Characterisation of *Per* mutant mice

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In memory of Claus Ramm

And God said, Let there be light: and there was light.
And God saw the light, that [it was] good: and God divided the light from the darkness.
And God called the light Day, and the darkness he called Night.
And the evening and the morning were the first day.
Gen 1:3-5

Abstract

The daily light-dark cycle of the environment is caused by the rotation of the earth. All kinds of organisms have evolved biological clocks in order to anticipate the rhythmic changes in their environment. The endogenous nature of these clocks has long been known, and because the period length of one complete cycle under constant conditions is about one day these rhythms are called circadian (*lat. circa* = about, *dies* = day). In 1971, the first step toward the discovery of the underlying molecular mechanisms which make the clock tick was made. The *Period* gene was discovered in the fruit fly (*Drosophila melanogaster*). Twenty years later, the mammalian homologues were discovered, and their role in the molecular machinery of the circadian system could be evaluated. In the present work, the impact of two genes of the *Period* family, *Per1* and *Per2*, were investigated by phenotyping mice with a targeted mutation in these genes, i.e. the $Per1^{Brd}$ and $Per2^{Brd}$ mice.

It could be shown that not only the circadian activity pattern is altered in these animals. but that the lack of the *Per1* also had severe consequences for several parameters, such as a lowered body weight, a lowered body temperature, and an altered emotionality and stress reaction. These changes in physiological and behavioural parameters were best explained by a disruption of the daily corticosterone rhythm with elevated daytime levels. In contrast, the lack of *Per2* seemed to have opposite effects. The $Per2^{-/-}$ animals tended to be heavier. In addition, they showed a higher pain threshold in the hot plate task. Furthermore, neither types of transgenic mice could entrain to a non-photic zeitgeber, i.e. temperature, while wildtype controls were able to do so.

Keywords: circadian, *Period*, mutant phenotyping

Zusammenfassung

Der tägliche Licht-Dunkel Wechsel, der durch die Rotation der Erde um sich selbst verursacht wird, hat dazu geführt, dass nahezu alle Lebewesen eine innere Uhr ausgebildet haben. Diese hilft ihnen, die zyklischen Änderungen in ihrer Umwelt zu antizipieren. Schon lange ist die endogene Natur dieser Uhren bekannt. Gibt es keine synchronisierenden Umweltfakoren, sogenannte Zeitgeber, zeigen sie einen Rhythmus mit einer Periodenlänge von etwa, aber eben nicht genau, 24 Stunden. Deswegen werden sie als circadian (*lat. circa* = ungefähr, *dian* = Tag) bezeichnet. Den zugrunde liegenden molekluren Mechanismen kam man 1971 das erste mal auf die Spur. Bei der Fruchtfliege (*Drosophila melanogaster*) wurde das *Period* Gen entdeckt. Die homologen Gene der Säuger konnten allerdings erst 20 Jahre später gefunden und ihre Rolle im molekularen Räderwerk der Uhr untersucht werden. In der vorliegenden Arbeit wird der Einfluss zweier Gene aus der *Period* Familie, *Per1* und *Per2*, auf viele verschiedene physiologische und Verhaltensparameter untersucht. Dazu werden Mäuse, in denen diese Gene gezielt ausgeschaltet wurden ($Per1^{Brd}$ and $Per2^{Brd}$), benutzt.

Es konnte gezeigt werden, dass nicht nur die Aktivitätsmuster dieser Mutanten sich von Kontrolltieren unterschieden, sondern dass ein Fehlen von *Per1* auch schwerwiegende Veränderungen in der Phyiologie der Tiere zur Folge hat. Beispielsweise sind die *Per1* defizienten Tiere leichter, haben eine tiefere Körpertemperatur. Ausserdem ist ihre Reaktion auf Stress verändert. Ihre Ursache scheinen diese Veränderungen in den vor allem tagsüber erhöhten Kortikosteron-Werten zu haben. Die *Per2* defizienten Tiere hingegen waren etwas schwerer als die Kontrollen. Ausserdem zeigten diese Tiere im Hot Plate-Test eine höhere Schmerzschwelle. In einem weiteren Versuch konnte gezeigt werden, dass keine der beiden *Per* defizienten Mäuse ihren Aktivitätsrhythmus an einen nicht-photischen Zeitgeber, in diesem Fall Temperatur, synchronisieren konnte.

Schlagworte: Circadian, *Period*, Phenotypisierung

Contents

1. Introduction

One of the most widespread properties of all organisms is a daily rhythm caused by the earth's rotation on its axis, determining day and night (Devlin and Kay, 2001; Edery, 2000). Obviously, sunlight is the most important source of energy for all kinds of species: both directly, in photosynthethic organisms from bacteria and prokaryotes to plants, and indirectly, in all kinds of animals via the food chain (Bloch *et al.*, 2001; Cahill, 2002; Hasegawa *et al.*, 1997; Lin *et al.*, 1999; Liu *et al.*, 1998; Pando and Sassone-Corsi, 2001; Roenneberg and Merrow, 2002b; Schultz and Kay, 2003). Hence, evolution should favour those individuals which can anticipate dusk and dawn in order to maximise e.g. their feeding time and/or minimise the risk of predation (Roenneberg and Merrow, 2002a, 2002b). Due to these circumstances, daily or so-called circadian (= about a day) rhythms are the most prominent among the rhythm of life. There is, however, a wide range of other rhythms with periods ranging from a few seconds for firing intervals of neurons involved in the breathing rhythm (Koshiya and Smith, 1999) to the circatidal (Palmer, 1995) and circalunar (Neumann, 1989), and the circannual rhythms e.g. of bird migration (Gwinner, 1996). Even rhythms with a period of several years can be observed, e.g. in the population dynamics of hare and lynx in Alaska (*cf.* Begon *et al.*, 1991; MacLulick, 1937). Among these rhythms, the daily ones are ubiquitous, and it has therefore become convention to name the period length compared to the length of a day. If the period length is under 24 hours the rhythm is called ultradian, if it is above 24 hours then it is called infradian.

1.1 The circadian system

In general, a biological clock should fulfil two criteria. First, a clock must convert a non-periodic source of energy into a self-sustaining periodic output, and second, the periodicity generated by the clock must time some biological events (Moore-Ede *et al.*, 1982, p. 21). In addition, the parts of the clock should not be able to be regarded as a clock

themselves. In order to be assigned as circadian, a biological rhythm generated by such a clock should meet the following four criteria:

(1) As expressed by the term *circadian*, the period length should be approximately 24 hours.

(2) The rhythm should persist under constant conditions, i.e. be free-running.

(3) It should synchronise to or in, other words, be entrainable to environmental cues, so-called zeitgebers (Aschoff, 1951). As stated above the most prominent of these zeitgebers is the light-dark cycle (Pittendrigh and Daan, 1976a). There are, however, more possible environmental and even social cues that animals are able to entrain to. It has been shown that organisms can entrain to temperature (Hoffmann, 1968; Laemle and Ottenweller, 1999; Liu *et al.*, 1998; Rajaratnam and Redman, 1998), food availability (Challet *et al.*, 1998; Mistlberger, 1994; Weber and Spieler, 1987), social cues (Levine *et al.*, 2002), sound (Menaker and Eskin, 1966), and atmospheric pressure (Hayden and Lindberg, 1969). Even a magnetic field can alter the heart rate nadir in humans (Griefahn *et al.*, 2002).

(4) Finally, the rhythm should be temperature compensated with a Q_{10} of about 1 (Anderson *et al.*, 1985; Ruoff *et al.*, 2000; Zimmerman *et al.*, 1968; but see: Gibbs, 1981).

Historical highlights

The existence of circadian rhythms is a long-known fact that was first described by Androsthenes in the 4th century B.C. He reported the cyclic leaf movements of *Tamarindus indicus* (Bretzl, 1903 *cf.* Moore-Ede *et al.*, 1982). Thereafter, it took nearly 2000 years until, in 1729, de Mairan showed, by putting plants in a closed cupboard, that the daily leaf movements of *Mimosa pudica* are controlled endogenously (Marchant, 1729 *cf.* Moore-Ede *et al.*, 1982). Another 30 years later it was shown that this endogenous rhythm is temperature independent (du Monceau, 1759 Zinn, 1759 both *cf.* Moore-Ede *et al.*, 1982). In 1832, de

Candolle showed the first free-run of the leaf movements of *Mimosa pudica*, which has a periodicity of 22 to 23 hours in constant darkness.

One of the first important studies with animals was carried out on bees by Beling (1929), who showed that bees have a so-called *Zeitgedächtnis* (memory for time), and can be trained to visit an artificial food source every 24 hours but not every 19 or 48 hours. In mammals, Richter (1922) had provided extensive insights into the clock functions of rats. He showed that the circadian rhythm can synchronise not only to light-dark cycles but to the time of feeding, as well. Subsequently it was shown in plants (in commercial lines of the bean *Phaseolus coccineus*) that many of the clock properties are genetically determined, including the ability to measure day length as a tool to detect seasonal changes and the length of the free-run (Bünning, 1935).

1.2 Circadian system of mammals

Anatomical structure

Although the phenomena caused by the biological clock had been well studied, the underlying anatomical correlates of the mammalian clock remained unknown until the second half of the last century. Lesioning studies pointed to the anterior-ventral hypothalamus as the location of the biological clock (Nauta, 1946 *cf.* Moore-Ede *et al.*, 1982). Then, in 1972, two independent groups showed by lesioning that area of the brain that the master clock is localised in the small, paired suprachiasmatic nuclei (SCN) of the anterior-ventral hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972).

Even stronger evidence was presented by SCN transplantation experiments (Ralph *et al.*, 1990). In work similar to the transplantation studies on the avian pineal gland (Zimmerman and Menaker, 1979), Ralph *et al.* (1990) showed that the circadian rhythm of an SCN-lesioned arrhythmic hamster can be restored by a transplanted SCN, establishing the circadian properties (i.e. period length and phase) of the donor animal.

In addition, an isolated SCN can generate a circadian rhythm *in vivo* (Inouye and Kawamura, 1979) and *in vitro* (Shibata and Moore, 1993; Yamaguchi *et al.*, 2003), and even single SCN neurons can produce such a rhythmicity (Welsh *et al.*, 1995). This means that every single one of the circa 11,000 to 12,000 neurons in the SCN of a rat (Guldner, 1983) generates an endogenous circadian firing pattern. How all these individuals neurons are synchronised among each other remains largely unknown, but first evidence points in the direction of neuropeptides (Colwell *et al.*, 2003; Cutler *et al.*, 2003; Yamaguchi *et al.*, 2003).

The intrinsic neurochemical composition of the SCN is quite diverse (Figure 1). Most of the retinal projections (see below) insert in the ventrolateral part (vlSCN) (van Esseveldt *et al.*, 2000). This so-called core region is characterised by vasoactive intestinal polypeptide (VIP), gastrin-releasing hormone (GRH) and the histidin isoleucin peptide (PIH), as well as calbindin (LeSauter *et al.*, 2002; Moore *et al.*, 2002). The core region can be distinguished from the so-called shell region, which is characterised by arginine vasopressin (AVP) and somatostatin (SS). In addition, γ-amino-butyric acid (GABA)-positive cells are widely distributed throughout the whole SCN (Piggins and Rusak, 1999; Romijn *et al.*, 1997). It was reported that GABA might be able to synchronise the firing pattern of the SCN neurons (Strecker *et al.*, 1997).

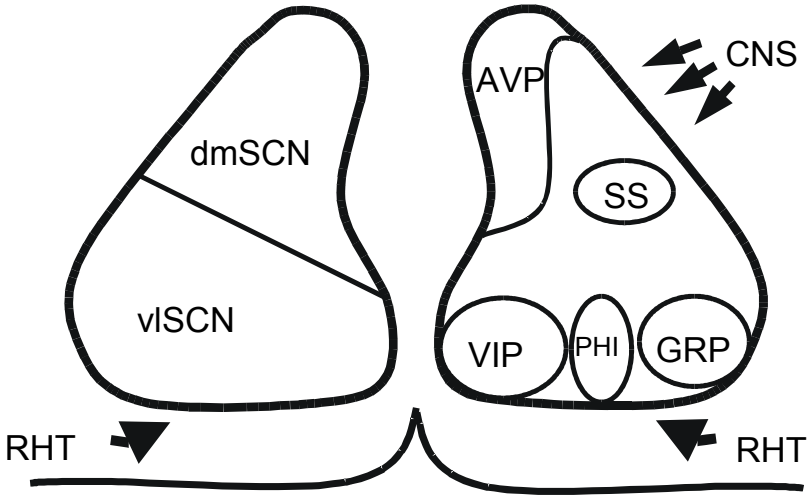

Figure 1. Simplyfied scheme of the distribution of neurotransmitters within the SCN. Abbrevations: VIP, vasactive polypeptide; GRP, gastrin-releasing peptide; PHI, peptide histidin isoleucin, AVP, arginin vasopressin peptide; SS, somatostatin; dmSCN, dorsomedial SCN; vlSCN, ventrolateral SCN (modified after Romijn *et al.*, 1997).

Clock input

As stated in the first chapter, one of the major time cues for the clock is the daily light-dark cycle of the environment. In mammals, light is perceived by the retina of the eyes and conducted to the SCN by the retino-hypothalamic tract (RHT, Moore and Lenn, 1972). Actually, only a small subset of retinal ganglion cells contributes to the RHT (Provencio *et al.*, 1998). The novel photopigment of these cells (Bellingham and Foster, 2002; Foster and Bellingham, 2002; Hattar *et al.*, 2002; Hattar *et al.*, 2003; Semo *et al.*, 2003) is exclusively found in pituitary adenylyl cyclase activating peptide (PACAP)-positive cells (Hannibal *et al.*, 2002). In contrast, the principle neurotransmitter of this pathway was once thought to be glutamate (Ebling, 1996). More recent studies, however, have proved PACAP to be co-localised with glutamate (Hannibal, 2002; Hannibal *et al.*, 1997), and further studies showed that both PACAP and glutamate are responsible for photic effects on the clock (Chen *et al.*, 1999; Hannibal, 2002; Hannibal *et al.*, 2001; Harrington *et al.*, 1999; von Gall *et al.*, 1998).

It is thought that the light input of the mammalian clock is exclusively perceived through the retina. There was a report (Campbell and Murphy, 1998) in which extraoccular

phototransduction via humural signalling, i.e. a light pulse behind the knee or at the back, was said to shift the body temperature and melatonin rhythm of human subjects, but this spectacular claim (Science News and Editorial Staffs, 1998) was later disproved (Wright and Czeisler, 2002).

In addition to this direct input to the SCN, there are two other main pathways for light. One is the pathway *via* the intergeniculate leaflet (IGL) of the lateral geniculate nucleus (LGN), i.e. the geniculohypothalamic tract (GHT, Morin and Blanchard, 2001). The predominant neurotransmitter of this pathway is neuropeptide Y (NPY, Mrosovsky, 1996a; Swanson and Cowan, 1975), and the other is the serotonergic (5-hydroxytryptamine, 5-HT) input via the Raphé nuclei (Meyer-Bernstein and Morin, 1996). Whereas the RHT obviously transmits the photic input to the SCN, the two latter pathways are responsible for the non-photic influences on the clock (Antle *et al.*, 1998; Lewandowski and Usarek, 2002; Mrosovsky, 1995, 1996a), as well as for multisynaptic photic influences on the clock (Piggins and Rusak, 1999).

All of these pathways lead to the ventrolateral part of the SCN (van Esseveldt *et al.*, 2000). In contrast, the dorsomedial part of the SCN, the shell, is the target of only a few afferent projections from the retina, but there are a number of hypothalamic projections including the preoptic, arcuate, ventromedial and dorsomedial nuclei (Card, 1999). The last seem to play a role in the output, i.e. in the production of the corticosterone rhythm, as well (Buijs *et al.*, 1999). Additional input stems from the lateral septum. These contribute mainly to the non-photic properties of the central pacemaker (Figure 2).

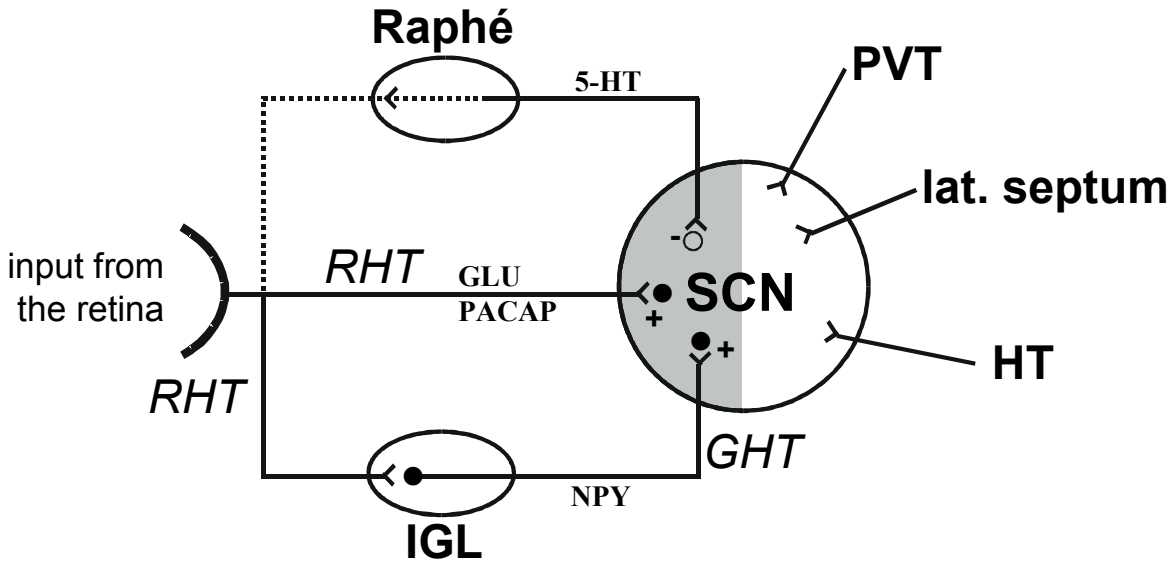

Figure 2. Input of the SCN. This scheme illustrates the main input pathways to the SCN. The excitatory photic information is transmitted via the retinohypothalamic tract (RHT) and the geniculohypothalamic tract (GHT) to the SCN, while serotonergic efferents from the Raphé nuclei negatively modulate photic signaling. The area shaded grey indicates the core SCN, and the shell is depicted as blank area with the non-photic input from other regions of the hypothalamus (HT), the lateral septum and the paraventricular thalamic nucleus (PVT). Abbreviations: + excitatory; - inhibitory; • light-responsive cells; o cells that may not be light-responsive (modified after: Card, 1999; Meijer, 1991).

