and where to find the most valuable food-items to the lowest costs in an environment, which may change over short periods of time'. Yet, it has been observed that animals appear to behave relatively efficiently in such environments. For instance, they collect food-items in such a way that a minimum amount of energy is spent to get a maximum benefit (Krebs & Davies, 1993; McFarland, 1999). The behavioural and physiological systems that underlie the temporal organization of the organism's behaviour and which operate to achieve this efficiency are referred to as motivational systems (Hughes & Duncan, 1988; Jensen, 1996; Spruijt *et al*., 2001). Motivational systems are represented in the central nervous system, among others, by the mesolimbic opioid-dopamine system (Berridge & Robinson, 1998, 2003; Spruijt *et al*., 2001). Key words in behavioural efficiency are 'liking', 'wanting' and 'weighing/choosing' (Berridge, 1996; Berridge & Robinson, 1998, 2003; van den Bos *et al*., 2002; van den Bos, 2004). The focus in this study is on 'wanting', which is reflected in the appetitive phase of motivated behaviour.

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'Wanting' (as defined by Berridge, 1996) refers to the disposition to act upon previously 'liked' commodities. The behavioural expressions of 'wanting' are, for example, anticipatory behaviour to a coming reward in Pavlovian conditioning paradigms (rats, van den Bos *et al*., 2003; van der Harst *et al*., 2003a, b; Von Frijtag *et al*., 2000; cats, van den Bos *et al*., 2003) and lever pressing behaviour under high requirement schedules in instrumental conditioning paradigms (for a review on rats see Salamone & Correa, 2002). The behavioural expression of 'wanting' is under the control of mesolimbic dopamine systems, in particular the (core area of the) ventral striatum (Blackburn *et al*., 1989; Berridge & Robinson, 1998, 2003; Bassareo & Di Chiara, 1999; Knutson *et al*., 2001a, b, 2003; de la Fuente-Fernández *et al*., 2002; Salamone & Correa, 2002; Phillips *et al*., 1991; Peciña *et al*., 2003).

We have previously suggested that, during or prior to the activation of anticipatory behaviour to a coming reward, the mesolimbic opioid system is activated and serves a role in overcoming, for example, fatigue, in order to achieve the goal (Spruijt *et al*., 2001). The ventral tegmental area (VTA), which contains the cell bodies giving rise to the mesolimbic dopamine system, contains opioid receptors, among them μ-opioid receptors (Mansour *et al*., 1995; Kitchen *et al*., 1997). It has been shown that μ-opioid receptor activation increases the release of dopamine in the ventral striatum (Devine *et al*., 1993; Di Chiara &

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Imperato, 1988; Leone *et al*., 1991; Schad *et al*., 2002). In line with our hypothesis and these data we have obtained preliminary behavioural data showing that low dose (0.1 μg/1.0 μl) injections of the opioid antagonist naloxone into the VTA decrease anticipatory behaviour in a Pavlovian conditioning procedure (Spruijt *et al*., 2001).

To test further the hypothesis that activation of μ-opioid receptors plays a role in 'wanting' behaviour, schedule induced food-anticipatory activity in μ-opioid receptor knockout mice was studied. Food related anticipatory behaviour was evoked by a feeding schedule of one meal a day and measured as running wheel activity (reviewed in Mistlberger, 1994). In the first experiment, we tested whether food anticipatory behaviour reflects the animal's physiological need to eat, i.e. its motivation (Spruijt *et al*., 2001). We hypothesized that a decrease in mealtime duration increases the amount of running wheel activity prior to food access. Therefore, we subjected mice to different schedules of daily mealtime duration and compared the amount of wheel running activity prior to food access across these schedules. As a tool to study the role of μ-opioid receptors we used μ-opioid receptor knockout mice (Schuller *et al*., 1999). We hypothesized that μ-knockout mice show little, if any, food-anticipatory activity. The μ-opioid receptor is a receptor for exogenous substances, such as morphine and heroin, but also for the endogenous opiate endorphin. Endorphins are cleaved from pro-opiomelanocortin (POMC), a precursor hormone that is highly expressed in hypothalamic neurones of the arcuate nucleus. We measured the expression of POMC in the arcuate nucleus to control for differences in endorphin levels between μ-knockout mice and wildtype mice during the feeding schedules. Finally, we measured indirectly whether the mesolimbic dopamine system is sensitive to activation *per se* by measuring the locomotor response to injections of the indirect dopamine agonist *d*-amphetamine (Pijnenburg *et al*., 1976; Zocchi *et al*., 1998) We hypothesized that μ-knockout mice show a normal or even a slightly enhanced (Park *et al*., 2001; Tien *et al*., 2003) locomotor response.

Materials and methods

Subjects

Mu-knockout receptor mutant mice were created by replacing exon 1 of the MOR-1 gene with a neomycin resistant gene (Schuller *et al*., 1999). Mutant mice were backcrossed to a C57BL/6 background (Charles River, l'Arbresle, France) for at least nine generations resulting in a line with a standardized background. All mice were bred in our own institutes (Rudolf Magnus Institute of Neuroscience, Ethology & Welfare) from heterozygote parents. Mice were weaned at the age of 4 weeks. At weaning or one day thereafter tail tips were taken to determine the genotype.

Genotyping occurred by polymerase chain reaction on genomic DNA from tail tips. The mutant product was 700 bp, the wildtype product 525 bp; the three primers used were:
outside the mutation site
(5′-GACTTTCCTGGCTGATGCAAACAACCT-3′),
within the mutation site
(5′-CATGGTTCTGAATGCTTGCTGCGGACT-3′)
and within the neomycin box
(5′-CTACCTGCCCATTCGACCACCAA-3′).

