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Ablation of the hypothalamic neuropeptide melanin concentrating hormone is associated with behavioral abnormalities that reflect impaired olfactory integration

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Abstract

Melanin-concentrating hormone (MCH) is an orexigenic hypothalamic neuropeptide. At least one receptor, MCH receptor 1 (MCHR1), is present in all mammals and is expressed widely throughout the brain, including cortex, striatum and structures implicated in the integration of olfactory cues such as the piriform cortex and olfactory bulb. Consistent with a potential role for MCH in mediating olfactory function, MCH knockout mice demonstrate abnormal olfactory behaviors. These behaviors include impaired food seeking by both genders while maintaining normal levels of locomotion, suggesting impaired olfaction. Males also exhibit increased aggression while females show defects in several olfactory mediated behaviors including mating, estrous cycle synchronization and maternal behavior. These findings suggest that hypothalamic inputs through MCH play an important role in regulating sensory integration from olfactory pathways.

1. Introduction

The neuropeptide melanin-concentrating hormone (MCH) was discovered in 1983 in teleost fish as a 17-amino-acid cyclic peptide, which induced color change by causing aggregation of melanosomes in scales (Kawauchi et al., 1983). Following this initial observation in fish, MCH expression was reported in the mammalian hypothalamus. In the mammalian brain, MCH is expressed in the lateral hypothalamic area (LH) and the zona incerta (ZI). Neurons from these areas go on to make monosynaptic projections throughout the brain (Elias and Bittencourt, 1997). In rodents the sole receptor for MCH, termed MCHR1, is also widely expressed in the areas to which the MCH neurons project including the striatum, piriform cortex, olfactory tubercle, olfactory bulb, as well as within the hypothalamus (Saito et al., 2001). This broad projection pattern of MCH neurons suggested that the MCH system may be involved in the regulation of a wide array of physiological functions (Pissios et al., 2006).

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A role in the regulation of feeding and energy homeostasis was initially described in studies which showed that MCH expression was increased with fasting in WT mice and also was elevated at baseline in congenitally obese *ob/ob* mice (Qu et al., 1996). Further studies demonstrated that intracerebroventricular injections of recombinant MCH peptide into the lateral ventricle of rats caused a robust induction of eating behavior (Qu et al., 1996, Rossi et al., 1997). Deletion of the MCH gene in mice leads to a lean phenotype largely secondary to increased energy expenditure (Shimada et al., 1998, Kokkotou et al., 2005). Conversely, mice in which MCH was overexpressed developed mild obesity (Ludwig et al., 2001). Mice with targeted ablation of MCHR1 (Marsh et al., 2002) also displayed a lean phenotype and resistance to diet-induced obesity (DIO). MCH also appears to be involved in regulating the mesolimbic dopamine system as MCHKO mice have increased dopaminergic tone and increased sensitization to amphetamine induced locomotion (Georgescu et al., 2005, Pissios et al., 2008).

Here we provide evidence that MCH also regulates aspects of many olfactory mediated behaviors. In elucidating the potential role of MCH in food intake we found that MCHKO mice have significant difficulty in locating food odors. Furthermore, MCHKO mice showed impairment in behavior typically mediated by pheromones such as abnormalities in mating behavior, impairments in synchronizing estrous cycling and impaired maternal behavior which was associated with significantly increased mortality in the offspring of MCHKO mothers. Pheromone mediated olfactory defects were not specific to females as we also found that MCHKO males are excessively aggressive as assessed by a social interaction paradigm. Thus we propose that enervation of olfactory structures by MCH is critical for the integration of olfactory stimuli.

2. Methods

2.1 Animals

WT and MCHKO littermates were bred from mice originally generated and characterized by our group (Shimada et al., 1998). Mice used in this study had been backcrossed onto the C57BL6 background for at least 15 generations. Colonies are maintained as het × het breeders, progeny are genotyped and housed as described for each procedure in the Beth Israel Deaconess Medical Center (BIDMC) animal facility at 22°C ambient temperature on a 12:12 L:D cycle. Mice were fed standard laboratory chow (Diet F6, Harlan-Teklad, Madison, WI) for the duration of the studies. The BIDMC Institutional Animal Care and Use Committee approved all procedures detailed herein.

