REPRODUCTION IS IMPAIRED IN FEMALE MICE WITH A MUTATED BIOLOGICAL CLOCK

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ABSTRACT
Circadian rhythms play a major role in the effective functioning of animals and the proper timing of reproductive functions is one of the strategies that ensure optimal chances for survival. The core mechanism of the endogenous clock is based on two interlocking transcriptional/ translational feedback loops involving different sets of clock genes, including *Per1* and *Per2*. In this study we used *Per1*- and *Per2*-deficient female mice to determine the effect of the endogenous clock on reproductive function(s). Although the young *Per*-mutant mice appear to be quite normal in their fecundity, we find that puberty is advanced and that middle-aged females show an irregularity and acyclicity of the estrous cycle leading to a lower reproductive success in comparison to the control group.
KEY WORDS: circadian rhythm, *Per*-genes, puberty, estrous cycle, fertility,

INTRODUCTION
The mammalian endogenous circadian clock is located in the suprachiasmatic nuclei of the hypothalamus (Moore and Eichler 1972, Stephan and Zucker 1972). Such an endogenous clock allows animals to anticipate environmental conditions, in order to be able to perform behavioral patterns at advantageous times during day or night and throughout the year. There are many environmental cues such as light, ambient temperature, rainfall and availability of food that animals can use to synchronize to daily and seasonal changes. While these environmental variables can be notoriously unpredictable and thus unreliable, changes in day length throughout the year provide a robust environmental signal with, literally, astronomical precision and thus high predictive value. The synchronization of breeding cycles with the appropriate season or time of day usually depends on the measurement of day length by the pineal gland (Reiter 1993). Thus, seasonally breeding animals such as hamsters and ground squirrels show reaction to changes of the photoperiod by switching from a period of reproductive activity (long day during summer) to periods of reproductive quiescence (short day during winter) (Steinlechner and Niklowitz 1992, Gorman and Zucker 1995). Many data from different rodent species demonstrate that oestrus-related events such as timing of pro-oestrus, surge of LH and FSH release, ovulation, increase in progesterone secretion, and onset of sexual receptivity are precisely timed by the internal circadian system and occur at specific times of day (Rusak and Zucker 1979, Eskes 1984, Turek 1985).

In recent years, a basic description of the molecular clockwork in the suprachiasmatic nuclei (SCN) has been achieved. It consists of a sequence of interlocking positive and negative feedback loops of gene transcription and translation. Today at least 15 genes are thought to be involved in generating this molecular pacemaker, among which *Clock*, *Bmal1*, *Per1/Per2* and *Cry1/Cry2* form the core of the clockwork. It has recently been shown that mutations in the *Clock* gene disrupt estrous cyclicity and interfere with successful pregnancy (Miller et al 2004, Kennaway et al. 2005). The aim of the present study was to determine whether deletions in the *Per* genes, and hence, a disruption of the endogenous clock have an effect on reproductive functions of female mice.

MATERIAL AND METHODS

Animals: We used homozygous B6.129S7-*Per1*^{tm1Brd} (*Per1*$^{(-/-)}$) (Zheng *et al.* 2001), B6.129S7-*Per2*^{tm1Brd} (*Per2*$^{(-/-)}$) (Zheng *et al.* 1999), and wild-type females B6X129.S7 as a control group for our experiments. They were housed individually in polycarbonate cages type III containing wood shavings as bedding material. They were maintained at 22 ± 1°C under a light-dark cycle of 12h light : 12h dark (LD 12:12). In the 1st experiment reported here, 10 females of each genotype were followed closely from the day of weaning (day 21) until reaching full sexual maturity, i.e. until vaginas had opened and a regular oestrus cycle occurred. In the second experiment 10 young virgin females (2 months of age) and 10 middle-aged virgin females (7-9 months of age) of each genotype (*Per1*$^{(-/-)}$, *Per 2*$^{(-/-)}$ and wild-type) were used. Ten males of each strain were kept in the same room. For copulation each female in pro-oestrus was placed in the cage of a male for one day and then put back in her own cage. During the non-reproductive and reproductive phases all females were weighed daily. Towards the end of the gestation period the cages of pregnant females were checked daily for the presence of offspring. The day on which young were found was recorded as the date of birth (day 1). To determine the reproductive success of the three strains litter sizes were recorded from the first and second parturition immediately post partum and on the day of weaning.

Assessment of onset of puberty: Puberty onset was determined by daily examination for vaginal opening. To avoid exposure to the odor of males, and thus impact of males on the onset of puberty, all females in LD and LL were maintained in an experimental room without any male. The daily examination began on postnatal day 21 and continued until an opening of the vagina was observed. From this day on we started taking daily vaginal smears for eight successive weeks to examine the onset of regular oestrus cycles of 4-5 days.