All animals were housed under a 12-h light : 12-h dark cycle. Before experiments started the dark period started at 07:00 h. During experiment 1, 2 and 3 the start of the dark period was shifted to a later hour (see below). All mice were housed in iso-sex groups ($n = 2$–4 in each group) in Macrolon type II cages with at least a tissue as enrichment in a temperature controlled room ($T = 21 \pm 1$ °Celcius; humidity at least 50%). A radio provided background noise throughout the day. Food and water were available *ad libitum*. Experiments were conducted when mice were aged 3–5 months.

Experimental procedures

The Animal Ethical Committee of Utrecht University approved all experiments.

Experiment 1 Duration of food access and food-anticipatory activity

Fifteen adult female wildtype C57BL/6 mice were used for this experiment. At least 2 weeks prior to the start of the experiments mice were placed on an altered light-dark cycle. The dark period started at 12:30 h. Hereafter animals were singly housed in transparent running wheel cages (dimensions 26 × 12 × 16 cm, l × w × h). These cages contained a running wheel (diameter 14 cm; width 9 cm) and a water bottle. The bottom was covered with sawdust. Food was placed on the bottom of the cage. The mice were adapted to the running wheel for at least 1 week. The activity in the running wheel was registered by a little magnet and a counter that was activated by the magnet when it passed the counter during a revolution of the running wheel. Running wheel activity was read from the counter at 12:30 h daily during this week and expressed as daily distance moved in kilometers (up to one decimal accuracy, i.e. 100 m). During the second week the feeding regime was changed such that food was only available for 2, 3 or 4 h from 12:30 h onwards ($n = 5$ mice per meal duration condition). Activity was scored at 10:00 h, 12:30 h, 14:30, 15:30 or 16:30 h. Throughout the experiment animals were weighed and their food intake monitored. If mice lost too much weight (> 18%) during the second week they were given some additional food at the end of the feeding period.

Experiment 2 Mu-knockout mice and food-anticipatory activity

Fifty-seven adult female wildtype, heterozygote and μ-knockout mice C57BL/6 mice were used for this experiment. The experimental conditions were as for experiment 1. We selected the food access schedule for this experiment based on experiment 1. During 4 h of food access we found relatively low levels of food anticipatory activity. As we expected a decrease in food anticipatory activity in the knockout mice the 2- or 3-h schedules were preferable. However, mice with 2 h food access showed a more rapid decline in body weight than animals with 3 h of food access (probably due to an increase in the total daily running during food restriction for the 2 h condition). Therefore, the 3 h schedule was used in this experiment.

Experiment 3 POMC expression

Adult female wildtype ($n = 5$) and μ-knockout ($n = 5$) C57BL/6 mice were used for this experiment. Brains were removed 2 h prior to the second day of the scheduled 3-h food access. They were then frozen in cold isopentane (−30 °C for 20 s). Pre-treated 16-μm cryostat sections from mouse hypothalamus were hybridized with ^{33}P-labelled antisense RNA probes according to van der Kraan *et al*. (1998). A 350 bp rat POMC cDNA fragment (from bp +97 to +447 relative to transcription initiation) was used as the template for synthesis of the RNA probe for *in situ* hybridization. POMC mRNA expression levels in the arcuate nucleus were quantified using MCID-M5 (Imaging Research, Ontario, Canada).

Experiment 4 d-amphetamine induced activity

Thirty-five adult female wildtype, heterozygote and μ-knockout C57BL6 mice were used for this experiment. Animals were kept on

their initial light-dark cycle period for this experiment. All testing was carried out during the dark period between 10:00 h and 16:00 h. Mice were placed in transparent test cages (dimensions 62 × 26 × 33 cm, l × w × h). The test cage contained saw dust (Lignocel type 3/4; cf. home cage) as bedding material. Test cages were cleaned between sessions for different mice. Mice were allowed to habituate for 30 min. Hereafter, they received subcutaneous injections of vehicle (0.9% NaCl) or *d*-amphetamine (*d*-amphetamine sulphate dissolved in 0.9% NaCl). A dose of 4 mg/kg (0.5 mL/100 g bodyweight) was used, which has been shown to induce a reliable increase in activity in CB57BL/6 mice (Zocchi *et al*., 1998). All *d*-amphetamine solutions were prepared beforehand at the dispensary of the Veterinary Faculty and frozen until use. The behaviour of the mice was recorded for at least 80 min. Throughout the experiment the behaviour of the mice was recorded by Ethovision (Noldus Information Technology BV, Wageningen, The Netherlands) on a PC at a sample rate of five samples per second. Littermates were tested simultaneously (four maximum).

Dependent variables

Experiment 1 and 2

Daily weight and food-intake of the mice were recorded. During the second week of experiments, daily running wheel activity was scored, i.e. the activity from 12:30 h on one day to 12:30 h on the next. Day 0 was defined as the 24 h prior to the first restricted feeding period. Days 1–4 were the four 24 h periods during which food restriction occurred. The running wheel activity prior to feeding was scored from 10:00 to 12.30 h, i.e. during a 2.5-h period. The running wheel activity during feeding was scored from 12:30 to 14:30, 12:30 to 15:30 or 12:30 to 16:30 h, depending on the specific test condition. All data are in kilometres, accurate to 100 m. Group scores are expressed as mean ± standard error of the means (SEM) unless otherwise indicated.