2.2 Buried food paradigm

Thirteen 12–16 week old male mice (n=6 MCHKO and n=7 WT) were used in food odorant testing. A buried odorant paradigm was used to assess the olfactory performance in response to an attractive food odor. Approximately 200mg of peanut butter (Skippy Smooth, USA) was placed in a microfuge tube with a 2mm hole pierced in the cap. A control microfuge tube contained 200 microliters of water. Microfuge tubes were place in a cage and were covered with 10 cm of odorless bedding (Alpha Dri, Shepherd Specialty Papers, USA).

Prior to the start of the testing period all mice were individually housed for a period of one week in cages identical to the testing cage. The day prior to the test mice were fasted overnight to encourage food seeking behavior. Mice were allowed to acclimate in the test cage for an hour prior to the onset of testing after which mice were removed briefly for placement of microfuge tubes containing either water or peanut butter. For placement of tubes cages were divided in two virtual halves with each tube taped to the bottom of the cage in the center of the respective sections. All tests were performed in standard mouse holding cages (191mm × 292mm × 127mm, Mouse holding cages, Allentown, PA).

Monitoring of the mice began immediately following re-introduction of the mouse to the cage, using automated video tracking software (Ethovision, Noldus Information Technology, USA). The latency (seconds) for the mice to find and expose the buried tube containing the odorant was scored. Recording was terminated either when the mice found and exposed the tube, or at 20min when the test period was concluded. One half of the cage containing the control tube filled with water was designated the placebo zone. The half in which we placed the peanut butter filled tube was termed the odorant zone. We also assessed the percentage of time that the mice spent in each zone.

2.3 Open field testing

Individual WT (n=16) and MCHKO (n=15) mice were placed in a fresh standard cage and recorded by video camera for a period of 1 h. Total distance traveled was measured using tracking software (Ethovision, Noldus Information Technology, USA).

2.4 Resident intruder paradigm

Following 1 week of acclimation to single housing conditions, 12 week old male WT (n=8) and MCHKO (n=8) mice were tested for social dominance and aggressive behavior in the resident–intruder paradigm. An intruder mouse (age, weight, and sex-matched C57BL6 mouse) was placed in the resident's home cage. Each test session (15 min duration) was recorded on videotape using a camera placed 150 cm above the home cage. Following the recording period, latency till first attack and number of attacks were hand coded and quantified from videotapes. Tests were immediately terminated if either mouse drew blood or caused visible injury.

2.5 Evaluation of estrous cycling

Estrous cycles of both WT (n=10) and MCHKO (n=10) female mice were evaluated with regard to onset and duration and synchronization of the cycle. The onset of the estrous cycle was defined as the first appearance of cornified cells in a cyclic fashion on vaginal smears. For estrous synchronization mice were housed in groups of 5 and vaginal smears were collected daily and assessed to determine synchronization of cycle in each group of females.

2.6 Video analysis of mating behavior

The mating activity of the mice was evaluated using a video monitoring system. Five pairs of animals of each genotype (WT male + WT Female vs. MCHKO male + MCHKO female) were set-up at the beginning of the dark cycle and filmed for 6 hours. Attempted mounts and

successful mounts scored by an unbiased observer reviewing the tapes at 2X speed during the testing period.

2.7 Maternal behavior

To evaluate the maternal behaviors of the mice, female WT (n=13) and MCHKO (n=9) mice were allowed to mate with male WT mice for a period of 3 days. Following this mating period male mice were removed from the cage and the female mice were subsequently monitored for signs of pregnancy daily by visual examination and weight measurements. The date of birth of pups was considered postpartum Day 0. Once pups were born they were counted (n) and weighed. Data is presented as total litter mass (g) and also as the mean mass of each pup (g) per day for a period of 14 days following birth. Pup mortality is presented as percentage of initial litter which died between days 0–14 post-partum.

2.8 Statistical analysis

Statistical evaluation of the results was performed using Minitab 15.1 statistical software (Minitab Inc, PA, USA). One-way analysis of variance (ANOVA) was used to dissect statistical differences for the factor genotype with repeated measures ANOVA used for time series data. All data are presented as mean values ± SEM, with *n* referring to the number of mice in each group.