Clock output

The ultimate reason for the evolution of a biological clock seems to be the benefit which derives from its output (Green, 1998). Although the anatomical projections of the SCN are well studied (Figure 3), our functional understanding of the output pathways is at best fragmentary (Piggins and Rusak, 1999). Furthermore, it has been shown by transplantation studies that behavioural rhythmicity in an SCN-lesioned animal can be restored even if the graft of the donor is encapsulated in a polymer coating which prevents any innervation, which indicates that a diffusable substance can convey the circadian signal (Silver *et al.*, 1996). However, endocrine rhythms, such as the pineal melatonin rhythm, are not restored in such experiments (Piggins and Rusak, 1999).

There are four main pathways which are used for initiation of hormonal secretion:

(1) Direct contact to neuroendocrine neurons containing gonadotropin-releasing hormone (GnRH) or corticotropin-releasing hormone (CRH), thereby regulating the blood levels of luteinizing hormone and the secretion of adrenocorticotropic hormone (ACTH) from the pituitary (Kalsbeek *et al.*, 1996), respectively.

(2) Indirect contact to neuroendocrine neurons *via* intermediate neurons such as the medial preoptic nucleus (MPN), the dorsomedial hypothalamic nucleus (DMH), which also contributes to the regulation of the corticosterone rhythm, or the sub-paraventricular nucleus (sPVN).

(3) Projections to the autonomic paraventricular nucleus (aPVN) to influence the autonomic nervous system, preparing the endocrine organs, such as the adrenal or the pineal glands, for the arrival of hormones, and thereby contributing to the release rhythm of corticosterone (Buijs *et al.*, 1999) and melatonin, respectively.

(4) Influencing its own feedback. As stated above, little is known about the roles of the SCN projections to the extra-hypothalamic paraventricular nucleus of the thalamus (PVT), sub- paraventricular nucleus of the thalamus (sPVT) or lateral geniculate nucleus (LGN) (Buijs and Kalsbeek, 2001). There is, however, evidence for a multisynaptic pathway to all the major sympathetic outflow systems (Ueyama *et al.*, 1999).

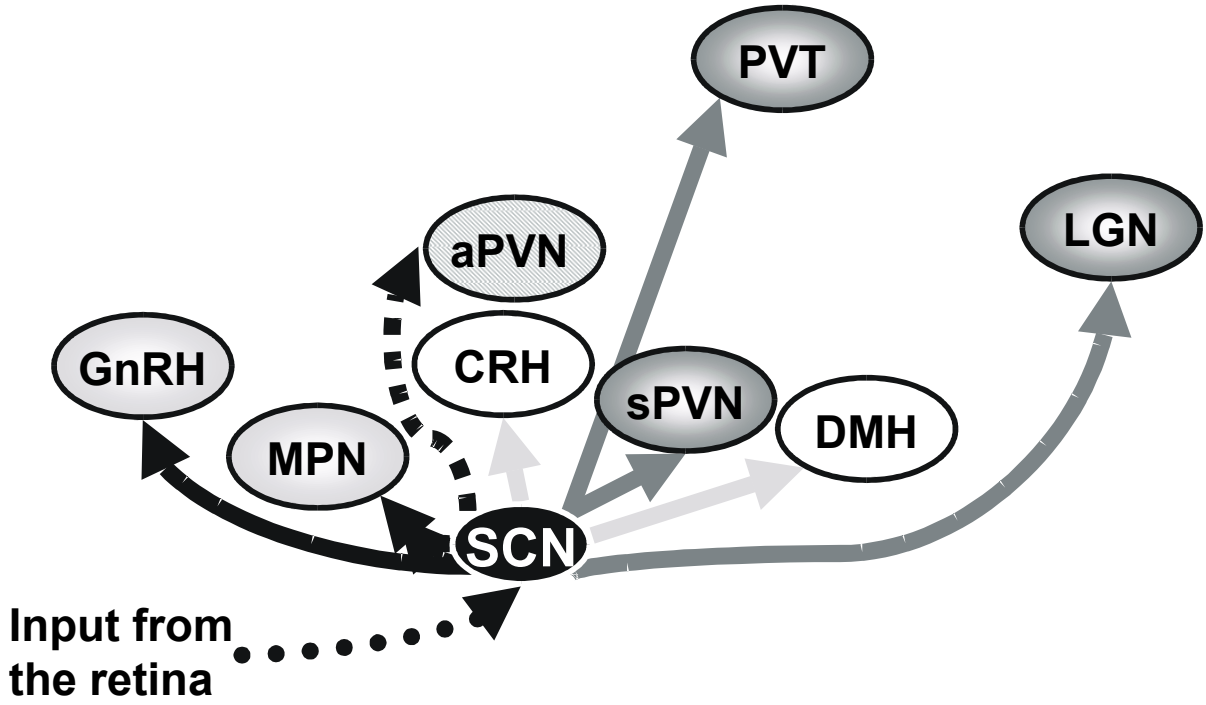

Figure 3. Scheme of the output pathways of the SCN. See text for detailed information. The different arrows indicate control of blood levels of melatonin (dashed), corticosterone (light grey), and luteinising hormone (black) via the respective nuclei. Abbrevations: CRH, corticotropin-releasing hormone; GnRH, gonadotropin-releasing hormone; MPN, medial preoptic nucleus; DMH, dorsomedial hypothalamic nucleus; sPVN, sub-paraventricular nucleus; aPVN, autonomic paraventricular nucleus; PVT, paraventricular nucleus of the thalamus; sPVT, sub-paraventricular nucleus of the thalamus; LGN, lateral geniculate nucleus (modified after: Buijs and Kalsbeek, 2001).

Molecular clockworks

The molecular basics of the circadian system were first studied in fruit flies (*Drosophila melanogaster*). More than 30 years ago Konopka and Benzer (1971) found the first so-called clock gene, which caused – by the way – the first behavioural phenotype. The gene was called *Period*, because both the period of the rest activity cycle and the mating song of the fly are disturbed (Konopka and Benzer, 1971). Those investigators found three different alleles of *Period* in *Drosophila* which were all mapped to the same locus, but it took another 13 years until the molecular tools for cloning and sequencing the gene were available (Bargiello *et al.*, 1984; Reddy *et al.*, 1984; Zehring *et al.*, 1984). Since then, seven other genes have been described that contribute to the fly's clock (Panda *et al.*, 2002). Most of these (*Vrille*: George

and Terracol, 1997; *Shaggy*: Martinek *et al.*, 2001; *Double-time*: Price *et al.*, 1998; *Cycle*: Rutila *et al.*, 1998; *Timeless*: Sehgal *et al.*, 1994; Stanewsky *et al.*, 1998; *dCry*: Todo *et al.*, 1996) were found by forward genetics (going from the behaviour to the gene). as was done with *Period*. Only *dClock* (Allada *et al.*, 1998) was discovered by a BLAST search against the mouse PAS (*Period* / aryl hydrocarbon receptor nuclear translocator / singleminded) sequence. Furthermore, tremendous gains have been made in our understanding of the molecular machinery of biological clocks in other model organisms such as *Neurospora*, *Arabidopsis* and mammals.

The first mammalian clock gene (*Tau*) was found as a naturally occurring mutation in the Syrian hamster (Lowrey *et al.*, 2000; Ralph and Menaker, 1988). Only a few years later, the first induced mutation was discovered in an N-ethyl-N-nitrosourea (ENU) screen. The gene discovered was called *Clock*, which means "circadian locomotor output cycle kaput" (Vitaterna *et al.*, 1994). However, it became obvious that *Clock* was not the whole story. Thanks to the advances in molecular biology, and to the available sequences from known clock genes or common clock gene features, such as the occurrence of a PAS domain or the presence of specific promoter sequences, so-called E-Boxes (CACGTG), it has been possible to discover a number of mammalian clock genes. At present, we know of 13 different clock genes which are involved in the feedback loops of the core clock (Table 1), and recent quantitative trait loci analysis studies suggest that there are even more candidates (Hofstetter *et al.*, 2003; Salathia *et al.*, 2002; Yoshimura *et al.*, 2002). Additionally, there are the so-called non-canonical clock genes or clock-affecting genes (Lakin-Thomas, 2000), which are characterised by altering the behavioural phenotype, although they have no (known) function in the core clock. For example, the deletion of the neuronal cell adhesion molecule (NCAM) leads to a shortened τ and occasionally to loss of behavioural rhythmicity in constant darkness (Shen *et al.*, 1997).

Table 1. Names and functions of the mamalian clock genes. Abbrevations: *Bmal1,* Brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1; *Cry, Cryptochrome*; *Csnk1ε,* caseine kinase 1-epsilon; *Dbp,* D-box binding protein; Dec, Differentiated embryo-chondrocyte expressed gene; *Mop3,* member of PAS superfamily 3; *Per, Period*; *Tim, Timeless*; *RevErbα*, nuclear receptor subfamily 1, group D, member 1.

Name	Function	Reference
Bmal1 (Mop3)	Transcriptional activator	(Bunger *et al.*, 2000; Hogenesch *et al.*, 1998)
Clock	Transcriptional activator	(King *et al.*, 1997; Vitaterna *et al.*, 1994)
Cry1	Negatively regulates CLOCK-BMAL1	(Todo *et al.*, 1996; van der Horst *et al.*, 1999)
Cry2	Negatively regulates CLOCK-BMAL1	(Todo *et al.*, 1996; van der Horst *et al.*, 1999)
Dbp	Link to downstream processes	(Lopez-Molina *et al.*, 1997)
Dec1	Negatively regulates CLOCK-BMAL1	(Honma *et al.*, 2002)
Dec2	Negatively regulates CLOCK-BMAL1	(Honma *et al.*, 2002)
E4BP4	Link to downstream processes *via* CREB	(Newman and Keating, 2003).
Per1 (RIGUI)	Negatively regulates CLOCK-BMAL1	(Sun *et al.*, 1997; Tei *et al.*, 1997)
Per2	Negatively regulates CLOCK-BMAL1 and positively regulates BMAL1	(Albrecht *et al.*, 1997b; Shearman *et al.*, 1997)
Per3	Uncertain	(Bae *et al.*, 2001; Shearman *et al.*, 2000)
RevErbα	Negatively regulates BMAL1	(Preitner *et al.*, 2002)
Tau (Csnk1ε)	Promotes degradation of PER1	(Lowrey *et al.*, 2000; Ralph and Menaker, 1988)
Tim	Possible dimerisation partner of PER2	(Tischkau *et al.*, 1999; Zylka *et al.*, 1998; but see Gotter *et al.*, 2000)

How these genes and their products are interlinked with each other is currently being studied extensively, and our understanding becomes more and more complex with every newly discovered clock gene (Roenneberg and Merrow, 2003). There remain, however, some open questions concerning the details of the interactions between the genes involved, and the different function of these genes in the clockworks of the different taxa (Field *et al.*, 2000).

For example, the function of the *cryptochromes* is different in plants (Dunlap, 1999) and *Drosophila* (Froy *et al.*, 2002; Hall, 2000), where it is used as a photopigment, than in mammals, where it is an essential part of the core clock (but see: Miyamoto and Sancar, 1998; van der Horst *et al.*, 1999; Zordan *et al.*, 2001). The fundamental principle, however, seems to be similar throughout all kinds of species: the (post-) transcription/translation feedback loop (TTL, Dunlap, 1999; Liu *et al.*, 1999). The rhythmic transcription of one or more clock genes produces rhythmic levels of clock RNA(s) that, in turn, produce rhythmic levels of clock protein(s). The clock protein(s) are negative elements that inhibit the transcription of their own genes, possibly by interfering with positive elements required to activate transcription of clock genes. When clock RNA and subsequently protein levels fall, transcription is activated and the cycle repeats. Rhythmic output could be controlled by either the negative or the positive elements acting on output gene expression (Figure 4).

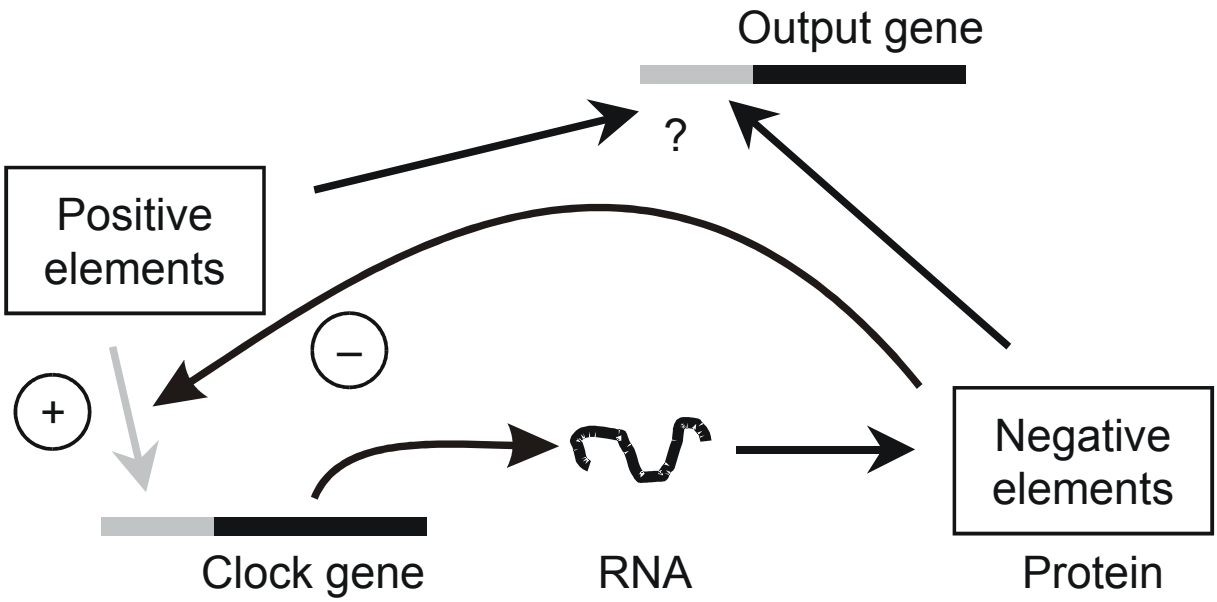

Figure 4. The transcription/translation feedback loop is characterised by negative elements which feed back on their own transcription *via* regulating positive elements and therefore producing an oscillation (from Lakin-Thomas, 2000).

Let us now focus to the mammalian genes of the *Period* family Three genes of this family have thus far been described: *Per1* (Albrecht *et al.*, 1997b; Shearman *et al.*, 1997), *Per2*$^{-}$ (Zheng *et al.*, 1999), and *Per3* (Shearman *et al.*, 2000).

The third gene of the family (*Per3*) seems to be less important for the proper function of the clock. A study on a functionally null allele of a *Per3*-deficient mouse revealed only subtle effects. The *Per3* $^{-/-}$ mice could entrain to a zeitgeber and remained rhythmic under constant darkness, but tended to have a shorter free-running period (Shearman *et al.*, 2000).

The two-oscillator model

In 1976, Pittendrigh and Daan proposed the model of the morning (M) and evening (E) oscillator (Pittendrigh and Daan, 1976b). Without knowing the underlying molecular mechanisms, they predicted a model of two coupled oscillators or principal groups of oscillators, one attached to dawn and the other to dusk. One of the major advantages of this model is that a clock built of these components should be able to entrain to various day lengths during the seasonal cycle of the year.

More than 30 years later Daan *et al*. (2001) revised the original model and proposed molecular correlates of the two oscillators. The *Period* genes were proposed as part of the negative limb of the molecular feedback loop. More precisely, the *Per1* gene is identified as part of the morning oscillator, whereas the *Per2* gene contributes to the evening oscillator. This is, however, an oversimplified version of the model. At least the *cryptochrome* genes (*Cry1* and *Cry2*) must be included. *Cry1* is believed to form heterodimers with *Per1*, while *Cry2* forms these dimers with *Per2*. Thus, *Cry1* contributes to M and *Cry2* to E.

From this model, several predictions can be derived and tested – thanks to the available transgenic mouse models (Figure 5). Daan *et al.* formulated the following four predictions which compare the known behaviour of wildtype mice with that of those lacking either E or M: (1) In M-deficient animals, the phase response curve (PRC) should show decreased phase advances during the early subjective night, and increased phase delays in the late subjective night, and *vice versa* in mice lacking E. As yet there are no studies available which have investigated all parts of a PRC. However, single phase-shift experiments have been carried

out, and the results of phase-shifts at ZT14 and ZT22 are in accordance with these prediction (Zheng *et al.*, 2001). (2) In mice lacking M, the free-running period in constant light should be shorter, the brighter the light intensity (Aschoff's rule) In contrast, mice lacking E should behave in contradiction to Aschoff's rule and exhibit a shorter τ in higher light intensities. Even in high light intensities, both mutant animals should stay rhythmic. This prediction was first confirmed by Steinlechner *et al.* (2002). For this purpose, they used *Per1* and *Per2*-deficient mice as models for the lack of M or E, respectively. (3) There should be no after effects on τ of the T-cycle length in mice deficient for either M or E. Dernbach (2003) looked for after effects of non-24-hour T-cycles. He used *Per1* and *Per2*-deficient mice as models, but his results did not support the prediction. (4) There should not be two daily peaks of multi-unit activity in electrophysiological recordings from horizontal SCN slices in either of the deficient mice. This prediction has thus far remained untested.