Experiment 3

The dependent variable was POMC mRNA expression (c.p.m.). Group scores are expressed as mean ± SEM unless otherwise indicated.

Experiment 4

The dependent variable was the distance moved (in meters) extracted from the Ethovision files. All group data are expressed as mean ± SEM unless otherwise indicated.

Statistics

The data from the different experiments were analysed by paired *t*-tests, and one-way, two-way or three-way analysis of variance (ANOVA) depending upon the specific dataset using SPSS version 9.0 for Windows. Factors are indicated in the Results section. *Posthoc* testing was carried out whenever appropriate, and is indicated in the Results section. Significance was set at $P \leq 0.05$; $0.05 < P \leq 0.10$ was taken to indicate a trend, whereas $P > 0.10$ was taken as not significant (NS). Unless otherwise indicated all statistics are two-tailed.

Results

Experiment 1

Mice with daily scheduled food access 2 h per day (in the first 2 h of the dark phase, their habitual activity phase) ate significantly less than mice with 4 h of food access per day [mean ± SEM 30.6 ± 2.0% vs. 57.6 ± 2.6% of their *ad libitum* daily food intake; one-way ANOVA (including 3 h) $F_{2,12} = 31.098$, $P \leq 0.001$, *posthoc* Student–Newman–Keuls test] and exhibited a significant increase in wheel running activity in the hours prior to food access (Fig. 1A). Mice with three (34.9 ± 3.1%) or 2 h of food access had similar amounts of food intake (statistics, as mentioned see above) and expressed similar levels of anticipatory behaviour prior to food access. Relative food intake during restricted food access was highly correlated with the amount of wheel running prior to food access (Fig. 1B; $r = -0.69$, $n = 15$, $P \leq 0.005$).

Experiment 2

Seven mice were discarded from the final analysis in the running wheel experiment; three (one individual of each genotype) because of technical problems with the running wheels, four (two wildtype mice, one heterozygote mouse and one μ-knockout mouse) because of not showing substantial running wheel activity (< 25 km total activity over 2 weeks). This left $n = 16$ wildtype (WT), $n = 16$ heterozygote mice (HT) and $n = 18$ μ-opioid receptor knockout mice (KO) mice for final data analysis.

Figure 2A shows the total daily running wheel activity for the different genotypes over the course of five days. The data show that no difference was present between the different genotypes, neither over the course of the experiment [two-way ANOVA, genotype, day (repeated measure): genotype × day $F_{8,188} = 1.062$, n.s.] nor independent of day (genotype $F_{2,47} = 0.046$, n.s.). All mice showed a decrease over the course of the experiment ($F_{4,188} = 34.513$, $P \leq 0.001$). However, a clear effect was found in the 2.5 h before feeding (Fig. 2B), i.e. although KO mice showed an increase in running wheel activity prior to food access during scheduled feeding this increase in activity was clearly lower than that of the HT and WT mice [two-way ANOVA, genotype, day (repeated measure), genotype × day $F_{8,184} = 2.550$, $P \leq 0.012$; genotype $F_{2,46} = 3.837$, $P \leq 0.029$]. The opposite was found for the running wheel activity during the 3-h feeding period, i.e. KO mice tended to show a lower decrease in running wheel activity than WT and HT mice [two-way ANOVA, genotype, day (repeated measure), day $F_{4,188} = 69.590$, $P \leq 0.001$; genotype × day $F_{8,188} = 1.668$, n.s.; genotype $F_{2,47} = 5.668$, $P \leq 0.006$]. HT mice especially, showed a strong decrease in running wheel activity. When the two periods were compared as running wheel activity per hour (Fig. 2D), it turned out that KO mice did not adjust their running wheel activity to the same extent as WT and HT mice: whereas WT and HT mice showed a clear shift over the days from more running wheel activity during feeding to more running wheel activity prior to feeding, KO mice showed only a partial shift, i.e. from more running wheel activity during feeding to similar running wheel activity prior and during feeding [three-way ANOVA, genotype, day (repeated), prior–during feeding (repeated): genotype × day × prior–during feeding: $F_{8,184} = 2.318$, $P \leq 0.022$].

No differences were observed between the different genotypes for either body weight or food intake during the experiment (Fig. 2E and F). All genotypes showed an equal decrease in body weight following food restriction, on average 82% of the free-feeding weight [two-way ANOVA, genotype, day (repeated measure): genotype × day $F_{8,188} = 0.388$, n.s.; day $F_{4,192} = 669.486$, $P \leq 0.001$]. With respect to food intake mice rapidly adjusted their food intake during the food restriction period to consuming more food following the first restriction day. It appeared that during the first day WT mice showed somewhat less consumption than HT and KO mice but caught up after this initial day [two-way ANOVA, genotype, day (repeated measure):