3. Results

3.1 Buried food paradigm

In our initial tests using peanut butter, overnight fasted WT mice explored the cage and 6 of the 7 mice tested (Figure 1A) were able to locate the tube containing the peanut butter during the observation period (494s±173s to expose the peanut butter tube), in contrast only 1 of the 6 MCHKO mice was able to find and expose the peanut butter tube (Figure 1A) in a time of 696s. The remaining 5 MCHKO mice were unable to locate the tube for the duration of the testing period (1200s). This defect was also reflected in the zone preferences of the mice, WT mice spent significantly more of the test period in the half of the cage containing the PB (Placebo Zone vs. Odorant Zone; 62.4% vs. 37.6%; P=<0.05; Figure 1B) while the MCHKO mice had no discernable zone preference (Placebo Zone vs. Odorant Zone; 53.4% vs. 46.6%; NS; Figure 1B).

3.2 Open field testing

The MCHKO mice struggled to find the peanut butter tube in the buried food paradigm, therefore, we conducted open field testing to assess exploratory behavior to determine if this defect was responsible for the difference we saw. After introduction to the new cage WT and MCHKO mice both explored to a similar extent, furthermore, the activity decreased at a similar rate over the course of the trial (WT vs. MCHKO, Repeated measures ANOVA, NS; Figure 1C). There was also no significant difference in the total distance moved over the duration of the testing sessions between genotypes (10777 ± 781 vs. 11742 ± 740; NS; Figure 1D).

3.2 Resident intruder paradigm

When compared to WT mice the MCHKO males showed a highly elevated level of aggression with the finding that as a group 75% (6/8 mice) of MCHKO mice displayed aggressive behavior during the 15 minute observation period period versus only 25% (2/8) in the WT group (Figure 2B). When compared to WT mice the MCHKO mice also had a much faster initial aggressive response with the first attack occurring significantly earlier in the course of the trial (WT vs MCHKO, 789.5s ± 73.4s vs. 334.5s ± 98.36s, p=<0.01; Figure 2A).

3.3 Mating behavior

WT (n=5 pairs) and MCHKO (n=5 pairs) mice were set-up for breeding and observed for 6h during the dark period using a video monitoring system. During this period, WT pairs demonstrated 10±3 attempted mounts, whereas MCHKO mice engaged in significantly increased attempts at mounting (28±3 attempts). However, MCHKO females appeared to be much less receptive than WT females and escaped from the mounting position with increased frequency when compared to WT. Successful mounting (assessed by vaginal plug formation in female mice) was seen in 4 out of 5 WT females, however, the success ratio was lower in the MCHKO females with plug formation evident in only 1 of the 5 mice.

3.4 Evaluation of estrous cycling

Typically female mice housed together synchronize estrous cycles which requires integration of pheromonal signals. Estrous cycles of both WT and MCHKO female mice were evaluated with regard to duration and synchronization of the estrous cycle. The onset of the estrous cycle was defined as the first appearance of cornified cells in a cyclic fashion on vaginal smears. Cycle length did not differ significantly between WT and MCHKO mice (5days ±0.19days vs. 5.2days±0.17days, NS). Estrous cycle synchronization, as defined by the percent of days in which all females in a cage were simultaneously either in the estrous or the diestrous phases of the cycle, was calculated for both female WT and MCHKO mice. While WT females had synchronous cycles 90% of the time, KO females showed cycle synchronization only 50% of the time. Therefore, although the estrous cycle in the MCHKO females was normal, they were unable to synchronize their cycles with those of other females in their immediate vicinity.

3.5 Maternal behavior

Mortality of pups was significantly lower among WT neonates as only 5 pups out of 92 died over the first 3 days (5.4%) when compared to that of the MCHKO neonates where 17 of 71 pups were cannibalized over the first 3 days. Mean litter mortality was 7.3%±4.2% in WT animals compared to 31.1%±13.6% in MCHKO mice (p=<0.05; Figure 4C). Two of the MCHKO mothers cannibalizing their entire litters the day following birth, while only 1 of 13 WT mothers cannibalized half of her litter over the same initial 3 days. At birth WT mothers had a mean litter size of 7.1±0.4 pups which was not different to the litter size seen in the MCHKO mothers (7.1±1; Figure 5A). Once fully cannibalized litters were excluded at 14 days after birth the mean number of pups surviving per WT litter trended higher than that of the litters seen from MCHKO mothers (6.5±0.4 vs. 5±1.2; p=0.2; Figure 4A).

The day following birth mean pup weight of WT and MCHKO pups was not significantly different (1.4g±0.05g vs. 1.3g±0.04g). On the final day of the study the mean pup weight of the WT pups trended higher than that of the MCHKO pups but the difference did not reach significance (6.3g±0.49g vs.5.8g±0.5g, Figure 4B). Previous studies have demonstrated that in mice, there is an inverse correlation between litter size and pup weight i.e. mothers with smaller litters have larger offspring (Hammond et al., 1996). However, while MCHKO mothers have smaller litter sizes than the WT mothers their pups also trend to be smaller.