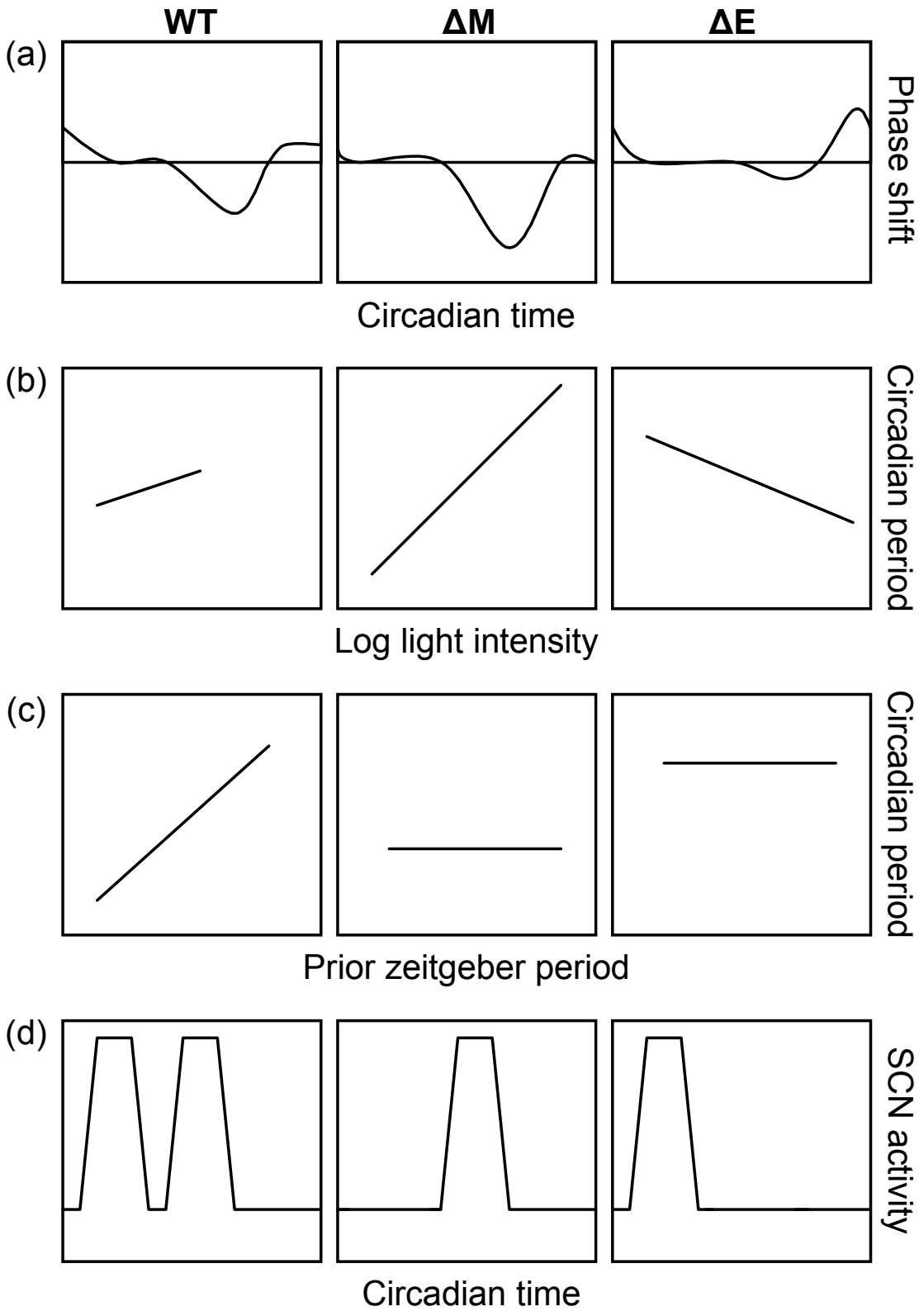

Figure 5. Summary of predictions from the two oscillator model. (a) Phase response curve for brief light pulses in DD. (b) Change of free-running period (τ) with increasing light intensities. (c) After effects of prior zeitgeber period on τ. (d) Expression of multi-unit activity of the SCN (from Daan *et al.*, 2001).

Even more putative oscillators

In recent publications, it was hypothesised that the redox state of the cell might contribute to an additional oscillator in the forebrain (Reick *et al.*, 2001; Rutter *et al.*, 2001). It seems that NPAS2/BMAL do form dimers in the forebrain, but not CLOCK/BMAL. For example, Pitts and colleagues (Pitts *et al.*, 2003) provide first experimental evidence for a food entrainable oscillator (FEO) which is not dependent on a functional CLOCK protein.

Another oscillator is found in neurons of the mitral cell layer of the olfactory bulb (Granados-Fuentes *et al.*, 2004a; Granados-Fuentes *et al.*, 2004b). The neurons of this tissue have all properties of those in the SCN. They are coupled intrinsic oscillators which are temperature compensated and able to entrain to a zeitgeber. Olfactory bulbectomy, however, does not affect the wheel-running pattern of rats. Therefore, it seems that this oscillator is "involved in rhythms outside the canonical circadian system" (Granados-Fuentes *et al.*, 2004b). In contrast, however, it has been reported that olfactory bulbectomy changes activity and body temperature pattern of a nocturnal lemur (Perret *et al.*, 2003), indicating that the circadian system is indeed involved.

And even more clocks and functions

As stated in the beginning of the introduction, there are rhythms with a period shorter or longer than 24 hours. Those are also represented on the molecular level by transcriptional translational feedback loops. In mice, for example, a 2-hour clock was found that times the formation of the somites and which is driven by WNT and NOTCH (Dale *et al.*, 2003; Pourquie, 2003).

There are also other functions which are linked to clock genes. Fu *et al.* (2002) showed that an altered *Per2* gene can cause increased susceptibility for cancer in a certain mouse strain. This implies a link between the clock and the cell cycle.

1.3 Mouse phenotyping

The recent bio-medical research deals with a whole variety of natural or induced mutations in rodents. For example, the number of publications that cite targeted mutations in mice has been increased from 1990 to 1995 by more than 1200% (Simpson *et al.*, 1997). Phenotyping can help in the characterisation of spontaneous mutants and in locating the mutated gene by revealing something about the function of the gene (e.g. Chwalisz *et al.*, 2003), and creating a targeted mutant is a powerful new tool to dissect the molecular mechanisms of complex phenotypes (Anagnostopoulos *et al.*, 2001; Crawley and Paylor, 1997; Karl *et al.*, 2003c; van der Staay and Steckler, 2001) such as the biological clock. As the underlying mechanisms of genetic networks are often poorly understood, it is equally important to have a proper characterisation of targeted mutations. As described by several authors (e.g. Gerlai, 1996), a null allele can cause several compensating up and down regulations of other gene(s) that might result in a complex, but secondary, phenotype which is not directly related to the gene of interest, and thus does not reveal anything about the function of the respective protein (Crawley, 2000).

Genetic background

One of the most critical aspects of the current research is the issue of the genetic background. Thus, it is important (1) to give a proper description of the genetic background of the animals used in a study, (2) to use a simple background, i.e. a standard strain such as C57BL/6J (B6), in order to give other scientists the chance to re-evaluate and expand the experiments done, and (3) to use a standard strain that allows comparison of results between different laboratories (Banburry Conference on Genetic Background in Mice, 1997).

Since many targeted mutants are generated with the various embryonic stem-cells (ES cells derived from various 129 sub-strains (Simpson *et al.*, 1997) and B6 foster mothers, the resulting transgenic offspring is an unknown mixture of these two strains. Even by

backcrossing the transgenic founders to B6 in order to remove the 129 part, there will be a flanking region on both sides of the mutated gene which can include several modifying genes. Thus, it has been suggested that one should use a 129 strain for crossing with the chimaeric mice and therefore generate a congenic strain on a 129 background (van Gelder and Hogenesch, 2004). If it is necessary to use the B6 strain, than one has to backcross for at least 10 generations (~ 99.8% identity) in order to meet the criterion for a congenic strain (Silver, 1995). A less time-consuming modification is a marker-assisted backcross. This method –so-called speed congenics (Wakeland *et al.*, 1997) – cuts in half the number of generations needed to achieve ~ 99.8% identity. There is, however, the problem of closely linked loci. In such case, the use of a 129 strain should be preferred (van Gelder and Hogenesch, 2004).

Behavioural phenotyping

As described above, the disruption of a gene can lead to a complex phenotype which can be directly related to the targeted gene and/or may have secondary consequences. In order to dissect those complex structures it is recommended to monitor the general health of the mutants prior to the specific behavioural tests (Crawley, 2000; Crawley and Paylor, 1997; Karl *et al.*, 2003c).

For behavioural phenotyping, it is essential to establish a high level of standardisation. A recent study conducted in three different laboratories revealed differences in the results although all laboratories were using the identical experimental protocol (Crabbe *et al.*, 1999). This means that even subtle changes in handling or the environment can have drastic effects on the results of a behavioural test (Wahlsten, 2001).

1.4 Aims and scope of this study

In this study, various aspects of the phenotype of the *Per1*$^{-/-}$ and *Per2*$^{-/-}$ mice were determined. Due to time and budget limitations, this study cannot be called comprehensive. Nevertheless, a wide range of parameters were under examination:

In order to achieve a proper phenotyping, first observations were made on the animals' general health, and some basic behavioural tests were made concerning sensory and motor abilities, as well as emotionality. Furthermore, some basal physiological parameters were recorded, such as body weight and body temperature.

As these tests gave some indications of an altered stress response of the transgenic mice as compared to that of wildtype, the hypothalamic pituitary adrenocortical (HPA) axis was examined in more detail. Therefore, the levels of fecal corticosterone metabolites were determined in order to determine basal unstressed values of the daily glucocorticoid rhythm, which reflects the activity of the HPA axis (Raber *et al.*, 2000). In addition, a stress-induced hyperthermia test was used to determine the reaction of the HPA axis upon an acute stressor.

Some of the *Per1* $^{-/-}$ mice developed severe skin problems which could not be tracked to any parasite or microbiological infection. In order to exclude an autoimmune disease the immunresponse of the animals was investigated by a challenge test in which the cytokine response was determined after a LPS challenge. Furthermore, the unspecific immune response was tested by using blood macrophages for a phagocytosis assay.

Moreover, some classical circadian experiments were carried out such as the response to a non-photic zeitgeber. The entrainment capabilities of the genotypes were tested with respect to the zeitgeber. Furthermore, activity patterns in constant light of various intensities were investigated for animals lacking a part of the morning as well as the evening oscillator, namely the *Per2*^{Brd} / *Cry2* $^{-/-}$ mice. Another prediction of Daan *et al.* (2001) concerning the two oscillator model was considered in the (re-)evaluation of the aftereffects of the non-24-hour T-cycles.

In a pilot experiment, two new techniques were established. Firstly, a newly developed inexpensive lickometer device, the Lick-O-Mat, was used to monitor the drinking activity of the animals. Secondly, an old protocol using feedback lighting (Ferraro and McCormack,

1984; Ferraro *et al.*, 1984) was rediscovered and a software-based application for it was developed.

In summary, the aim of the present study was to provide a proper characterisation of the *Per1* $^{-/-}$ and *Per2* $^{-/-}$ mice in order to give insight into the putative alterations in the physiology and behaviour of these animals. In this way, it should be possible to interpret previous and future experiments on the basis of solid data.

2. Animals, Material and Methods

2.1 The animal model

All procedures including animals were in accordance with the animal law of the Federal Republic of Germany and the guidelines of the European Union. The experiments are approved by the district government of Hannover.

We used B6.129S7-*Per1*^{tm1Brd}, B6.129S7-*Per2*^{tm1Brd} (abbreviation: *Per1*^{Brd}, *Per2*^{Brd}) and double mutants bred out of these mice which were kindly made available by U. Albrecht. These mutants are referred to below as *Per1*$^{-/-}$, *Per2*$^{-/-}$, and *Per1*$^{-/-}$ / *Per2*$^{-/-}$, respectively. For a detailed description of the *Per1*^{Brd} mutants see Zheng *et al.* (2001) and for the *Per2*^{Brd} mutants see Zheng *et al.* (1999). In brief, the *Per1*-deficient animals were generated by replacing 15 of the 23 exons of the *Per1* gene with a hypoxanthine phosphoribosyltransferase (*Hprt*) gene, thus creating "most likely" (Zheng *et al.*, 2001) a null allele of this gene (Figure 6).

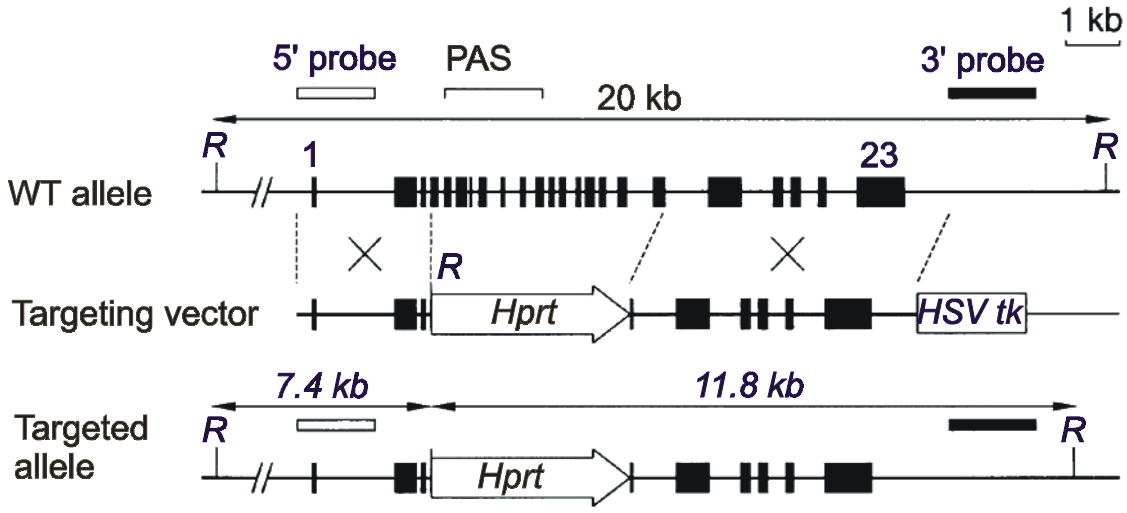

Figure 6. Genomic structure of the murine *Per1* gene, the targeting vector, and the predicted structure of the targeted allele. Exons are indicated by vertical black bars with the first and last exons numbered. Abbreviations: *Hprt*, hypoxanthine phosphoribosyltransferase gene; kb, kilobase pairs; *R*, *Eco*RI; *HSV tk*, Herpes simplex virus thymidine kinase gene; WT, wildtype (from Zheng *et al.*, 1999)

In the case of the *Per2*-deficient mice a part of the highly conserved PAS B and the entire PAC subdomain were deleted, resulting in a significantly shortened protein that lacks

possible dimerisation sites (Figure 7). However, the shortened transcript still cycles to a certain degree and it cannot be ruled out that the *Per2* allele has some residual function.

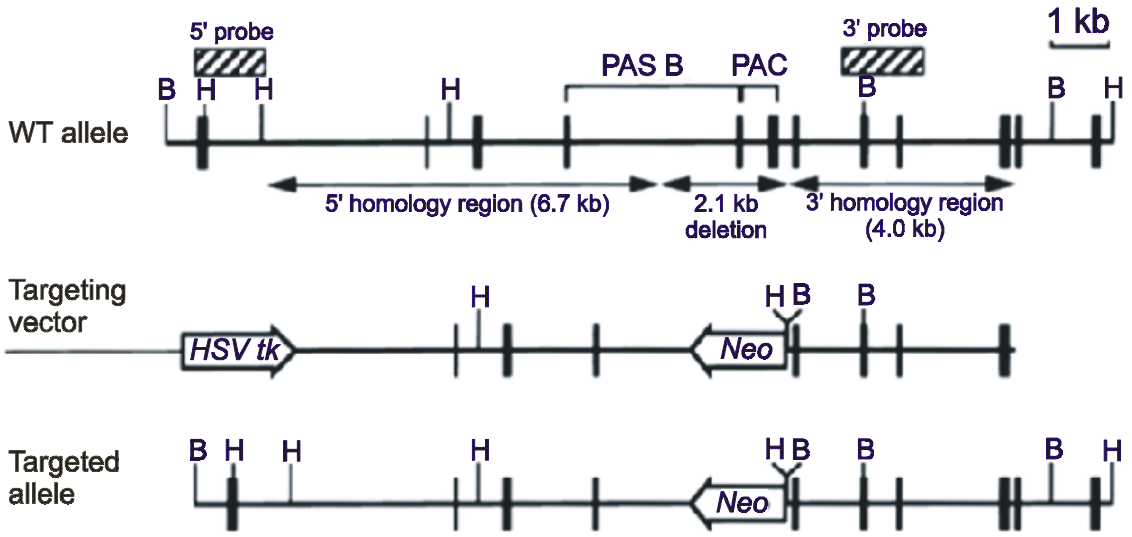

Figure 7. Genomic structure of a portion of the mouse *Per2* gene, the targeting vector and the predicted structure of the targeted allele. Exons are indicated by vertical black bars. Abbreviations: B, *Bam*HI; bp, base pairs; H, *Hin*dIII; *HSV tk*, Herpes simplex virus thymidine kinase gene; kb, kilo base pairs; *Neo*, neomycin resistance gene; PAS B, a dimerisation site present in *Per*, *Arnt* and *Sim*; PAC, protein subdomain; WT, wildtype (from Zheng *et al.*, 2001).