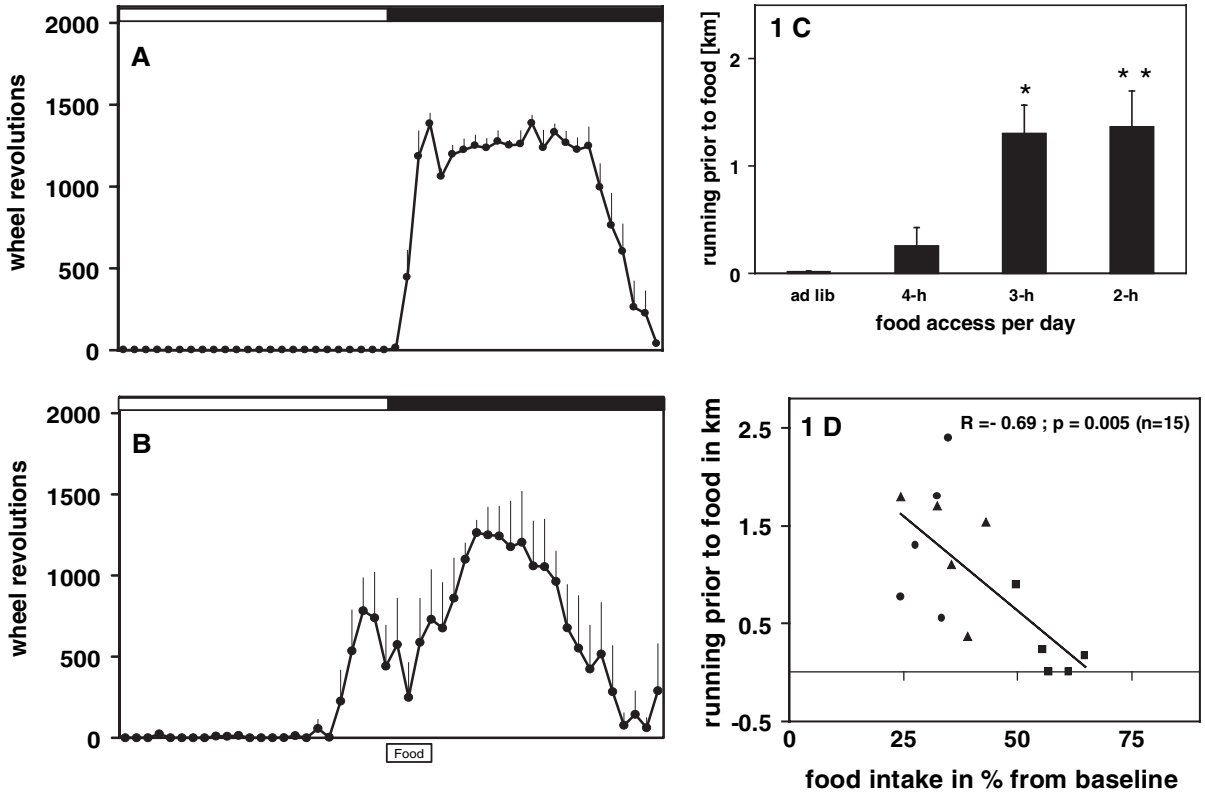

FIG. 1. (A) Nocturnal rodents with voluntary access to running wheels and *ad libitum* access to food exhibit a circadian wheel running rhythm with high levels during the dark phase, their habitual activity phase, and almost no running during the 12-h light phase of the 24-h light-dark cycle. (B) Daily scheduled food availability results in a re-distribution of wheel running activity over the 24-h day. Scheduled food access is preceded with high levels of behavioural activity in the hours prior to food access (box with food below the *x*-axis indicate hours of food access). (C) Averaged (3 days) running wheel activity prior to food access of mice (2.5 h; $n = 5$ mice per group) subjected to food restriction and 2, 3 or 4 h of food access. Activity prior to food access was compared to baseline *ad libitum* activity using paired *t*-tests. As no differences were found between different baseline conditions, the mean of baseline activity is shown for all 15 mice, paired *t*-tests were run however, on the original group data. Values were 4 h, $t = 1.496$, n.s.; 3 h, $t = 4.043$, $P \leq 0.02$; 2 h, $t = 4.798$, $P \leq 0.01$; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. (D) Pearson product-moment correlation of averaged (3 days) running wheel activity prior to food access and averaged (3 days) relative food intake of mice. Different symbols indicate different groups; ■ 4 h of food access; ▲ 3 h of food access; ● 2 h of food access.

genotype × day: $F_{6,138} = 2.564$, $P \leq 0.022$; day: $F_{3,138} = 291.717$, $P \leq 0.001$]. During *ad libitum* feeding conditions no differences were found between wildtype, heterozygote and μ-knockout mice in food intake, 4.73 ± 0.16, 4.74 ± 0.23 and 4.49 ± 0.24 g (mean ± SEM), respectively (one-way ANOVA, $F_{2,47} = 0.457$; n.s.).

Experiment 3

Figure 3A–C shows the expression of POMC in the arcuate nucleus of μ-knockout mice and wildtype mice during scheduled feeding. No differences were observed between μ-knockout mice and wildtype mice ($t = 1.553$, d.f. = 8, n.s.).

Experiment 4

In Fig. 4A–C it can be seen that the 4 mg/kg dose of *d*-amphetamine enhanced locomotor activity compared to vehicle [three-way ANOVA, genotype, drug, time bin (repeated measure): drug × time bin $F_{15,435} = 6.360$, $P \leq 0.001$] in each genotype (genotype × drug × time bin $F_{30,435} = 0.544$ n.s.). Based on the three-way ANOVA, the increase in locomotor activity following *d*-amphetamine did not differ between genotypes. When comparing the locomotor response to *d*-amphetamine within genotypes, it should be noted that the stimulant effect was most prominent in the HT and KO mice: drug × time bin for WT ($F_{15,120} = 1.505$, n.s.), HT ($F_{15,180} = 3.662$, $P \leq 0.001$) and KO ($F_{15,135} = 2.624$, $P \leq 0.002$).