4. Discussion

The present study demonstrates that deletion of MCH in mice gives rise to pronounced abnormalities in behaviors that are mediated through both classical and pheromonal olfactory stimuli. In mice, olfaction regulates a broad range of behaviors including mating, rearing of offspring, aggression/territory defense and the spatial location of attractive and aversive odors. Given the vital role played by olfaction in determining mouse behaviors very little is known about the central pathways regulating olfaction (Takahashi et al., 2005). In rodents inputs into the olfactory system are supplied by both the olfactory ganglion and the VNO (Touhara and Vosshall, 2009). When we tested the MCHKO mice in a simple food seeking paradigm we found that they have significant impairments in their ability to locate a food odor. That this most basic food seeking response is significantly perturbed suggests that MCH may play a role in the integration and processing of multiple olfactory stimuli. We then determined that this impairment in food seeking behavior was not secondary to reduced locomotion/exploration as MCHKO mice performed normally in the open field test. Thus we went on to test an array of olfactory behaviors in the MCHKO mice to determine the extent of the olfactory defects caused by a lack of MCH.

It is known that olfaction is important in mediation of aggressive behavior and that removal of the olfactory bulb leads to a complete absence in spontaneous aggression in mice (Ropartz, 1968, Fortuna and Gandelman, 1972). In the current study MCHKO mice were found to be significantly more aggressive towards intruder mice than WT in the resident intruder paradigm.

Increased aggression in male MCHKO mice may reflect an inability of MCHKO mice to accurately identify conspecifics. Indeed MCHKO males are sufficiently more aggressive towards cage mates that they require to be housed individually and resist handling vigorously (AC Adams & E Maratos Flier, Unpublished data).

We also found that several behaviors dependant on olfaction in females were also disrupted. As chemosensory cues from members of both sexes have effects on the estrous cycle of female mice (Parkes and Bruce, 1961) we examined estrous cycling in the MCHKO females. We showed that the estrous synchronizing effect seen in group housed females is absent in the MCHKO females when compared to WT. However, other parameters such as length of the cycle were unchanged, these results taken together reinforce the notion that MCH does not play a role in the endocrine regulation of reproduction but rather plays a role in the perception of the olfactory reproductive environment of the mice.

Olfaction has previously been reported to be important in mating, specifically olfaction is required to detect and integrate chemical cues released by members of the opposite sex. For example male sheep, cattle, and goats detect chemicals present in the anogenital region of females that allow them to assess the females' estrous state (Rekwot et al., 2001). In 1956 Whitten reported that anosmia or the inability to smell due to olfactory bulbectomization rendered inexperienced male and female mice of breeding age unable to mate and in fact caused retarded gonadal development (Whitten, 1956). Bulbecomy induced anosmia also led to a cessation in mating activity of sexually experienced males indicating that functioning olfactory circuits are critical for successful breeding.

In many species, olfaction mediates critical communication among conspecifics, binding chemical cues emitted by another animal –pheromones –and sending information about these cues to the brain. These cues influence both the behavior and physiology of the receiving animal. In the context of maternal behavior, olfaction is largely known for its role in promoting maternal defense (offspring protection) in mice; lesioning it eliminates this behavior entirely (Bean and Wysocki, 1989). Other studies have also shown a role for olfactory responses in mediating the time lactating mice spend on-nest (Kimchi et al., 2007). Interference with the function of the olfactory bulb either by removal or chemical lesions in mice has been shown to dramatically effect maternal behavior in virgin females, leading to high levels of cannibalism, failure to build nests and a subsequent failure to feed surviving pups (Herrenkohl and Sachs, 1972).

MCHKO females exhibit mothering deficits similar to those seen in bulbectomized mice. In addition mothering behavior has previously been demonstrated to be mediated by dopamine (Byrnes et al., 2002, Champagne et al., 2004, Gammie et al., 2008). We have previously shown that MCHKO mice have altered dopaminergic tone (Pissios et al., 2008), hence this may also be contributing to the poor pup survival in the MCHKO litters. We observed a significantly elevated level of pup mortality in MCHKO litters indicative of poor mothering. Furthermore even though WT mothers were rearing more pups when compared to MCHKO mice pup weight was still lower in the knock outs. This may reflect poor mothering, possibly due to the aforementioned reduction in time on nest as seen in mice with anosmia. Another possibility is the increased metabolic rate in MCHKO mice (Shimada et al., 1998) which is associated with significantly reduced body mass in adulthood may manifest itself at an early age.