In addition, double mutants out of the two transgenic mice were generated by intercrosses. All genotypes were maintained homozygous. Therefore, no littermates of the transgenic animals were available as controls. Instead, a F2 generation between the background strains B6 and 129S7 was used as control group. The (B6x129S7)F2 control animals are called wildtype (WT) animals.

In addition, $Per2^{-/-}$ / $Cry1^{-/-}$ animals were used for a constant light experiment. The animals were bred by using the F2 population of $Cry1^{+/-}$ / $Cry2^{+/-}$ and $Per2^{Brd}$ animals (for a detailed description see Oster *et al.*, 2002; van der Horst *et al.*, 1999).

Finally, C57BL6/J mice obtained from the Zentrales Tierlaboratorium (Hannover, Germany) were used for the following experiments. *Per1* expression was measured after a heat pulse for the evaluation of temperature as a zeitgeber. The newly developed lickometer

device was evaluated by comparing the actograms derived from passive infrared detectors and the licking activity.

Genetic monitoring

The genotype of the animals was randomly tested for correctness. In order to test the animals for the *Per1*^{Brd} allele, tissue from the tip of the ear was sampled and stored until analyses in –20 °C. For the genotyping we used southern blotting as described by Oster (2003). The templates for the probes were equivalent to those used by Zheng *et al.* (2001). The procedure for the determination of the *Per2*^{Brd} animals was analogous. The templates for southern blotting are described in Zheng *et al.* (1999).

2.2 Environmental conditions

Animals were fed *ad libitum* with standard maintenance diet (Altromin 1324, Altromin, Germany) with tap water also available *ad libitum*. The animals were held in LD 12:12 with illuminance above 300 lx. The standard room temperature was 21±1°C, and a relative humidity of 65%. Animals were held in standard macrolon cages type II (length: 20.7 cm, height: 14.0 cm, breadth: 26.5 cm, base: 363 cm^2) on standard bedding (Altromin, Germany). Unless stated otherwise, these are the conditions used in all experiments.

2.3 Recording of total locomotor activity

The total cage activity was recorded using a passive infrared detector (PID, Conrad Electronics, Germany). The PIDs were simply attached to the cage lid. The signals were detected by an I/O interface card (PIO48 II, BMC, Germany) and stored in a personal computer with a software initially programmed by T. Ruf. For a detailed description of the software and hardware used, see Dernbach (2003). An important point, however, is the switching characteristic of the PID itself. For between one and four seconds after a single event, the number of recorded events varies between the individual PIDs. Therefore, a

insensitive time of five seconds was implemented in the software. This results in a maximal number of 17, 280 events every 24 hours. Thus, it was possible to compare the recording patterns of different individual PIDs and even roughly to quantify the activity patterns of the animals.

2.4 Recording of running wheel activity

A running wheel system developed in the working group of S. Steinlechner was used consists of a metal running wheel with a diameter of 15 cm. The bars of the running wheel were wrapped with a metal gauze in order to prevent slipping and resulting injuries. An additional handmade 5 cm high clear plastic surrounding was added to the cages because the macrolon type II cages would otherwise have been too low for the running wheels. Due to this construction, the cage lid including the food-hopper with the drinking bottle was also elevated by 5 cm. The running wheel was fixed to the cage lid and the entrance to the running wheel was about 3 cm from the cage floor. A permanent magnet was attached to the outside of the running wheel. Each passing of this magnet by a reed relay (Conrad Electronics, Germany) on the cage top was counted as a single wheel revolution. The signal was recorded via an I/O-interface card by a personal computer and stored to hard disk in one-minute bins. The software used was practically identical to the version for the PIDs (see previous chapter).

2.5 Recording of drinking rhythm

Drinking activity was monitored by a lickometer device developed by the author, the so-called Lick-O-Mat, which works on the following principle: the mouse is a high resistor, so that when it licks on the nipple of the water bottle, the circuit between cage floor and water bottle is closed, and a computer counts via an I/O interface card the number of contacts between tongue and water bottle nipple. The same software was used as for the recording of total cage activity. A circuit diagram and the parts lists are given in Appendix D. Due to the

fact that the Lick-O-Mat needs two poles a wiremesh cage was used of the same size as a macrolon type II cage.

2.6 Telemetric temperature recording

Temperature-sensitive transmitters (Model X, MiniMitter, USA) were implanted in the peritoneal cavity of our mice. The animals were anaesthetised with pentobarbital (60 mg/kg body weight). The lower ventral part of the abdomen was shaved and a 1 -1.5 cm long incision was made in the skin and the peritoneum. The transmitter was then inserted into the peritoneal cavity of the mouse and the peritoneum and the skin were sewn shut. The animals were given two weeks to recover from the surgery. The signals of the transmitters were received by a low-cost pocket radio (Conrad Electronics, Germany) and digitised by a 12-bit AD/DA computer card (BMC, Germany). Data were stored every minute on a personal computer by software programmed by T. Ruf. For a detailed description of the telemetric recording see Dernbach (2003).

2.7 Behavioural phenotyping

Behavioural phenotyping can be subdivided into several categories (Crawley, 1999, 2000; Karl *et al.*, 2003c): (1) basal phenotyping (general health, sensory and motor functions), and (2) special behavioural domains (e.g. emotionality and nociception).

Behaviour in emotionality tests like the open field is dependent on the prior experiences of the animals (Walsh and Cummins, 1976). Therefore, the open field was the first test performed, followed by the tests for neurological reflexes (postural reflex, eye blink reflex, ear twitch reflex, whisker orientating reflex, pupil reflex) and nociception (hot plate), and finally, for motor abilities (wire hang, pole test).

Feeding and water consumption

While the animals were maintained in their home cages 24-hour food and water consumption was measured by weighing the food in the food hopper and the water bottle, every 24 hours for 5 days.

Reflexes

The following reflexes were tested (Crawley and Paylor, 1997; Karl *et al.*, 2003c):

(1) *Balance:* The animal is placed in an empty cage and the cage is moved rapidly from side to side and up and down. The normal behaviour would be to stretch out all four legs in order to maintain an upright position.

(2) *Eye blink reflex:* The eye of the animal is lightly touched with a cotton-tip swab. The normal response would be to blink.

(3) *Ear twitch reflex:* The tip of the ear is slightly pinched with tweezers. The normal response of the animal would be to twitch the ear.

(4) *Whisker orientating reflex:* The animal is placed in an empty cage and its whiskers are lightly touched with a cotton-tip swab. The normal response would be a stop in the continual movement of the whiskers; frequently, the head of the mouse turns towards the side of the stimulus, as well.

(5) *Pupil reflex:* The animal is placed in a dimly lit room until its pupils are fully dilatated. Then, the beam of a penlight is directed in the eye of the animal and the constriction of the pupil is observed, as is the re-dilation of the pupil after removal of the light beam.

Wire hanging

The wire hang test is designed to measure neuromuscular strength (Paylor *et al.*, 1998). The animal is placed on a standard type III cage wire mesh lid. The lid is gently waved in mid-air so that the mouse grips the lid. Then the lid is turned upside down approximately

30 cm above the soft bedding material in the home cage of the mouse. The latency to fall in the cage is measured with a cut-off time of 60 s.

Pole test

The pole test measures bradykinesia, which is the ability to make slow movements (Ogawa *et al.*, 1985). A wooden stick (length 50 cm, diameter 1.5 cm) is installed vertically on a metal base (Sedelis *et al.*, 2000). The stick is wrapped in a plastic gauze to improve the grip of the mice on the stick, and a ball (diameter 3 cm) is fixed upon the upper end of the stick. The animal is placed directly below the ball looking in the direction of the ball; the time it takes the mouse to turn round reach the base of the stick is measured. If the animal slides rather than climbs down the pole or if the animal does not move at all, both parameters are recorded as 120 s and the animal is returned to its home cage.

Open field

The open field is based on the free exploration of an unknown environment. It is useful for the assessment not only of emotionality and exploration behaviour but also the activity of the animals. The input for the system came from a CCD camera (Ikegami, USA) and the sessions were stored on video tapes on a standard video recorder (Toshiba, Japan). In addition, the sessions were digitised by a frame grabber (Picolo, Euresys, USA, resolution 384 x 288 pixel) and quantitatively analysed with Etho Vision Software Version 2.0 (Noldus, The Netherlands). The following parameters were used for the automated detection system: *detection method* subtraction absolute, *sample rate* 12 samples/s, *image filter* erosion/dilatation 5 pixel, *contrast* 88, *brightness* –12.

The open field test was always done between ZT13 and ZT15 under dim red light (< 1 lx). The open field was a glass cube (40 x 40 cm floor space, 50 cm height) which was placed in a white box. For the analyses, we subdivided the open field in 16 10-cm squares. After the mouse was placed in the middle of the open field its behaviour was recorded over a

10-min period). Thus it was possible to quantify the time spent and the distance moved in each of the 16 squares of the open field. In addition, the defecation rate was recorded by counting the fecal boli after each open field session.

2.8 Hot plate

The hot plate is used to investigate pain sensitivity in mice (Crawley, 1999; Karl *et al.*, 2003c).

The animals were placed on a 30-cm square metal surface with a temperature of 52,5 °C which is surrounded by 28-cm high plexiglas walls, a so-called analgesia meter (Columbus Instruments, USA). The animals remained on the hot plate until they showed one of the following behaviours: (1) lick hind paws, (2) raise hind paws, or (3) jump. The time of the first occurrence of one of these behaviours was recorded and the animal was immediately removed from the hot plate. In order to prevent tissue damage, a cut-off time of 25 s was set (Bannon *et al.*, 2000).

In order to exclude the effects of stress-induced analgesia (Amit and Galina, 1986), a second set of animals was tested in a habituated design. For this purpose, the animals were tested as described above. After a washout period of three days, the animals were habituated to the hot plate by placing them in the apparatus for three minutes on four subsequent days without heating the plate (Karl *et al.*, 2003b).

2.9 Glucose tolerance test

A glucose tolerance test was carried out following the method described in Rohl *et al.* (2004) and Cooney *et al.* (2004).

After an overnight fasting (14 h), the blood glucose level of the animals was measure at ZT 1 using an automatic glucose monitor (Glucometer Elite, Bayer, Germany). Directly after the determination of fasting blood glucose level, the animals were injected intraperitoneally with glucose (2 g/kg body weight in distilled water). The blood glucose level was re-

determined 30, 60, and 120 min after the injection. The blood samples (5 µl) were collected from a small incision made at the distal part of the tail of the conscious and unrestrained mice.

2.10 Stress-induced hyperthermia

The stress-induced hyperthermia experiment was carried out following published procedures for single house mice (Peloso *et al.*, 2002; van der Heyden *et al.*, 1997). The rectal temperature of a mouse was measured by inserting a thermistor probe of a digital thermometer (Therm 2244-1, Ahlborn, Germany) 2 cm into the rectum of the mouse. Prior to insertion into the rectum the probe was lubricated with petroleum jelly. The temperature was recorded after the it had been constant for 10 s. The accuracy of the measurement was 0.1 °C. Given that the T_b is strongly dependent on the time of day (Benstaali *et al.*, 2001; Connolly and Becker-Lynch, 1981; Refinetti, 1999; Tankersley *et al.*, 2002), all experiments were carried out between ZT 2 and ZT 4, because T_b is minimal at that time (Benstaali *et al.*, 2001). A repeated measurement design was chosen. The animals used in this experiment were housed singly. Twenty-four hours prior to the experiment the animals were placed in fresh cages. On the day of the experiment, the basal temperature of the first animal was taken no later than 5 min after the experimenter entered the room. Subsequently, the temperature was determined 15, 30, 90 and 180 min after the basal measurement. After each temperature measurement the animals were put back in their respective home cage.

In order to validate the test for this investigation, a pre-test was made with seven male C57BL6/J mice. As Figure 8 shows, the B6 mice showed a clear rise in temperature, which was most prominent after 30 min with a mean rise of 0.8 ± 0.1 °C. The temperature had normalised after 90 min. In light of these findings and the values obtained from the literature (van der Heyden *et al.*, 1997), the times for this study were chosen in that part of the curve with the steepest slope (15 min), at the maximum level (30 min) and after a recovery from

arousal (90 min) as stated above. The 180 min measurement served as an additional control for total recovery.

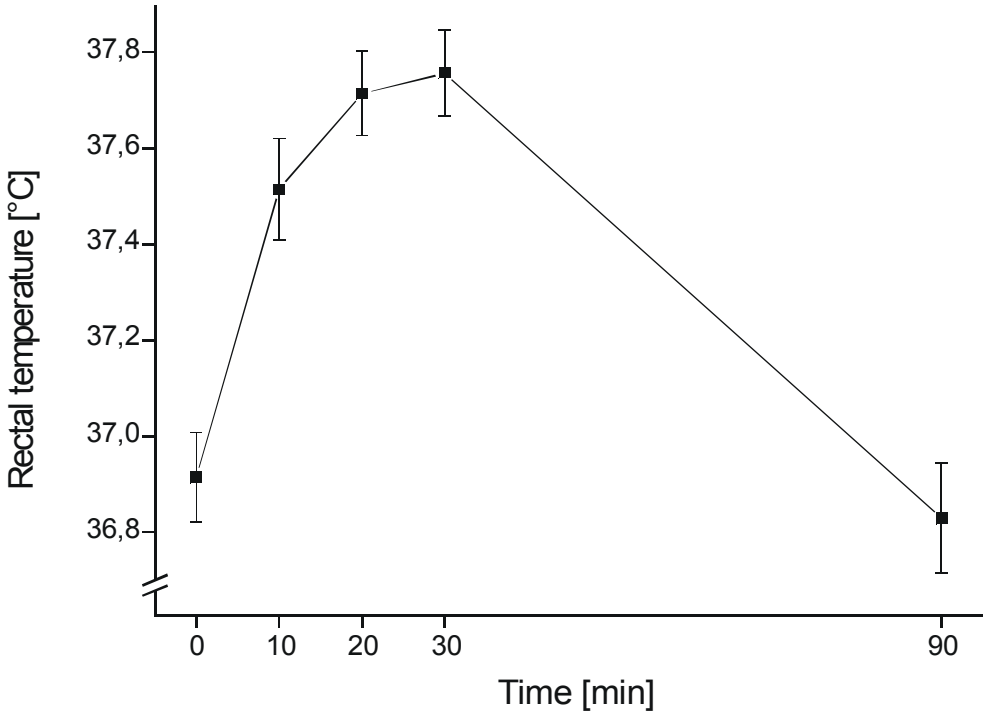

Figure 8. Course of rectal body temperature of C57BL6/J mice in a stress-induced hyperthermia test. Group mean (n = 7) and sem are given.

2.11 LPS stimulation

In order to gain information about the immune function of the animals we injected lipopolysaccherids (LPS) into the peritoneum of the animals and determined the changes in T_b and cytokine levels (TNF-α, IL-1β). Two experiments were carried out.

(1) Three weeks before the experiment. the mice were implanted with transmitters for core body temperature recording as described above. On the day of the experiment, the mice were injected intraperitoneally with LPS (*Escherichia coli* serotype 0111:B4, 3mg/kg body weight) purchased from Sigma-Aldrich (Germany) in pyrogene-free PBS at ZT 6. Immediately before and 90 min after the injection the animals were bled by the retro-orbital venous plexus. The blood samples (circa 70 µl) were collected in heparinised micro-haematocrit tubes (Brand, Germany). After centrifugation (6000 U/min for 10 min) plasma

and cell fractions were stored separately at -20°C. For the detection of tumour necrosis factor α (TNF-α) we used an enzyme-linked immunosorbent assay (ELISA; Pierce Endogen, USA). The assay range was 50 – 2450 pg/ml and both intra- and inter-assay variabilities were < 10%.

(2) Two changes were made compared to experiment 1. First, the animals were not implanted with transmitters, and second, the second blood samples were taken three hours after the LPS injection. For the detection of interleukin 1-beta (IL-1β) we used an ELISA (Pierce Endogen, USA). The assay range was 15.6 – 1000 pg/ml and both the intra- and the inter-assay variabilities were was <10%.

2.12 Phagocytosis assay

The phagocytotic activity of leukocytes was assessed by a quantitative phagocytosis assay (Phagotest, Orpegen Pharma, Germany). This assay measures the percentage of leukocytes which have ingested bacteria. Immediately after the mice were killed by cervical dislocation, the mice were decapitated and blood samples were bled into lithium-heparinised cups (1 ml, Kabe, Germany). For the test, the samples (100 µl) were incubated with fluorescein (FITC)-labelled opsonised *Escherichia coli* for 10 min either at 37 °C or – in case of the negative controls – on ice. Phagocytosis was stopped by placing all samples on ice. In addition, the FITC fluorescence of the non-internalised bacteria was quenched. Then, the erythrocytes were lysed and the DNA of cell fragments and bacteria aggregations stained. Finally, the samples were analysed by flow cytometry (FACS, FC500 Cytomics, Beckman Coulter, USA). For measurement, a live gate was set in the FL2 channel in order to exclude bacteria.

2.13 Hormonal rhythms in feces

Hormonal rhythms of corticosteroids were measured in the mice. Because bleeding a mouse every three hours is very stressful, and the blood volume in mice is quite low, urine and fecal samples were taken, which is relatively unstressful for the animals.