Examination of oestrus cycle: To distinguish the different phases and length of the oestrus cycle as well as to identify the oestrus status for successful copulation with a male Vaginal smears were taken daily for 6 months in the mice's activity phase, i.e. 1 – 2 h after lights off. The smears were obtained by inserting a cauterized metal loop into the vagina not further than 1mm so as to minimize the possibility of inducing pseudopregnancy. The vaginal smear was transferred to a drop of saline solution on a microscopic slide and fixed in MeOH for 2 min before staining with methylene blue solution.. After 1.5 months each female in pro-oestrus was placed for 24 hours in a cage with a male and was then checked for a vaginal plug to verify copulation.

The smears were classified into different oestrus stages according to the description of Nelson *et al.* (1982). Prolonged di-oestrus and permanent oestrus for at least 15 days were considered as anoestrus. An oestrus cycle of > 6 days was considered as prolonged and one of < 3 days as irregular.

Embryonic implantations in uterus: At the end of the experiment all remaining females of 10-13 months of age (wild-type females N = 7, *Per1*$^{(-/-)}$ N = 6 and *Per2*$^{(-/-)}$ N = 10) were sacrificed by CO_2 and their uteri were removed. The uteri were stained using 10% ammonium sulphide solution in accordance with the description of Kopf *et al.* (1964) for counting the implantation scars.

RESULTS AND DISCUSSION

Onset of puberty and the first regular oestrus cycle: Under LD conditions *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females display significantly advanced vaginal opening (U-test *Per1*$^{(-/-)}$: $p < 0.001$; *Per2*$^{(-/-)}$: $p < 0.001$) as well as an earlier appearance of the first regular oestrus cycle (U-test: *Per1*$^{(-/-)}$: $p = 0.002$; *Per2*$^{(-/-)}$: $p < 0.001$) compared to the wild-type females (Table 1).

In the absence of a mature male we observed under LD conditions an advanced onset of puberty in *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females compared to wild-type females. This is characterized

by accelerated vaginal opening and accelerated appearance of the first regular oestrus cycle. Normally female laboratory mice housed with an adult male display the first ovulation at about 37 days of age whereas females in the absence of a mature male are sexually mature at about 57 days of age (Vandenbergh 1967). Even though in the present study the wild-type females with the genetic background B6.129S7 were housed in the absence of a male they displayed much earlier sexual maturity at an age of 36.5 ± 1.39 SEM days than the mice investigated by Vandenbergh (1967). Recent studies on different mouse strains have shown that genetic factors modulate the timing of puberty by 50 - 80% (Eaves *et al*. 2004). Thus, this difference in the timing of puberty onset in female mice can be a consequence of the genetic differences among the various inbred strains.

Table 1

Onset of puberty and sexual maturation in Per-mutant and wild-type mice

Genotype	***Per1*$^{(-/-)}$**	***Per2*$^{(-/-)}$**	**Wild-type**
Vagina opened on day	26.0 ± 1.05	28.9 ± 0.66	36.5 ± 1.39
1st regular oestrus cycle on day	34.5 ± 2.45	34.5 ± 1.13	46.8 ± 1.70

Reproductive success: All primiparous and multiparous females in both age classes and of all three strains were successful in becoming pregnant. Young adult *Per1*- and *Per2*-mutants produced significantly larger litter sizes - on average 7.9 and 7, respectively, than the middle-aged *Per* mutants (U-test: *Per1*$^{(-/-)}$: Z= 2.26 p > 0.05; *Per2*$^{(-/-)}$: Z = 2.83, p > 0.05). Furthermore, the litter size of young adult *Per*-mutants did not differ from the litter size of the middle-aged wild-type (Kruskal-Wallis-ANOVA: Ch^2 = 3.76, FG = 2, p = 0.15). Almost every pregnant middle-aged wild-type female gave birth and was a successful breeder in contrast to *Per1*- and *Per2*-mutant females. Primiparous wild-type and *Per1*-mutant females produced the same litter size as multiparous females, i.e. they did not differ in their reproductive outcome. However, only 33.3% of multiparous *Per1*-mutant females bred successfully, whereas 90% of the wild-type multiparous females bred. In contrast, *Per2*-mutant females as primiparous females did not raise a single pup successfully. The pups were obviously eaten by their mothers. As multiparous females they produced significantly smaller litter sizes than middle-aged wild-type females did (U-test: Z = 2.26, p > 0.05).

Oestrus cycle: All wild-type females exhibited regular oestrus cycles (100% over 6 weeks). A four day oestrus cycle in wild-type females occurred significantly more often than in *Per*-mutant females (Kruskal-Wallis-ANOVA: Ch^2 = 10.05, FG = 2, p < 0.001) (Fig. 1). However, *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females were acyclic in 37.07% and 44.85% of this time, respectively. The remaining time was characterized by cyclicity of four, five and longer than 6 days, while *Per1*-mutants exhibited more often 4 day cycles than prolonged cyclicity (ANOVA: $F_{2, 27}$ = 4.01, p < 0.05, Post hoc-test. p = 0.04).