5. Conclusions

MCH has previously been shown to be important in the regulation of reward and motivated behavior (Duncan et al., 2005, Shimazaki et al., 2006, Pissios et al., 2008). Here we show a novel component of MCH signaling in regulation of olfactory mediated behaviors. That loss of a specific neuropeptide has such a profound effect on a wide variety of behaviors is not surprising, given the widespread distribution of MCHR1 and the widespread monosynaptic projections made by MCH neurons throughout the brain. Future studies aiming to gain insight into the mechanism of the apparent olfactory dysfunction induced by loss of MCH may be better served by a more specific focus on specific regions of the brain either by using Cre-lox technology or use of microinjection of MCH modulating pharmacological agents.

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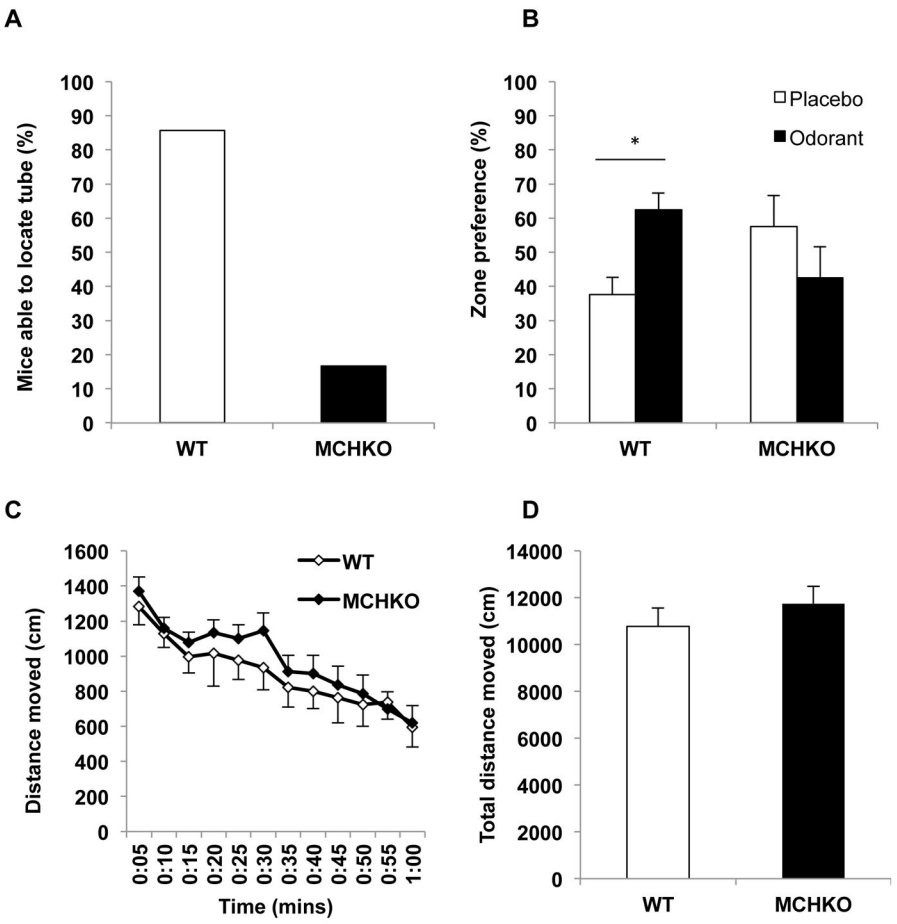

Figure 1.

A. The percentage of mice in each group able to locate the PB tube during the testing period was significantly higher in the WT mice than in the MCHKO mice further reinforcing the apparent olfactory deficit we see in the total time spent in the odorant zone.

B. Wild type mice (Open bars) spend a significantly longer time (% of total test time) in the PB (Odorant zone) of the cage when compared to the placebo zone consistent with the attractive odor of the PB. However, in the MCHKO mice (Black bars) there is no significant zone preference exhibited indicating an inherent inability to locate a buried food odorant.