Sampling

For urine and feces sampling, the animals were held in special metabolic cages. These consist of a cylindrical cage (17.5 cm diameter x 10 cm height) with a soft plastic mesh (mesh aperture 0.3 cm) floor. After an initial habituation period of the days in the metabolic cages, the urine and feces were collected for 48 hours as described below. During the test period the mice were offered apple juice (naturally cloudy apple juice, diluted 1:2 with tapwater) in order to increase the urination volume.

The urine is flows alongside the wall of the catchment funnel and is then led via a tube into a cup, and the feces fall directly through the middle of the funnel and are stored in a small plastic vessel. Both urine and feces, were sampled at three-hour intervals for a period of 48 consecutive hours. At the end of each three-hour sampling period, the downmost part of the catchment funnel and the connection tubes were washed with 1 ml demineralised water by a perfusion pump (Perfusor, Melsungen AG, Germany). This was done in order to make sure that there were no carry-overs between the sampling periods. Urine samples including the washing-water were transported by a peristaltic pump (3610, Colora Messtechnik, Germany) and collected in a cup. After the fecal boli were weighed, feces and urine were stored at 20 °C.

Detection of corticosterone metabolites in feces

The assay for the determination of corticosterone in feces can be divided in two parts: (1) the extraction procedure, and (2) the enzyme immunoassay (EIA). Both parts are

described in detail elsewhere (Palme and Möstl, 1997; Touma *et al.*, 2004; Touma *et al.*, 2003).

After homogenisation 0.05 g of each fecal sample was weighed and shaken on a multi-vortex (Buchler Instruments, Austria) for 30 min with 1 ml of 80% methanol. If less than 0.05 g feces were sampled at one particular time, the proportion of methanol was reduced accordingly. Then the samples were centrifuged for 10 min at 2500 × g and 500 µl of the supernatant were used as stock solution. Before freezing at -20 °C, the stock solution was diluted 1:10 with assay buffer (Tris/HCl 20 mM, pH 7.5), and the diluted solution was used for the determination of corticosterone metabolites.

The concentration of the corticosterone metabolites in the feces was determined by a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) as described by Touma (2003). The antibody used in this EIA binds to a 5α-3β,11β-diol of steroids, and crossreacts as follows: 5α-pregnane-3β,11β,21-triol-20-one (100%), 5α-pregnane-3β,11β,21-tetrol (110%), 5α-pregnane-3β,11β,17α,21-tetrol-20-one (45%), 5α-androstane-3β,11β-diol-17-one (230%). If the corticosterone or its metabolites was changed in one of the recognising positions, then the crossreactivity was below 1%. That was also the case in the tested gonadal steroids and their metabolites (e.g. progesterone, androsterone). The intra- and interassay coefficients of variation were 9.1% and 14.0%, respectively.

2.14 Drinking rhythms

Lick-O-Mat validation experiment

The first experiment with the Lick-O-Mat was made in order to validate the method. The total activity in the cage was recorded by PIDs as described above. In addition, the licking events at the tip of the water bottle were counted by the Lick-O-Mat. An Aschoff

type-II schedule (Mrosovsky, 1996b) was carried out and a 4-hour phase advance took place after a washout period. Here, C57BL6/J mice were used.

2.15 Constant light conditions for double-mutant animals

This experiment was designed as in Steinlechner *et al.* (2002). Prior to the experiment the animals were held in LD 12:12 for at least five weeks. After an initial period of ten days in constant darkness, the animals were held in LD 12:12 for a washout period of 20 days. The instantaneous arrythmicity in DD is a control for the double-mutant genotype. Then the animals were transferred to constant light with a low light intensity (5 lx) for 15 days, which was subsequently elevated to 200 lx for 25 days, and finally to 300 lx for 16 days.

For this experiment, the animals were held in macrolon type III cages (length: 42.5 cm, height: 18.5 cm, breadth: 26.6 cm, base: 800 cm^2), equipped with running wheels and PIDs as described above. The free-running period for the constant darkness and each of the following light intensities was calculated using ten days of data in a Lomb-Scargle periodogram analysis (see Statistical Analysis).

2.16 Non-24-hour T-cycle

For this experiment, the animals were held in heightened type II cages (height: 19 cm) equipped with running wheels (see above). The animals were housed in a ventilated and light-proof chamber. The light regime in the chamber was controlled via a steady-state relay (Sharp, Germany) by the same PC that recorded the running wheel revolutions. Three groups were used in this experiment, *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT, consisting of six, six and four animals, respectively. After an initial period of 17 days under an altered T-cycle (T = 22.5 hours). the animals were transferred to total darkness for 30 days. The free-running period in total darkness was determined directly after and 15 days after lights-off. Five subsequent days were used for each determination of τ. The phase angle was calculated by comparing onset of

activity at the first day in constant darkness with the theoretical offset of the light on that day. In this case, the onset of activity was determined by visual inspection.

2.17 Temperature as zeitgeber

Light is the dominant zeitgeber for all kinds of organisms (see Introduction). However, it has been reported that, in mammals, temperature can pose as a zeitgeber, as well (but see Hoffmann, 1968; e.g. Rajaratnam and Redman, 1998). Here, we studied both the output in terms of activity as well as the underlying mechanism in terms of induction of *Per1* gene expression.

Temperature cycles and activity

Prior to the experiment, the animals were held for at least 14 days under standard conditions (see above) with one exception: the ambient temperature was set in the thermoneutral zone of the animals, which is typically between 28 °C and 31 °C in mice (Gordon, 1993; Williams *et al.*, 2002), because in a pilot experiment without this initially high temperature phase, all animals failed to entrain to the presented temperature cycle. Two separate experiments were conducted. In both cases, the animals were transferred to constant darkness (dim red light < 1 lx), and the ambient temperature was set to 28 °C (warm) in the former light phase, and the temperature was set to 22 °C (cold) in the former dark phase (WC 12:12). The temperature was accurate to ± 1 °C, and the transition phase between cold and warm was no longer than 30 min.

(1) After two weeks in WC 12:12 with onset of the cold phase at 06:00h, the cold phase was phase-delayed for four hours. Afterwards the cold phase was shifted back to the old phase. The activity was measured by the use of PIDs as described above.

(2) The temperature cycle was absent during the first two weeks of constant darkness. Then the WC 12:12 was established with onset of the cold phase at 06:00h. Finally, the animals were retransferred to constant darkness without a WC cycle in order to

measure the free-running period of the animals. The running wheel activity was monitored as described above.

Temperature pulse and *Per1* expression

In order to assess the underlying mechanism of the temperature entrainment, the reaction of *Per1* mRNA abundance in the SCN was measured after a temperature pulse near the middle of the dark phase (ZT 17) with *in situ* hybridisation.

The animals were held in LD 12:12 at 21 °C ambient temperature. In the night of the experiment, the experimental group of animals was transferred in a heated chamber (31 °C) for 30 min, and subsequently put back in the same room as the control group. After an incubation time of one hour (at ZT 18.5) all animals were sacrificed in CO_2 and transcardially perfused with 0.01 M phosphate-buffered saline (PBS) and then fixed with 100 ml of ice-cold 4% paraformaldehyde (PFA) in PBS. Immediately after the fixation, the brain was removed and put in 4% PFA in PBS overnight.

The next day the brain was transferred into 30% sucrose solution for cryo-protection. Afterwards, the brains were cut in coronary sections of 20 µm thickness in a cryostat (HM 500 OM, Microm, Germany), mounted on a slide, and stored at -80 °C until hybridisation took place. The in situ hybridisation was performed as described in Albrecht *et al.* (1997a).

2.18 Feedback lighting

Mice were housed in heightened standard cages with running wheels and PIDs as described above. Each cage was put in a single ventilated and light-proof chamber. In addition, each chamber was equipped with a light sensor. The light in the chamber was switched on and off by an eight-channel relais interface card which was controlled by the same computer used for recording of total activity in the cage, wheel revolutions and light regime.

Prior to the start of the experiments mice were held for at least 14 days in LD 12:12. At ZT 6 of the first day of the light regime the lighting regime was switched to feedback. From then on, lights were turned off (1) or on (2) by the mouse running in the wheel. The criteria for lights off and lights on were evaluated with wildtype mice in pre-experiments (data not shown). For lights off the mouse had to turn the wheel more than eight times in less than two minutes. The lights were turned on again if there were no wheel revolutions during 15 minutes.

2.19 Statistical analysis

All values measured are given as means plus or minus standard error of means where appropriate. The data from PIDs, running wheels and Lick-O-Mat is plotted so that each cycle's activity is shown both to the right and below that of the previous cycle, a so-called double plotted actogram. If not stated otherwise, the x-axis is 48 hours starting on the left with 00:00 h (ZT 6).

For testing for statistically significant differences between the different genotypes under comparison, we used a one-way analyses of variance (ANOVA) with the factor "genotype" and the Scheffé *post hoc* test for pairwise comparison, where appropriate. In the case of multiple measurement of a single animal a repeated measurement design of the ANOVA was used. In case of a pairwise comparison of unpaired groups, the Mann-Whitney U-Test was used for non-parametrical comparison.

In case of repeated measures of a continuous variable, the area under the curve was calculated by numerical integration using the trapezoidal rule. The built-in algorithm of Origin 6.0 (Microcal Software, USA) was used to compute the results.

All statistical tests were carried out two-tailed and the level of significance was set at $\alpha = 0.05$. The programmes Microsoft Access 97 and Microsoft Excel 97 were used for

calculation of descriptive statistics. With exception of the rhythm detection, all testing of hypotheses was computed with the programme StatView 5.0 (SAS, USA).

The free-running period of the animals was determined with the programme Peanuts (kindly made available by T. Ruf) using the Lomb-Scargle algorithm (Lomb, 1976; Ruf, 1999; van Dongen *et al.*, 1999a; van Dongen *et al.*, 1999b) for rhythm detection in unevenly sampled data sets. Unless noted otherwise, ten days of data were used for the rhythm detection.

The on- and offset of activity were determined by visual inspection of the double plotted actogram.

3. Results

3.1 General health

In general, the *Per1* $^{-/-}$ and *Per2* $^{-/-}$ animals did not show any abnormalities compared to WT. However, there were two phenomena which seemed to occur only in the transgenic animals. Firstly, *Per1* $^{-/-}$ animals had severe skin problems. The animals in question started to scratch themselves on the neck, at the ears, and at the sides of the snout. This led to a total loss of hair on the neck and the snout and to partial loss of the pinna (Figure 9). Two of these animals were examined for parasites and bacteria without any positive findings. This is in agreement with the findings of two other studies in which *Per1* $^{-/-}$ animals are being held (U. Albrecht, pers. comm.). The histology of the affected areas showed a chronic eosinophilic dermatitis with epidermal hyperplasy.

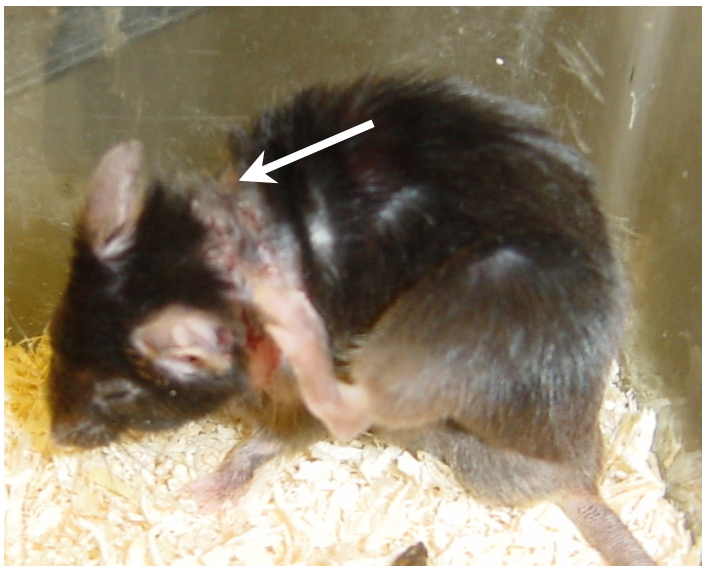

Figure 9. *Per1*$^{-/-}$ mouse with severe skin problems (*arrow*).

Secondly, some of the *Per2* $^{-/-}$ and double-knockout animals developed a rectal prolapse. In contrast, none of the WT animals showed such a prolapse. The reason for this prolapse remained unclear. The diagnostic findings showed no unusual bacteria or any parasites that could be blamed for these symptoms. In addition, the histology of the intestine showed no

signs of inflammation except in the prolapse itself (Figure 10). Preparation and haematoxylin-eosin staining of the intestine was done as described in Bleich (2003).

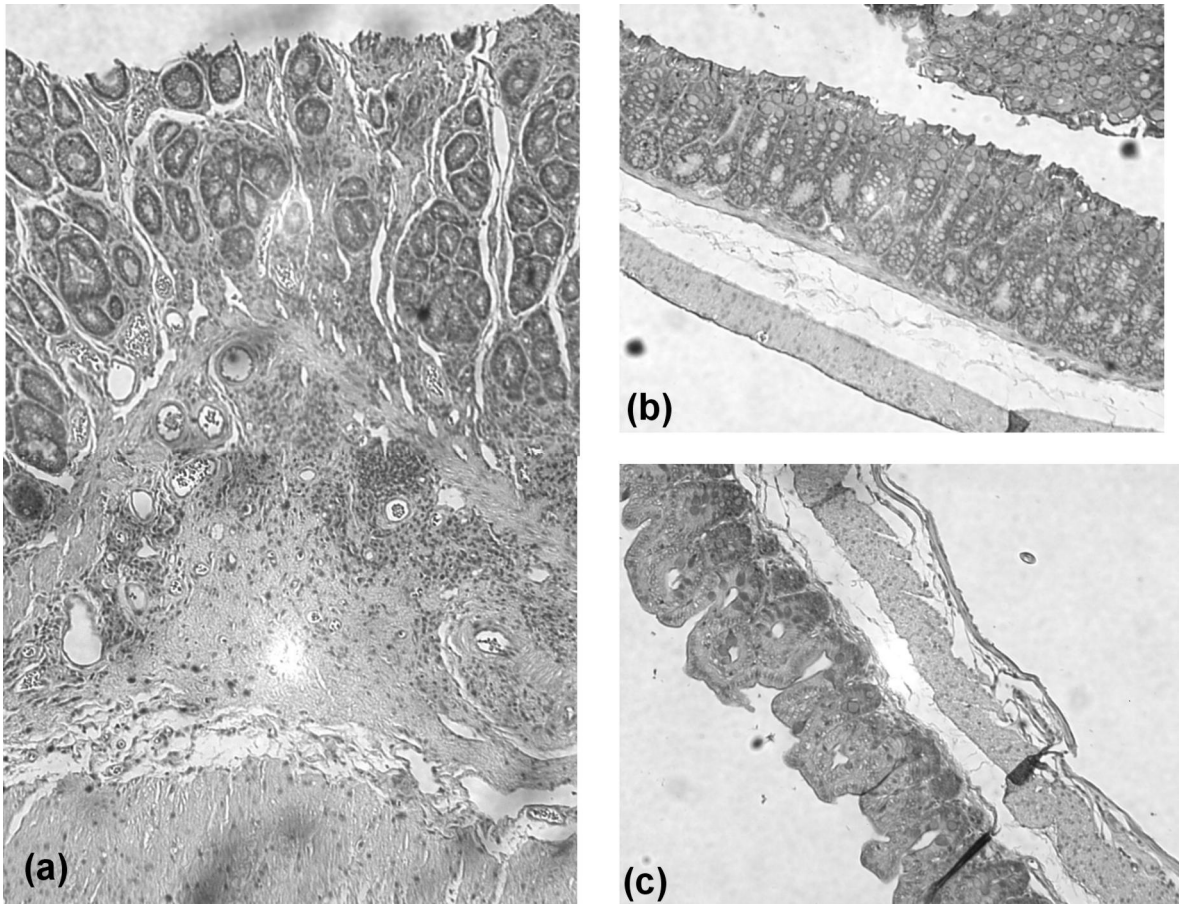

Figure 10. H-E staining of three sections of the ceacum and colon from a *Per2*$^{-/-}$ mouse with a rectal prolapse. (a) Rectal prolapse (x 100), (b) normal colon (x 50), and (c) normal caecum (x 50). For details see text.

3.2 Body weight

The body weight of all genotypes was measured in bi-weekly intervals. There were significant differences in body weight of the adult animals among the genotypes. Although these differences were not statistically significant during the growth phase (Figure 11), they were in the adult animals (older 3 months Crawley, 2000, p. 25). In addition, some data on young (< 3 months) *Per2* $^{-/-}$ and WT animals (kindly provided by U. Albrecht) were compared to our own data for *Per1* $^{-/-}$ animals (data not shown). Due to an insufficient database for the

Per1 $^{-/-}$ animals, the comparison did not reveal any significant differences in these young mice. There was, however, a trend to smaller body mass in *Per1* $^{-/-}$ mice.