Discussion

The present study revealed that running wheel activity prior to scheduled food access is related to the animal's motivation for food. Furthermore, mice lacking the expression of the μ-opioid receptor, i.e. μ-knockout mice (Schuller *et al*., 1999), do not adjust their schedule induced running wheel behaviour prior and during feeding time in the same way as wildtype mice. Rather than showing more running wheel activity before than during feeding, they show equal activity before and during feeding. Whilst all genotypes responded to *d*-amphetamine with increased locomotor activity, μ-knockout mice tend to have a stronger locomotor response to *d*-amphetamine than wildtype mice. Finally, μ-knockout mice do have a normal expression of POMC, the precursor of endogenous endorphin.

Schedule induced activity

As food intake during restricted food access is highly correlated with the amount of wheel running prior to food access, these data indicate that the intensity of food anticipatory activity highly reflects the animal's physiological need to eat, i.e. its motivation. Under

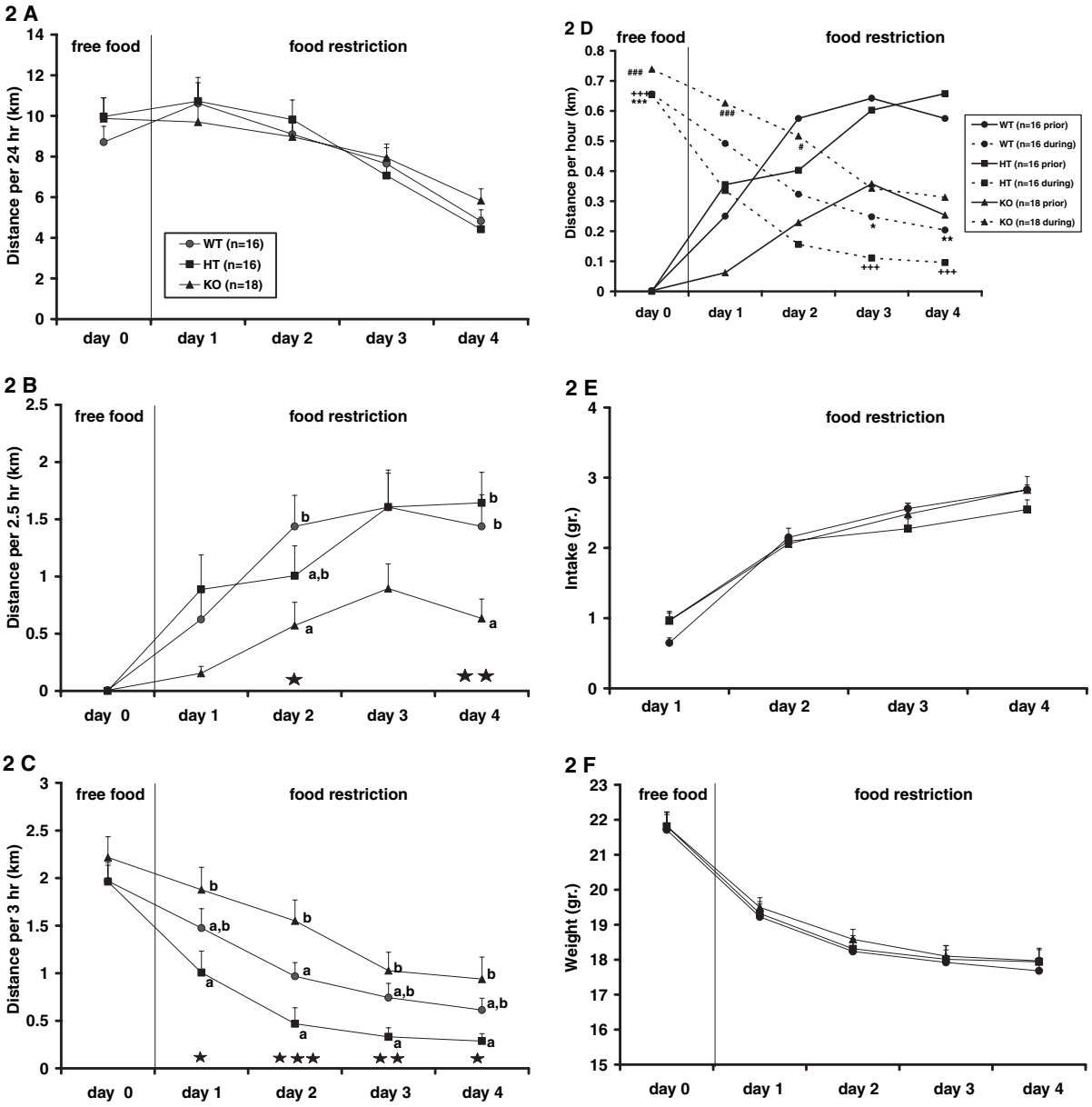

FIG. 2. (A) Mean daily running wheel activity during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (days 1–4) for wildtype, heterozygote and μ-knockout mice. (B) Mean 2.5 h running wheel activity prior to food access during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (days 1–4) for wildtype, heterozygote and μ-knockout mice. $*P \leq 0.05$, $**P \leq 0.01$ [one-way ANOVA; groups with the same characters are not different from one another (*posthoc* Student–Newman–Keuls)]. (C) Mean 3-h running wheel activity during food access during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (days 1–4) for wildtype, heterozygote and μ-knockout mice. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ [one-way ANOVA; groups with the same characters are not different from one another (*posthoc* Student–Newman–Keuls)]. (D) Mean hourly running wheel activity prior to and during food access during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (days 1–4) for wildtype, heterozygote and μ-knockout mice. SEMs are omitted for clarity; #, + , $*P \leq 0.05$, ##, ++ , $**P \leq 0.01$, ###, +++ , $***P \leq 0.001$ (paired *t*-test, during vs. prior; for KO, HT and WT, respectively). (E) Mean food intake during food access restricted feeding conditions, i.e. 3 h of daily food access (day1–4) for wildtype, heterozygote and μ-knockout mice. (F) Mean body-weight during *ad libitum* feeding conditions (day 0) and restricted feeding conditions, i.e. 3 h of daily food access (day1–4) for wildtype, heterozygote and μ-knockout mice.