C. In an open field test WT and MCHKO mice both explored to a similar extent, furthermore, the activity decreased at a similar rate over the course of the trial (WT vs. MCHKO, Repeated measures ANOVA, NS).

D. There was also no significant difference in the total distance moved over the duration of the testing sessions between genotypes (10777 ± 781 vs. 11742 ± 740; NS).

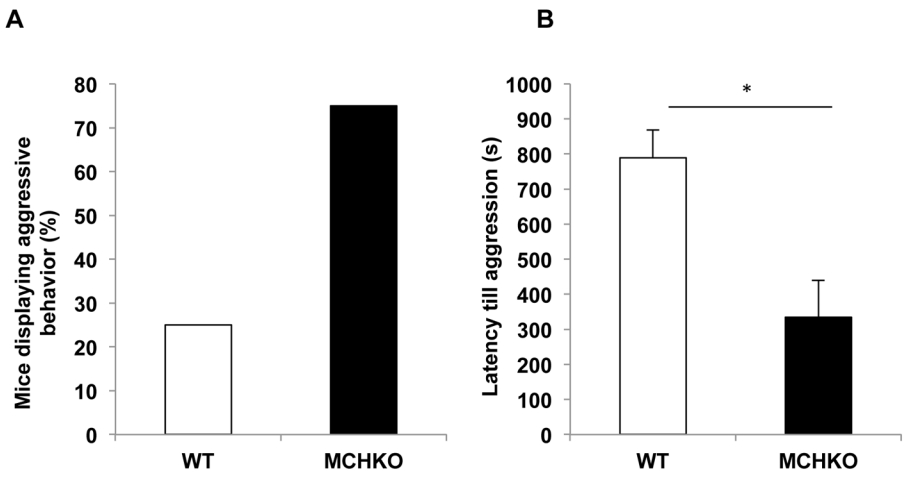

Figure 2.

A. The percentage of mice in the MCHKO group which displayed aggressive behavior during the testing period was significantly greater than in the WT group (75% vs. 25%).

B. The time until mice displayed aggression towards an intruder mouse placed in their cage was found to be significantly higher in WT mice when compared to their MCHKO counterparts (789.5s ± 73.4s vs. 334.5s ± 98.3s, p=<0.001), indicating a significantly heightened level of aggression towards intruder mice in the MCHKO mice.

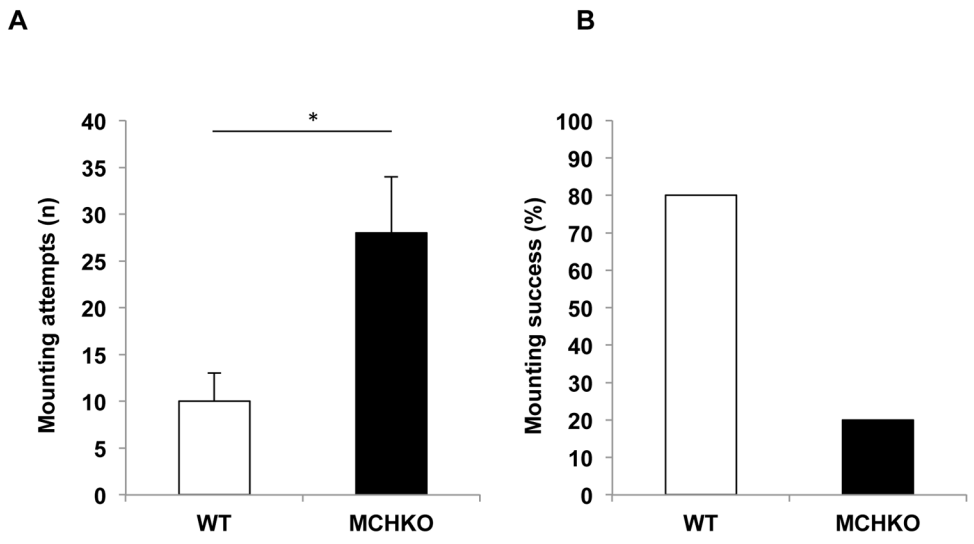

Figure 3.

A. During video recording sessions WT males were found to attempt significantly less mountings of females than that of the MCHKO males with their respective partners (10±3 vs. 28±6, p=<0.05). This difference may in part explain the reduced fertility of the MCHKO mice when bred in a homozygous manner.

B. In accordance with the number of failed mounting attempts the efficacy of the mating was found to be significantly higher in WT females when compared to MCHKO females (80% vs. 20% resulted in subsequent pregnancy respectively).