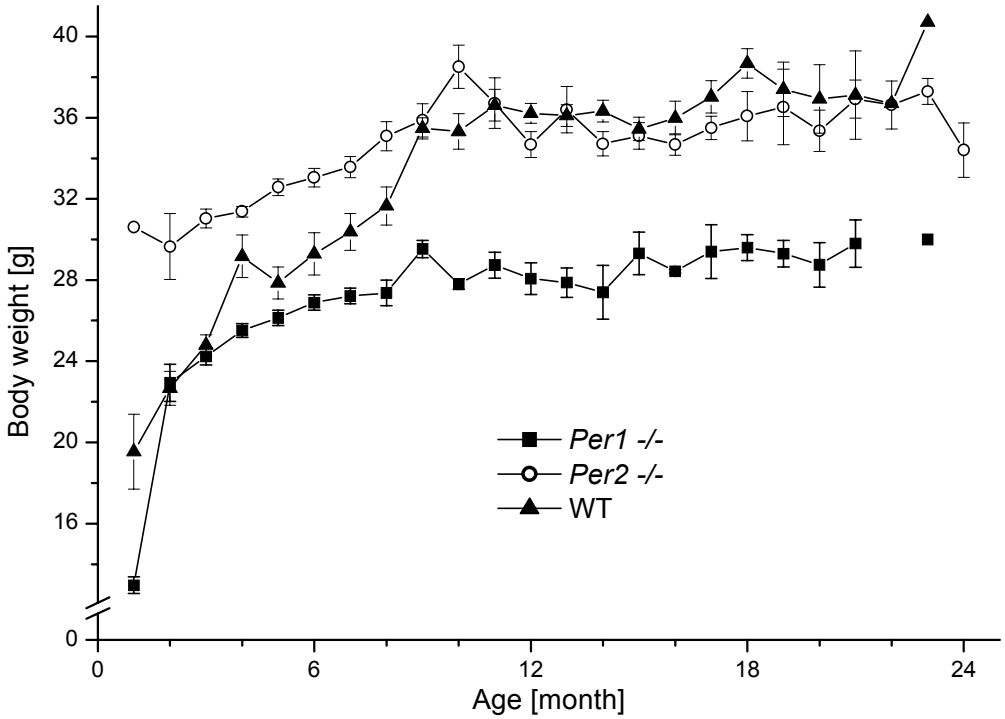

Figure 11. Development of the body weight of the three genotypes *Per1* $^{-/-}$ (n = 56), *Per2* $^{-/-}$ (n = 43), and WT (n = 73). Data are given as mean ± sem. For details see text.

For the adult mice, the differences are very obvious and highly significant ($F(2,133) = 45.552$, $p < 0.0001$). The *Per1* $^{-/-}$ mice (26.9 ± 0.4 g, n = 56) are smaller than WT (31.3 ± 0.8 g, n = 37) and *Per2* $^{-/-}$ animals (35.1 ± 0.7 g, n = 43) as is shown in Figure 12. *Post-hoc* comparison revealed highly significant differences between all genotypes under comparison (*Per1* $^{-/-}$ *vs.* *Per2* $^{-/-}$: $p < 0.0001$; *Per1* $^{-/-}$ *vs.* WT: $p > 0.0001$; *Per2* $^{-/-}$ *vs.* WT: $p = 0.0007$).

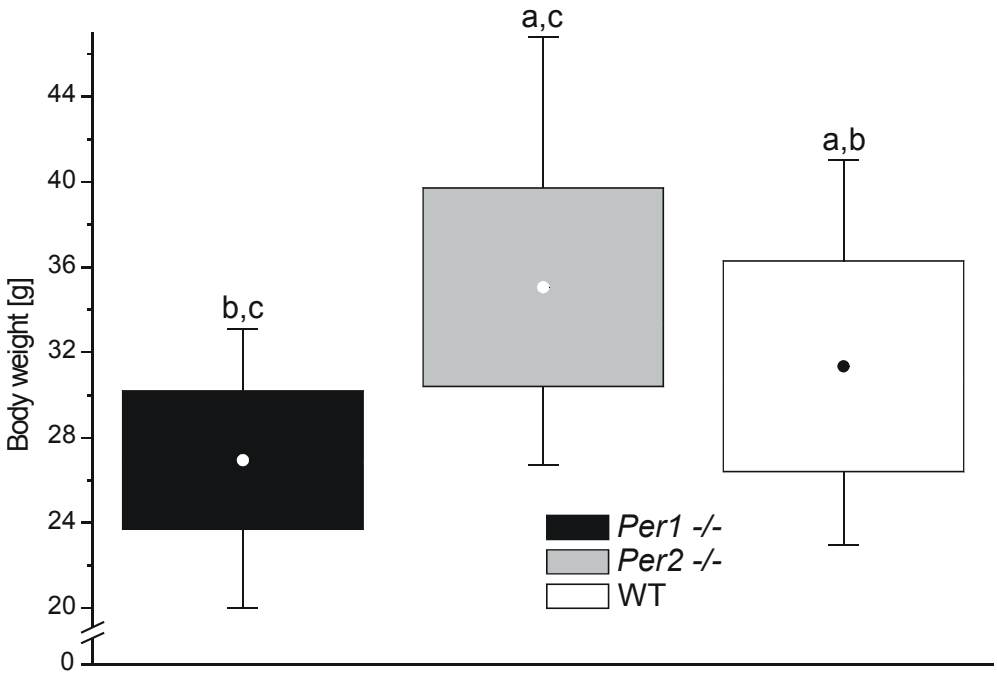

Figure 12. Box-plot of body weight of adult (older than 3 months) animals of the three genotypes *Per1* $^{-/-}$ (n = 56), *Per2* $^{-/-}$ (n = 43), and WT (n = 73). Values are given as mean (*circle*), SD (*box*), and range (*whiskers*). Statistical significances are indicated as a *vs. Per1* $^{-/-}$; b *vs. Per2* $^{-/-}$; c *vs.* WT. For details see text.

3.3 Body temperature

The telemetric recording of T_b at a constant ambient temperature (T_a) of 28 °C revealed a distinct daily temperature pattern with lower temperatures during the day and higher temperatures at night (Figure 13). The mesor of this oscillation was lowest for the *Per1* $^{-/-}$ (36.1 ± 0.1 °C) animals. The *Per2* $^{-/-}$ (36.6 ± 0.1 °C) and WT animals (36.6 ± 0.1 °C) showed a slightly higher T_b. The amplitude of this T_b rhythm for the *Per1* $^{-/-}$, *Per2* $^{-/-}$ and for WT animals, 1.1 °C, 1.3 °C and 1.4 °C, respectively.

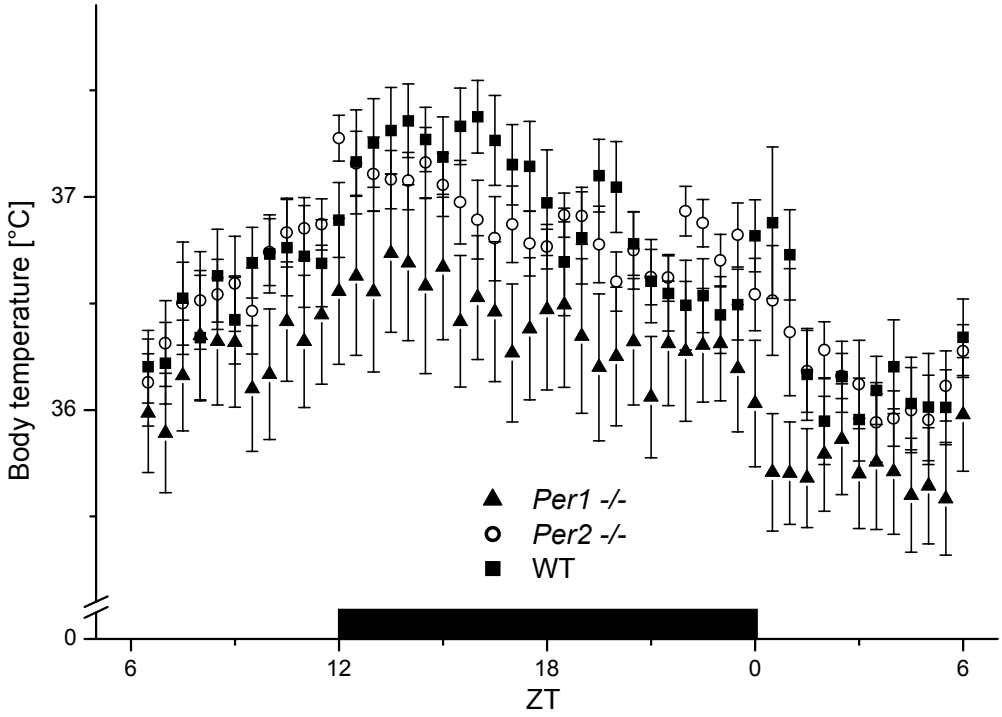

Figure 13. Mean time course of T_b over 24 hours under entrained conditions. Data are given as 30 min mean ± sem from *Per1* $^{-/-}$ (n = 7), *Per2* $^{-/-}$ (n = 8), WT (n = 8) animals from 10 consecutive nights. The black bar indicates the dark phase.

As stated above, the mean of the T_b of the genotypes differed constantly by about 0.5 °C. This difference, however, is not statistically significant ($F(2,20) = 1.749$, $p = 0.1996$, n.s.). If divided into the dark ($F(2,20) = 1.345$, $p = 0.2832$, n.s.) and light phase ($F(2) = 2.032$, $p = 0.1572$, n.s.) the seemingly obvious difference among the genotypes is still not significant. This might be due to the low animal numbers and the high variation in the values.

Finally, if one compares only the mean T_b of the first hour after lights on (ZT0 to ZT1), a statistically significant difference can be obtained ($F(2,20) = 4.072$, $p = 0.0328$). Subsequent *post hoc* comparison points out the difference between *Per1* $^{-/-}$ and WT ($p = 0.0407$, Figure 14).

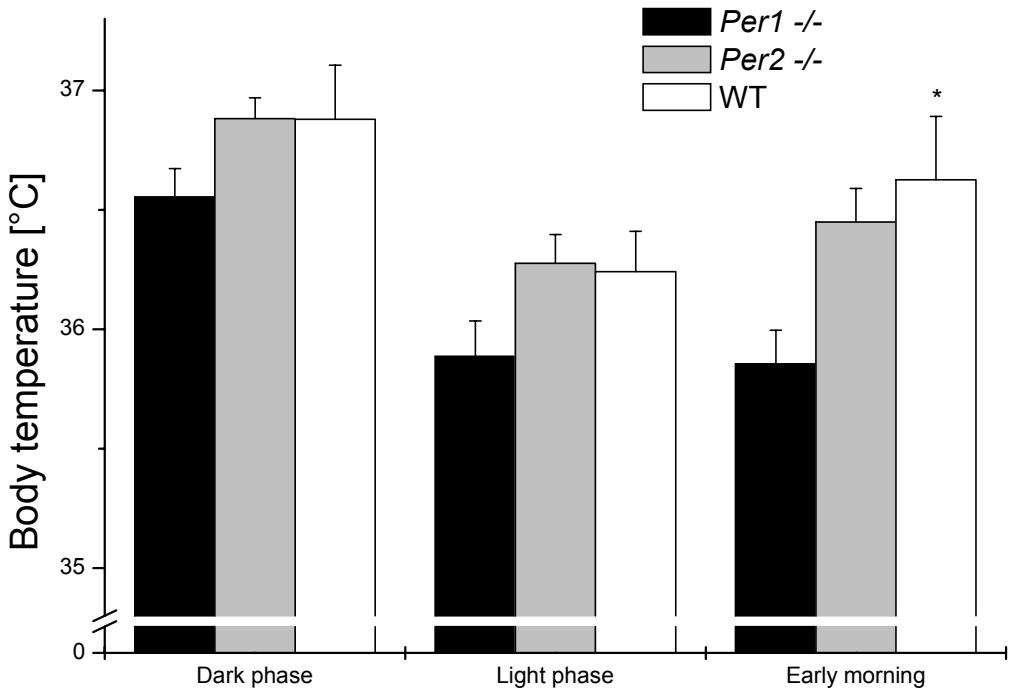

Figure 14. Mean T_b for dark phase, light phase, and first hour after light onset. Values are given as mean ± sem. Each value represents mean ± sem from *Per1* $^{-/-}$ (n = 7), *Per2* $^{-/-}$ (n = 8), WT (n = 8) animals from at least 5 consecutive nights. * statistically significant *vs. Per1* $^{-/-}$, $p < 0.05$.

The raw data of T_b recordings of a previous study (Steinlechner *et al.*, 2002), which were made with a slightly different setup (21 °C ambient temperature, macrolon type III cages equipped with running wheels, animals four months of age at the beginning of the experiment). These data were re-analysed in order to validate the low T_b recordings of *Per1* $^{-/-}$ animals. Figure 15 shows a daily T_b course under LD 12:12 conditions. For the *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals the amplitude of the T_b rhythm was 2.5 °C, 1.9 °C and 1.4 °C, respectively. It is noteworthy that the amplitude of the T_b rhythm in the *Per1* $^{-/-}$ animals is twice as high as in the experiment in the thermoneutral zone. The two other genotypes, however, do not show such a high increase in the amplitude of T_b in Steinlechner *et al.* (2002).

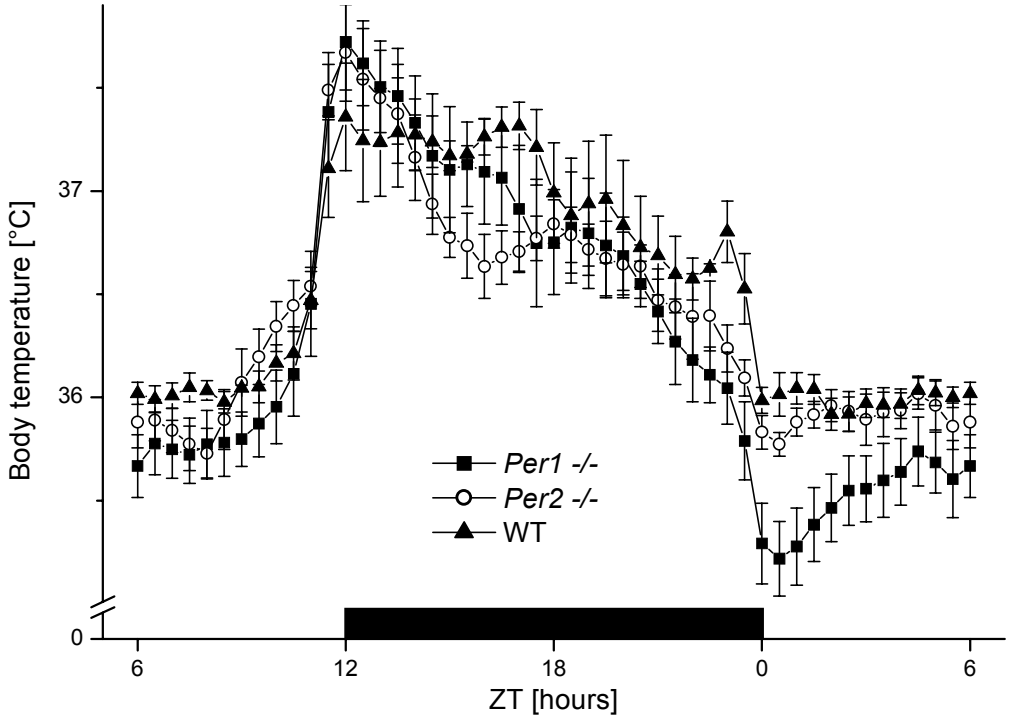

Figure 15. Time course of T_b over a 24–hour period under entrained conditions. The black bar indicates the dark phase. Data are given as 30 min mean ± sem. Group size: *Per1* $^{-/-}$ n = 6, *Per2* $^{-/-}$ n = 6, WT n = 4.

In contrast to the results of the present study, the *Per1* $^{-/-}$ animals of Steinlechner *et al.* (2002) did not show significant differences in T_b either as a 24-hour mean ($F(2,13) = 1.554$, $p = 0.2483$, n.s.) or in a night time mean ($F(2,13) = 0.476$, $p = 0.6318$, n.s.; Figure 16). During the light phase, however, in that study the *Per1* $^{-/-}$ animals exhibited a significantly lower T_b ($F(2,13) = 4.177$, $n = 0.0397$). The difference is most prominent in the first hour of the light phase ($F(2,13) = 11.7$, $p = 0.0012$). The *post hoc* comparison revealed no pair significances for the light phase mean, but there were pair significances for the first hour after lights on. The mean difference between *Per1* $^{-/-}$ and WT is 0.75 °C ($p < 0.0018$) and *Per2* $^{-/-}$ is 0.48 °C ($p < 0.0184$)

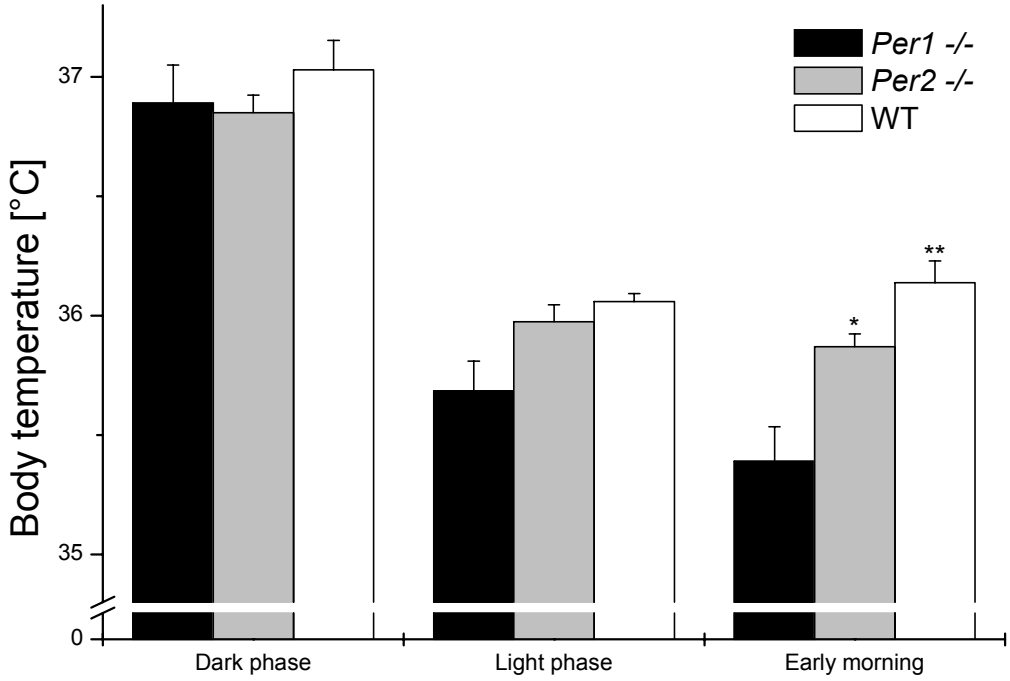

Figure 16. Mean T_b for dark phase, light phase, and early morning (ZT0 to ZT1). Each column represents mean ± sem of *Per1* $^{-/-}$ (n = 6), *Per2* $^{-/-}$ (n = 6), and WT (n = 4). Statistical differences *vs. Per1* $^{-/-}$ are noted as: * $p < 0.05$, ** $p < 0.01$.