laboratory conditions it seems paradoxical that animals become very active during times of low food availability, as food will become available without having to work for it. However, in view of behavioural efficiency, the increased expression of anticipatory behaviour during reduced food availability likely mirrors an important motivational behaviour in the survival of an organism in its natural environment. For example, in times of restricted and timely available food access, an organism requires to establish a proper balance between its reduced energy intake and daily energy expenditure. The observed increased expression of anticipatory behaviour may reflect exploratory behaviour in the search for food.

μ-knockout mice and anticipatory activity

Before discussing the differences around feeding time between μ-knockout mice and wildtype mice it is worth noting that the effects

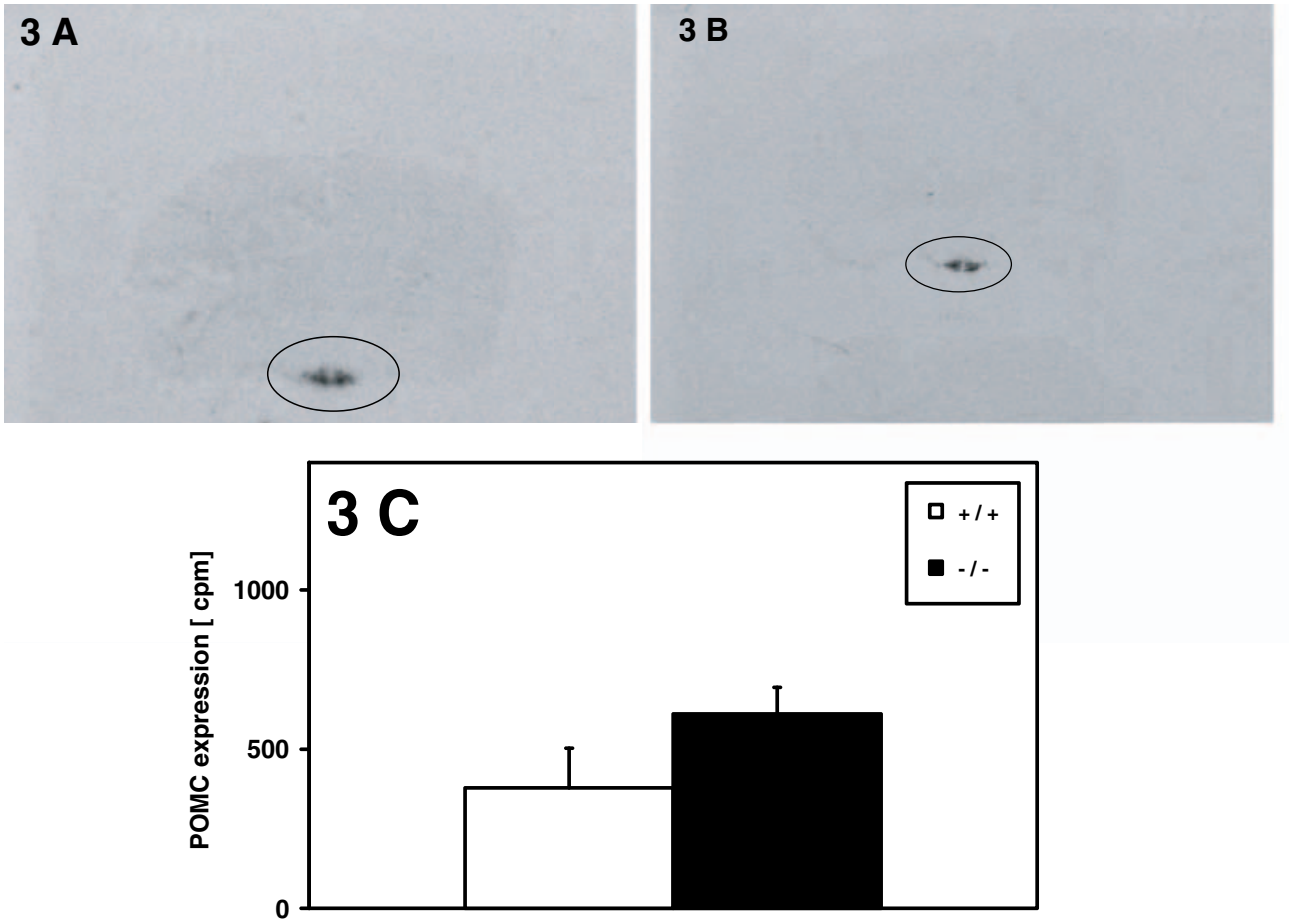

FIG. 3. (A) POMC expression in the arcuate nucleus of the hypothalamus (encircled) of wildtype mice prior to food access. (B) POMC expression in the arcuate nucleus of the hypothalamus (encircled) of μ-knockout mice prior to food access. (C) Mean POMC expression in the arcuate nucleus of wildtype and μ-knockout mice prior to food access.

observed during this period are not due to differences in general daily running wheel activity, weight or food intake changes *per se*, as no effects were observed on any of these parameters. The data show that, as in *ad libitum* fed mutant mice (Zhou *et al*., 2002), POMC gene expression is not affected in μ-receptor knock mice as a consequence of scheduled food access. These data show that the endogenous μ-opioid receptor affects food anticipatory behaviour, but does not impair endogenous endorphin expression levels during *ad libitum* and scheduled daily food access. Moreover, no differences were observed between μ-knockout mice and wildtype mice with respect to blood glucose levels prior to feeding (unpublished data) ruling out metabolic differences between genotypes underlying the differences in running wheel activity.