Implantations in uteri and live offspring: Per1$^{(-/-)}$ and *Per2*$^{(-/-)}$ females had a significantly higher number of embryonic scars in the uterus compared to the total number of their live offspring from the 1st and 2nd parturition (Wilcoxon-test: *Per2*$^{(-/-)}$: Z = 2.93, p < 0.05, N = 10; *Per1*$^{(-/-)}$: Z = 2.02, p < 0.05, N = 6). Wild-type females, however, did not differ significantly between the number of implantations and the number of live offspring (Wilcoxon-test: Z = 1.82, p > 0.05, N = 7). Moreover, *Per1*$^{(-/-)}$ females showed a significantly lower number of implantations than the control group (U-test: Z = 2.45, p < 0.05). Hence, primiparous as well as multiparous middle-aged mutant female mice *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ are characterised by a low reproductive success in comparison with the middle-aged wild-type females. These results indicate an accelerated reproductive ageing of the *Per*- mutants versus the wild-type strain. This conjecture is supported by the lower incidence of regular oestrus cycles in *Per*

mutants compared to wild-type females. *Per* mutants were acyclic for 41% of the observation period (1.5 months), while the wild-type females of the same age showed no acyclicity at all and the normal oestrus cycle of four days occurred significantly more often in the control group. Acyclic phenomena, e.g. persistent vaginal cornification or leukocytosis, characterize the age-associated decline of cyclicity in rats and mice (Nelson *et al.* 1982).

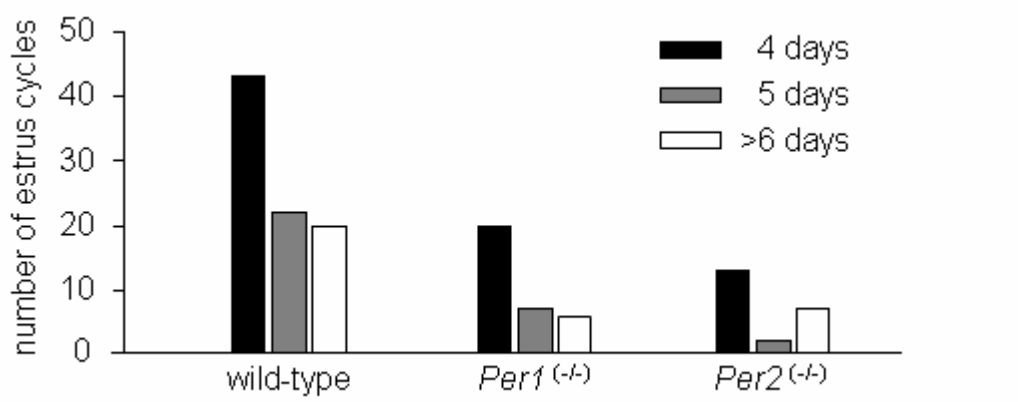

Figure 1: **Total number of oestrus cycles over 1.5 months in all non-reproducing females of each strain, N = 10 each.**

This decline in fecundity is usually accompanied by a decrease in the number of live pups. Thus, the lower reproductive success of middle-aged *Per* mutants may be due, at least in part, to changes in the length of the oestrus cycle and in its frequency. In addition, even mutant females who have a regular oestrus cycle also often failed to reproduce successfully. Since only 33% of middle-aged pregnant *Per1* and 50% of *Per2* mutants were successful in breeding they seem to be characterized by less fecundity compared to the control group where almost all pregnant females were successful breeders. Comparisons of implantation scars with the successfully bred offspring in the present study confirm that the decreased fecundity in middle-aged *Per* mutants is related to reproductive failures during gestation. Studies on rats have demonstrated that an induced or spontaneous delay of ovulation results in increases in abnormal development and subsequent death of embryos (Fossum *et al.* 1989). Thus, the uterus of aged animals appears to be responsible for post-implantation loss in those females with implantation sites (Parkening *et al.* 1978). Results from the present study reveal that the number of foetal implantation scars of *Per2* mutants does not differ from that of the control group. However, the total number of surviving pups in *Per2*- and *Per1*-mutants is significantly lower than the number of implantation scars. Hence, *Per*-mutants seem to suffer from post-implantation loss. The functional alterations causing decreases in both fertility and fecundity in middle-aged *Per1*- and *Per2*-mutant female mice are currently unknown. We can conclude, however, that age-related changes in the cyclicity of 9-12 month old *Per1*- and *Per2*-mutant females are qualitatively similar to those of 13-16 month old C57BL/6J female mice that were investigated by Nelson and co-workers (1982).

CONCLUSIONS

1. Successful reproduction requires precise temporal coordination among various endocrine and behavioral events.
2. We show here that a disruption of a core element of the biological clock, namely the *Per1* and *Per 2* genes leads to an impairment of reproductive functions in female mice.
3. Puberty is advanced in *Per1*- and *Per2*-mutant female mice.
4. Reproductive success is lower in middle-aged *Per1*- and *Per2*-mutant female mice.

5. Number of implantation scars and number of live offspring was significantly lower in *Per1*-mutant female mice.
6. In *Per2*-mutant female mice only the number of successfully reared pups was greatly reduced indicating that the problems arose after implantation of the embryo.

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