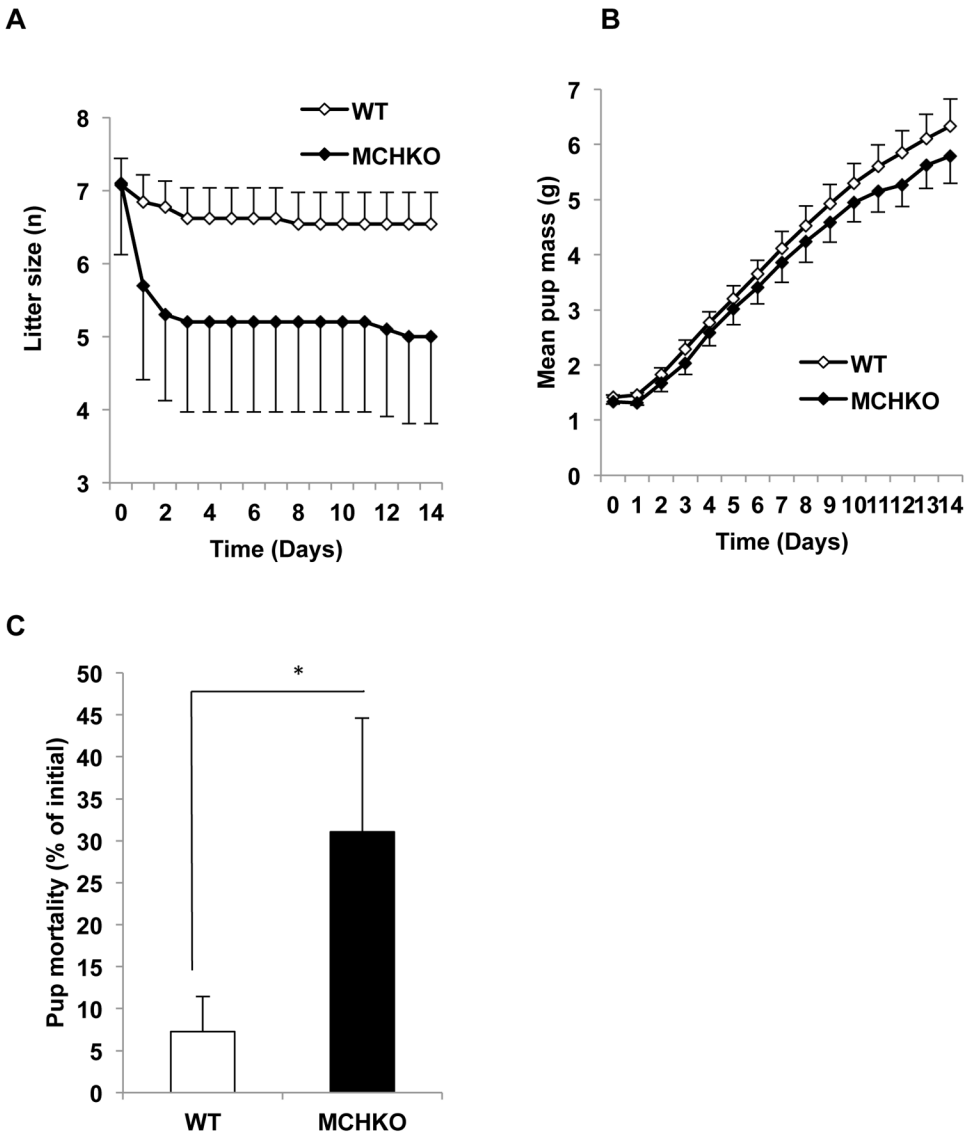

Figure 4.

A. Numbers of pups were not different between WT and MCHKO litters immediately following birth on day 0 (7.1±0.4 vs. 7.1±1, NS). However, at the end of the study period on day 14 WT litters trended to be larger than MCHKO litters (6.5±0.4 vs. 5±1.2, NS).

B. Mean weight of pups was found to diverge throughout the measurement period with WT mice being trending to be heavier on day 14 when compared to MCHKO pups (6.3g±0.49g vs.5.8g±0.5g, NS).

C. Concordant with the reduced litter size at the end of the monitoring period mortality was significantly higher in the MCHKO litters when compared to WT (7.3%±4.2% vs. 31.1% ±13.6%, p=<0.05).