The change from free food to restricted food induced a clear change in the running wheel behaviour of the wildtype mice. Whereas no running wheel activity was seen in the 2.5 h preceding the onset of the dark period under free food conditions, running wheel activity began to occur in this period when food was only available during the first 3 h of the dark period. These results are in line with those observed in other studies on C57BL/6 mice (Abe *et al*., 1989; Marchant & Mistlberger, 1997) and in general, on food-anticipatory activity under restricted feeding schedules (Mistlberger, 1994). In wildtype mice a strong decrease occurred in running wheel activity during the feeding hours, concurrently with the increase of running wheel activity before the feeding hours. Under free food conditions mice showed far more activity during the first hours of the dark period than in the hours before the onset of the dark period, whereas under restricted food conditions mice showed far more activity before the onset of the dark period than during the first hours of the dark period when food was available. Although several models exist to account for the changes seen in this running wheel behaviour, no model until now fully explains the changes in behaviour (see, e.g. Mistlberger & Marchant, 1995; Mistlberger *et al*., 2003). It has been shown that the development of food-anticipatory activity is neither dependent on the suprachiasmatic nucleus (Marchant & Mistlberger, 1997) nor the lateral hypothalamus (Mistlberger *et al*., 2003) but involves the hippocampus and cortical areas (Wakamatsu *et al*., 2001). Whatever the underlying mechanism, it is clear that a form of learning takes place. As we observed that during four days of the schedule the MOR-deficient mice developed food-anticipatory behaviour but at a lower overall level than wildtype mice and heterozygote mice, the data therefore suggest that μ-opioid receptors are not necessary for the development of food-anticipatory activity, i.e. for this learning process *per se*. Consistent with this, the decrease in running wheel activity during the first hours of the dark period, i.e. during the period that food was present, also occurred in the μ-knockout mice, however, halted to the point that an equal amount of activity was seen prior and during feeding.

In so far as food-anticipatory activity may be considered identical to anticipatory activity under a Pavlovian conditioning schedule, or