3.4 Behavioural phenotyping

Feeding

The amount of food and water consumed over a period of 24 hours was measured by simply weighing the food hopper and the water bottle each day at ZT9. In the analysis, there were significant differences among the genotypes in the feeding and drinking amounts. Because the genotypes differed in weight (see above), all values were presented both as the amount per individual mouse and as the amount per 30 g mouse. This hypothetical standard mouse was introduced in order to represent the intake per g bodyweight, and at the same time to illustrate the intake in relation to the actual intake per mouse. There were differences both in the amount of food consumption per mouse and in the amount of food consumption per 30 g of mouse (Figure 17). Similar results were obtained for water consumption (Figure 18).

If calculated as food intake per animal, the *Per2* $^{-/-}$ animals ate more (5.5 ± 0.2 g), while the *Per1* $^{-/-}$ (4.4 ± 0.1 g) and the WT (4.3 ± 0.2 g) animals had the same lower food intake (Figure 17). The ANOVA revealed a significant result ($F(2,28) = 16.277$, $p < 0.0001$), and the *post hoc* test turned out to be significant between *Per2* $^{-/-}$ and *Per1* $^{-/-}$ ($p = 0.0003$) as well as WT ($p = 0.0001$). When the body weight of the animals is taken into account, it becomes clear that the food intake per 24 hours was higher for both the *Per1* $^{-/-}$ (5.8 ± 0.3 g) and *Per2* $^{-/-}$ (5.4 ± 0.2 g) than for WT (4.5 ± 0.2 g). Again, the ANOVA indicate differences between the genotypes ($F(2,28) = 6.623$, $p = 0.0044$), which turned out to be significant between WT and *Per1* $^{-/-}$ ($p = 0.0051$) as well between WT and *Per2* $^{-/-}$ ($p = 0.0494$).

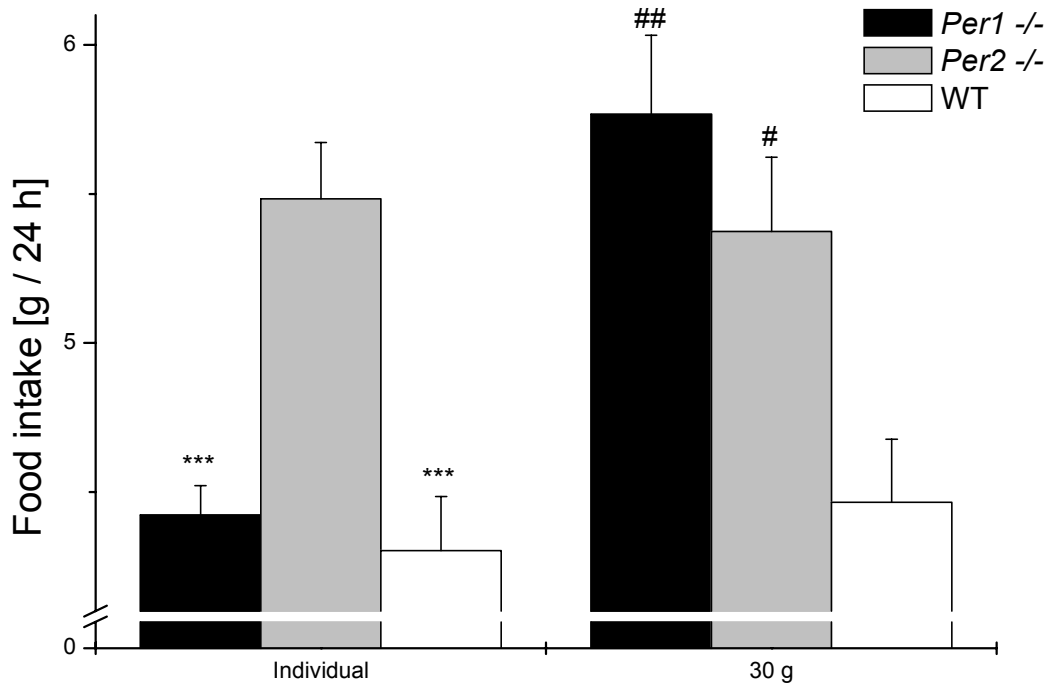

Figure 17. Twenty-four hour food consumption of *Per1* $^{-/-}$ (n = 10), *Per2* $^{-/-}$ (n = 12) and WT (n = 9), depicted as group mean ± sem. Significant difference *vs. Per2* $^{-/-}$ is indicated as *** $p < 0.001$ and *vs.* WT, as # $p < 0.05$, ## $p < 0.01$.

As indicated above, the results for water intake are similar to those for the food intake. The calculation for individual animals shows that the *Per2* $^{-/-}$ (6.6 ± 0.3 g) mice drank more than the *Per1* $^{-/-}$ (4.9 ± 0.3 g) and WT (4.7 ± 0.3 g) animals (Figure 18). Again, these

differences were significant in the ANOVA ($F(2,28) = 11.972$, $p = 0.0002$) and in the pairwise *post hoc* test among *Per2* $^{-/-}$ and *Per1* $^{-/-}$ ($p = 0.0018$) as well as *Per2* $^{-/-}$ and WT ($p = 0.0008$). The calculation per 30 g mouse shows that the WT (4.4 ± 0.4 g) animals consumed less water than either the *Per1* $^{-/-}$ (6.3 ± 0.3 g) or the *Per2* $^{-/-}$ (6.4 ± 0.4 g). Obviously, these differences were significant in the ANOVA ($F(2,28) = 11.997$, $p = 0.0009$) and subsequent *post hoc* comparison revealed significant differences among *Per1* $^{-/-}$ and WT ($p = 0.0047$) as well as between *Per2* $^{-/-}$ and WT ($p = 0.0025$).

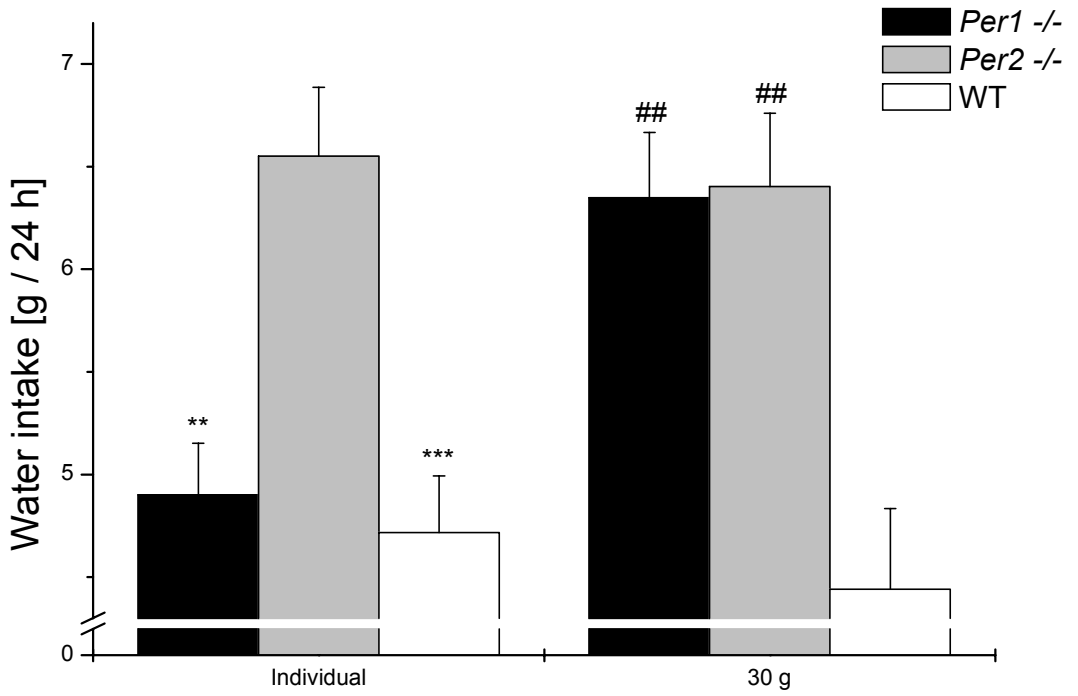

Figure 18. Twenty-four hour water consumption of *Per1* $^{-/-}$ (n = 10), *Per2* $^{-/-}$ (n = 12) and WT (n = 9) depicted as group mean ± sem. Significant difference *vs.* *Per2* $^{-/-}$ is indicated as ** $p < 0.01$, *** $p < 0.001$, and *vs.* WT as ## $p < 0.01$.

Reflexes

Table 2 shows the results for the testing of reflexes. No consistent deficiencies were found by any of these tests. Although there some animals did not respond to the auditory stimulus, this was very likely due to a stress-related phenomenon.

Table 2 Summary of neurological reflexes Abbreviations: + positive result

	Type of reflex				
Genotype	balance	eye blink	ear twich	whiskers	pupil
Per1 $^{-/-}$	+	+	+	+	+
Per2 $^{-/-}$	+	+	+	+	+
WT	+	+	+	7 of 9	+

Wire hanging

All *Per1*$^{-/-}$ (10 of 10) and WT (9 of 9) animals were easily capable of maintaing their grip on the wire for 60 seconds (cut-off time), but most of the *Per2*$^{-/-}$ mice failed in this task (10 of 12). The animals that lost their grip could not hang for more than 10 seconds without falling of. Interestingly, these were the heaviest animals of all under comparison here.

Pole test

Figure 19 depicts the result for all genotypes. The *Per2*$^{-/-}$ (n = 12) animals needed the most time to make the 180 ° turn (99.8 ± 11.0 s), and it took them the longest to reach the bottom of the pole (106.2 ± 9.3 s). In comparison, it took the *Per1*$^{-/-}$ (n = 10) and the WT (n = 9) animals 59.8 ± 11.9 s and 72.4 ±14.3 s, respectively, to make the 180 ° turn and they needed a total of 76.8 ± 9.9 s and 83.1 ± 12.3 s, respectively, to reach the ground. The large difference in mean group values is largely due to the fact that 9 out of 12 *Per2*$^{-/-}$ individuals did not even manage to turn 180 °. Neither parameter, however, revealed any statistically significant differences between the genotypes (time to 180 ° $F(2,28) = 3.001$, $p = 0.0659$, n.s.; time to ground $F(2,28) = 2.379$, $p = 0.1111$, n.s.).

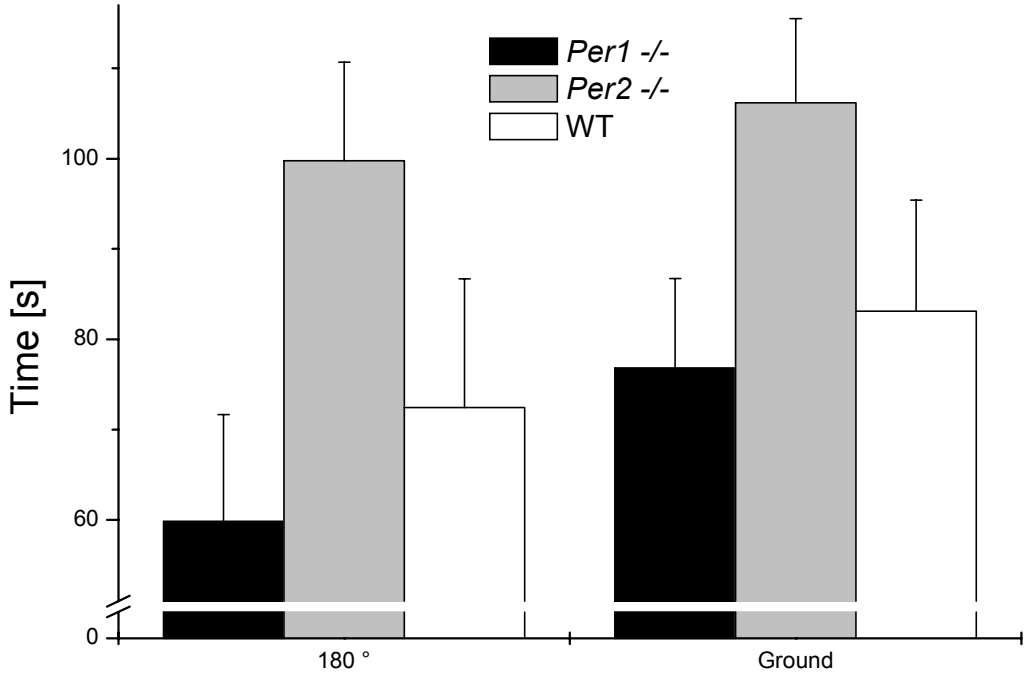

Figure 19. Results for the time to turn 180 ° on the pole (180 °) and to reach the ground (Ground) as group mean ± sem. Group size of *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT was n = 10, n = 12 and n = 9, respectively.

Open field

The results from the open field can be categorised as follows: (1) where the animals stop, and (2) how much the animals move in the open field. As expected, all animals showed a preference for the periphery over the central area of the open field (Figure 20). All animals spent more than 80% of the 10 min test time in the periphery of the open field. The mean time spent in the periphery was 546 ± 10 s, 525 ± 9 s, and 515 ± 14 s for *Per1* $^{-/-}$, *Per2* $^{-/-}$, and WT, respectively. These differences between the genotypes were not statistically significant ($F(2,29) = 1.940$, $p = 0.162$, n.s.).

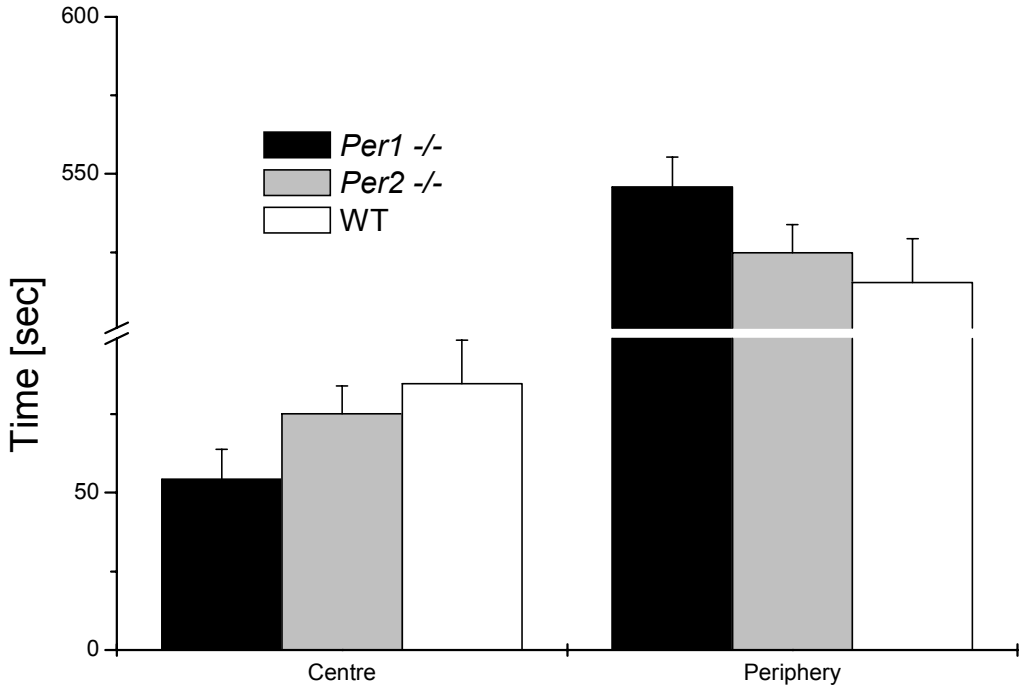

Figure 20. Place preferences of the mice for the different compartments of the open field. Data are shown as mean ± sem. Group size of *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals was 10, 12 and 9, respectively.

Another parameter of the open field data is presented in Figure 21, i.e. the latency of the animals to reach the periphery after the start of the experiment. The *Per1* $^{-/-}$ and WT mice needed 3.9 ± 1.5 s and 4.5 ± 1.8 s, respectively, while the *Per2* $^{-/-}$ (10.5 ± 2.6 s) took longer. Note that this trend did not reach the level of statistical significance in the ANOVA ($F(2) = 3.128$, $p = 0.0589$, n.s.), which might be due to the large variation. This is also reflected by the low power of the analysis (Power = 0.548).