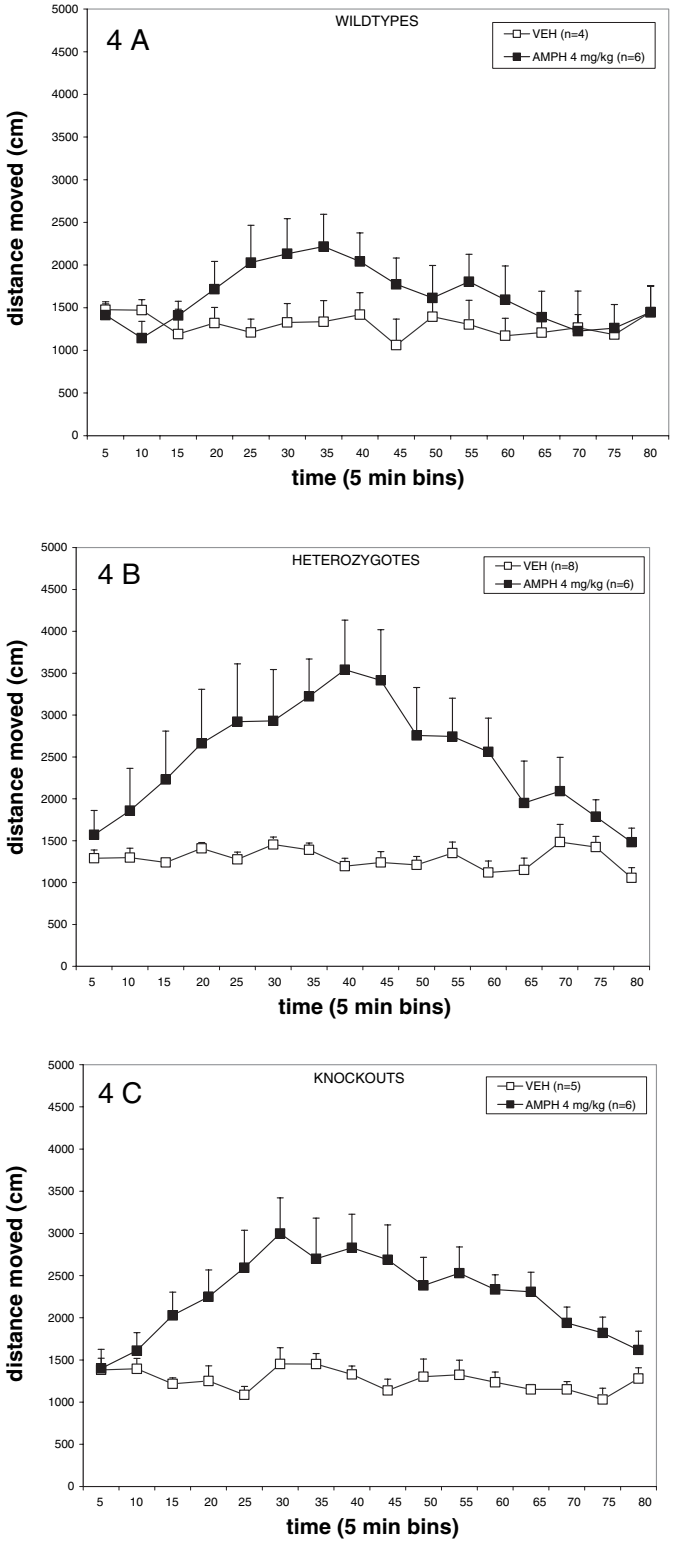

FIG. 4. (A) Mean distance moved after injection of vehicle or *d*-amphetamine (4 mg/kg) in wildtype mice during 80 min, expressed as 5-min bins. (B) Mean distance moved after injection of vehicle or *d*-amphetamine (4 mg/kg) in heterozygote mice during 80 min, expressed as 5-min bins. (C) Mean distance moved after injection of vehicle or *d*-amphetamine (4 mg/kg) in μ-knockout mice during 80 min, expressed as 5-min bins.

appetitive behaviour prior to consummatory behaviour (Spruijt *et al*., 2001) regulated by the mesolimbic dopamine system (Blackburn *et al*., 1989; Phillips *et al*., 1991; Berridge & Robinson, 1998; de la Fuente-Fernández *et al*., 2002), these data therefore suggest that μ-opioid receptors in the VTA, which the μ-knockout mice lack (Kitchen *et al*., 1997), are not necessary for the development of anticipatory behaviour *per se*. The overall change in schedule induced behaviour resembles that obtained by Parkinson *et al*. (1999) for cue-induced (CS) magazine visit behaviour observed in animals with lesions of the core-region of the ventral striatum: no change in total number of magazine visits, but a clear failure to specifically increase magazine visits while the CS announces the arrival of a reward resulting in similar activity before and after the reward (US). Both running wheel activity (Vargas-Pérez *et al*., 2003) and food restriction (Cadoni *et al*., 2003) have a profound effect on the core area of the ventral striatum, which is involved in anticipatory behaviour (Bassareo & Di Chiara, 1999). The present data would thus suggest that μ-opioid receptors in the VTA driving dopaminergic activity in the core area of the ventral striatum play a role in adapting reward related behaviour to the requirements of the environment; when it is opportune to behave in relation to the arrival of rewards, such as food (cf. Schultz, 2000; Phillips *et al*., 2003). In so far as food anticipatory activity and behavioural sensitization under dopaminergic stimulant drugs are dependent on the core area of the ventral striatum (Cadoni *et al*., 2003) the data of the present study fit in with the observation that μ-knockout mice do show a cocaine induced sensitized motor response *per se*, but at a lower overall level than wildtype mice (Yoo *et al*., 2003).

Although we conclude that μ-knockout mice have an impaired regulation of their anticipatory behaviour to rewarding stimuli, it may be suggested that the hedonic valuation of rewarding stimuli is reduced in these mice, which may explain the decrease in anticipatory activity. However, we observed in the present study that food intake levels between wildtype mice and μ-knockout mice were not different. If consumption is taken as a measure for hedonic valuation, then no differences exist between wildtype and μ-knockout mice for hedonic valuation. Whilst this may have been caused by the limited amount of time to eat, we have also observed that μ-knockout mice have a similar increased preference for sucrose (5% sucrose vs. water), another measure for hedonic valuation, compared to wildtype mice in a two-bottle free-choice paradigm; sucrose preference ratios after 3 days 0.95 ± 0.01 (wildtype mice) vs. 0.96 ± 0.01 (μ-knockout mice). Overall, these data are in line with a recent study in mice lacking endogenous β-endorphins (the natural ligand for μ-opioid receptors; Hayward *et al*., 2002), and suggest that the hedonic aspects of food consumption were not reduced in μ-knockout mice. Furthermore, it should be noted that the appraisal of food not only relies on reward systems as discussed here but also on food regulatory mechanisms in the hypothalamus that tend to dominate under conditions of deprivation or metabolic stress, such as in the present study, and thus overrule the hedonic effects of endorphins (Hayward *et al*., 2002). Unless more sensitive measures for the hedonic aspects of food consumption become available that prove differently, the data indicate that μ-opioid receptor signalling is at least involved in the appetitive phase of food motivated behaviours under the present conditions.

d-amphetamine induced activity

Overall μ-knockout mice tended to show a stronger *d*-amphetamine induced locomotor response than wildtype mice. This seems to be in line with the observation that μ-knockout mice have supersensitive dopamine receptors compared to wildtype mice (Park *et al*., 2001;

Tien *et al*., 2003). The dose of *d*-amphetamine should have produced a stronger response in wildtype mice (Zocchi *et al*., 1998). However, the present mice were tested without any prior manipulation, whereas those of Zocchi *et al*. (1998) were isolated for 48 h before testing. Short-term isolation may enhance the responsiveness to *d*-amphetamine (Kokkinidis & MacNeill, 1982; Cabib & Puglisi-Allegra, 1996). Whatever the explanation for this discrepancy may be, the differences between μ-knockout mice and wildtype mice on schedule induced behaviour are not due to a dysfunctioning dopamine system *per se*, but rather the way the mesolimbic dopamine system is activated under scheduled feeding.

Conclusion

The present data strongly suggest that μ-opioid receptors play a crucial role in adapting reward related behaviour to the requirements of the environment, when it is opportune to behave in relation to the arrival of rewards, such as food.

Abbreviations

AbbreviationsHT, heterozygous; KO, knockout; n.s., not significant; POMC, pro-opiomelanocortin; VTA, ventral tegmental area; WT, wildtype.

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