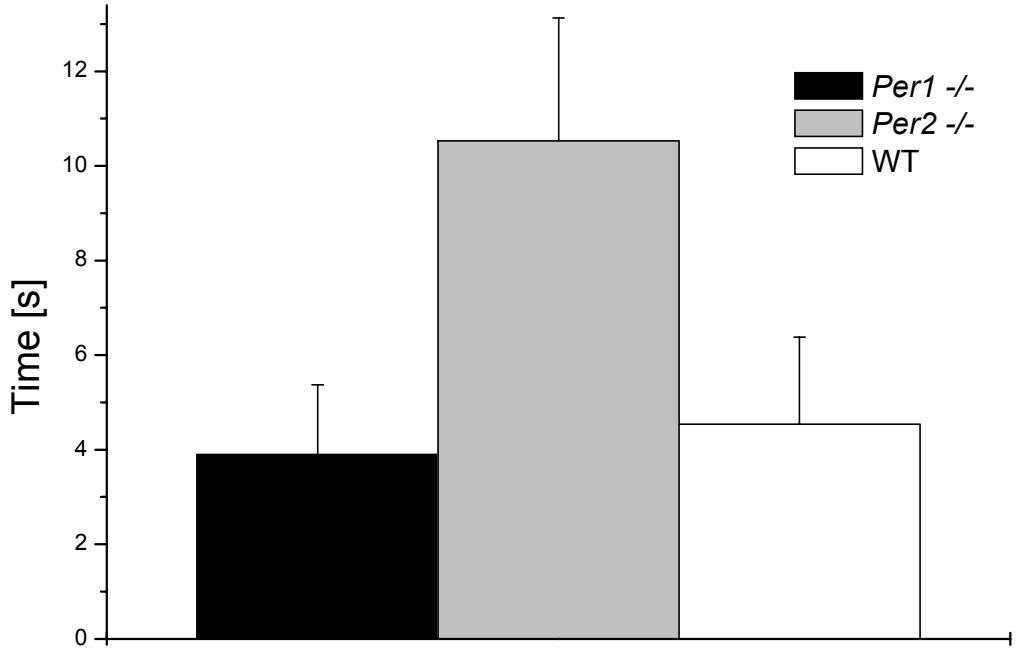

Figure 21. Latency to reach the pheripherie after the start of the open field experiment. Data are shown as mean ± sem. Group size of *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals was 10, 12 and 9, respectively.

Nevertheless, there were significant differences among the genotypes, namely in the travelled distance (Figure 22). The total distance travelled through the open field differed significantly between the genotypes ($F(2,29) = 4.086$, $p = 0.0273$). *Post hoc* comparison revealed a significantly shortened distance travelled by the *Per2* $^{-/-}$ (4581 ± 271 cm) compared to the *Per1* $^{-/-}$ (5637 ± 233 cm) mice ($p = 0.382$), but no significant differences between WT (5381 ± 325 cm) and any of the mutants (*vs. Per1* $^{-/-}$: $p = 0.8217$, *vs. Per2* $^{-/-}$: $p = 0.1406$). If the distance travelled in the periphery and in the centre are considered separately, there were significant differences between genotypes for the periphery ($F(2,29) = 5.043$, $p = 0.132$), but not the centre ($F(2,29) = 1.030$, $p = 0.3698$, n.s.). Again, the only pairwise significant difference was between *Per1* $^{-/-}$ and *Per2* $^{-/-}$ ($p = 0.145$).

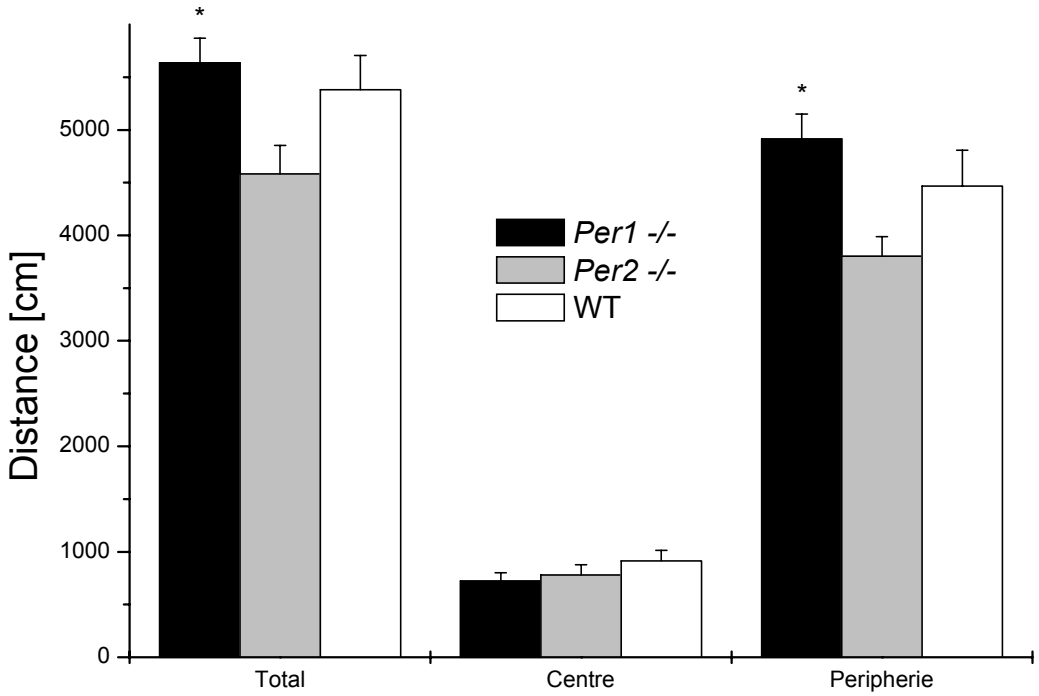

Figure 22. Distance travelled in a 10 min open field session. Group size of *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals was 10, 12, and 9, respectively. Data are shown as mean ± sem. Level of significance *vs. Per2* $^{-/-}$ is shown as * $p < 0.05$.

Additionally, the defecation rate in the open field was recorded as the number of fecal boli. Figure 23 shows that the *Per1* $^{-/-}$ defecated least (1.3 ± 0.7 boli, $n = 8$), and that the defecation rate was higher in WT animals (3.5 ± 0.9 boli, $n = 10$), and highest in *Per2* $^{-/-}$ (6.5 ± 0.5 boli, $n = 12$). These differences were found to be significant by ANOVA ($F(2,27) = 11.78$, $p = 0.0002$), and subsequent *post hoc* testing showed a significantly higher defecation rate in *Per2* $^{-/-}$ than in *Per1* $^{-/-}$ ($p = 0.0003$) and WT animals ($p = 0.0226$).

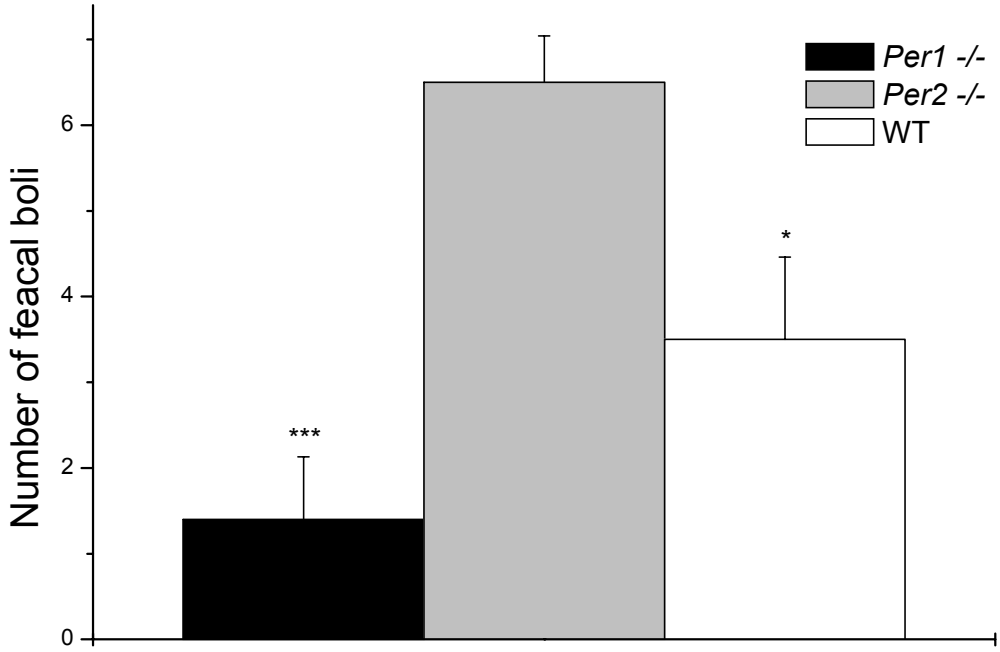

Figure 23 The defecation rate as number of fecal boli in the open field is given for each genotype as mean ± sem. Group size of *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals was 10, 12 and 9, respectively. Level of significance *vs. Per2* $^{-/-}$ is shown as * $p < 0.05$, *** $p < 0.001$

3.5 Hot plate

The hot plate test was done in order to look for abnormalities in nociception.

The genotypes differed in their responses to the unhabituated form of this task. Whereas WT animals endured only 10.7 ± 0.7 s on the hot plate, the *Per1* $^{-/-}$ and *Per2* $^{-/-}$ animals showed no signs of pain for 13.5 ± 3.0 s and 13.6 ± 0.7 s, respectively (Figure 24). There were significant differences in the ANOVA ($F(2,28) = 6.947$, $p = 0.0035$), and *post hoc* comparison showed that WT endured for shorter times than *Per1* $^{-/-}$ ($p = 0.142$) and *Per2* $^{-/-}$ ($p = 0.0081$). In contrast, the transgenic genotypes did not differ in this respect ($p = 0.9939$).

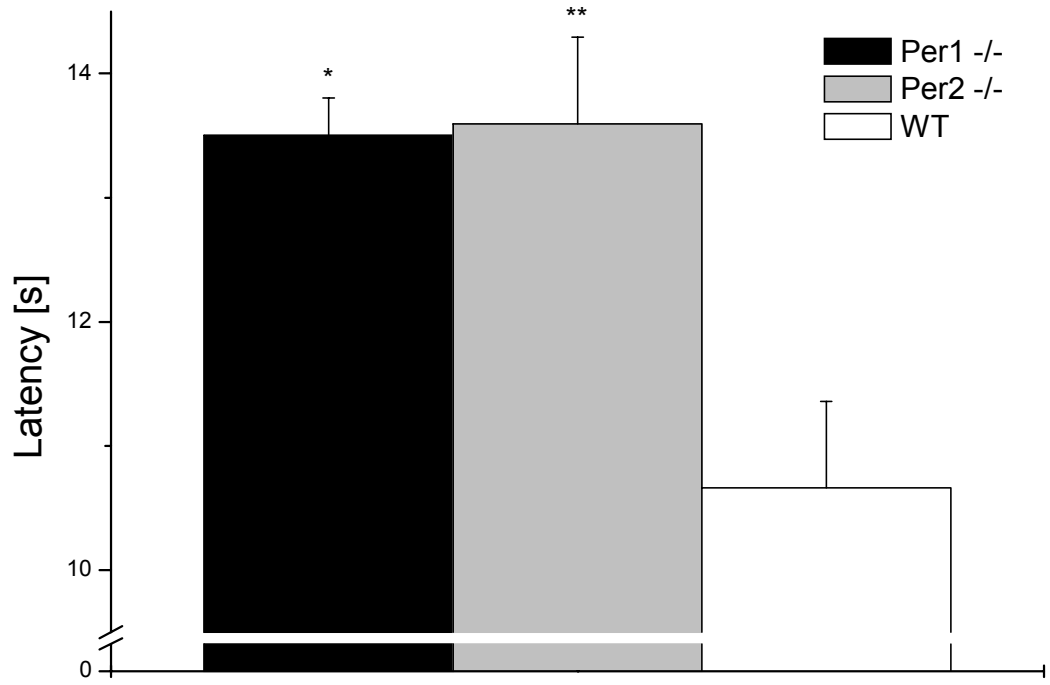

Figure 24. Nociception of naive mice in the hot plate task. The latency (to lick paws or to jump) after being placed on a 52.5 °C hot plate was measured. Group size was *Per1* $^{-/-}$ n = 10, *Per2* $^{-/-}$ n = 12, WT n = 9. Data are given as mean ± sem. Significant *vs.* WT * $p < 0.05$, ** $p < 0.01$

In addition, another set of animals (*Per1* $^{-/-}$ n = 8, *Per2* $^{-/-}$ n = 8, WT n = 7, only females) was tested in a habituated version of this task in order to exclude a "hyperalgesic-like response" (Karl *et al.*, 2003a), which might be due to stress. The animals were tested at ZT9 for basal (stressed) values and after 4 days of habituation, i.e. 3 min of exploration on the unheated hot plate. The basal test verified the results of the previous test, showing a higher latency in the naive *Per1* $^{-/-}$ (20.8 ± 1.4 s) and *Per2* $^{-/-}$ (23.5 ± 1.3 s) than in the WT (13.1 ± 1.0 s) animals. This difference was highly significant in the ANOVA ($F(2,20) = 17.982$, $p < 0.0001$), and subsequent *post hoc* testing showed the differences to *Per1* $^{-/-}$ *vs.* WT ($p = 0.0014$) and *Per2* $^{-/-}$ *vs.* WT ($p < 0.0001$). In the habituated (re-)test, the latency was decreased in all three genotypes (*Per1* $^{-/-}$ 14.6.1 ± 0.6 s, *Per2* $^{-/-}$ 17.3 ± 1.3 s, WT 11.0 ± 1.0 s). These differences were significant in a paired t-test (*Per1* $^{-/-}$ $p = 0.0091$, *Per2* $^{-/-}$ $p = 0.0036$, WT $p = 0.0235$). Although the genotypes again showed significant differences in

the habituated test ($F(2,20) = 9.541$, $p = 0.0012$), these differences show minimal attenuation. The p*ost hoc* testing revealed no differences between *Per1*$^{-/-}$ and WT ($p = 0.0711$) animals. The *Per2*$^{-/-}$ mice showed a less pronounced, but significantly higher latency ($p = 0.0012$). Taken together, these results show a clear attenuating effect of habituation on the latency in the hot plate task, which is most obvious in the *Per1*$^{-/-}$ and *Per2*$^{-/-}$. This effect abolished the difference between *Per1*$^{-/-}$ and WT, but not the elevated latency in *Per2*$^{-/-}$ as compared to WT mice.

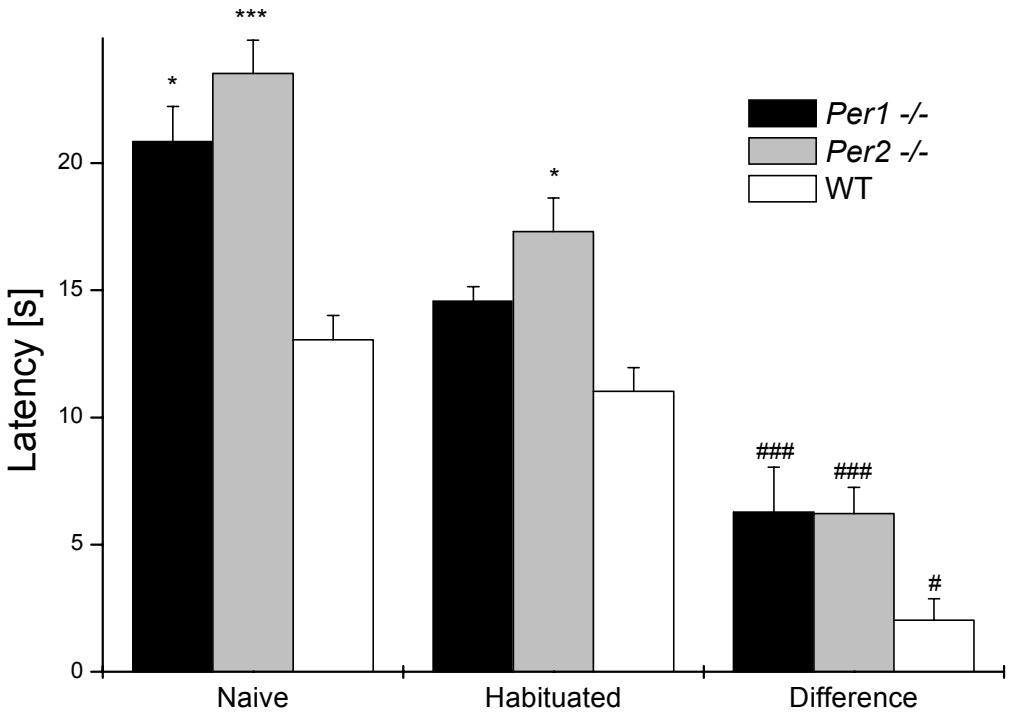

Figure 25. Nociception of the mice in the habituated hot plate task. The latency (to lick paws or to jump) after being placed on a 52.5 °C hot plate was measured in naive (*left*) and habituated (*middle*) mice. The level of significant difference in latency between the two sessions (*right*) in a paired t-test is given as # $p < 0.05$, ### $p < 0.001$. Significant differences *vs.* WT are given as * $p < 0.05$, *** $p < 0.001$. Group size was *Per1* $^{-/-}$ n = 8, *Per2* $^{-/-}$ n = 8, WT n = 7. Data are given as mean ± sem.

3.6 Glucose tolerance test

The glucose tolerance of the animals was tested by i.p. injection of glucose solution after an overnight fasting. Figure 26 shows the mean values of the initial fasting blood glucose levels and the subsequent measurements after i.p. administration of glucose solution. Group sizes were *Per1* $^{-/-}$ n = 17 (6 male, 11 female), *Per2* $^{-/-}$ n = 17 (6 male, 11 female), and WT = 20 (10 male, 10 female). Both *Per1* $^{-/-}$ and *Per2* $^{-/-}$ differed significantly from the wildtype animals in the basal level as well as in the tolerance test. The repeated measures ANOVA showed significant differences in the course of blood glucose level ($F(2,43) = 6.643$, $p = 0.0028$), and subsequent *post hoc* comparison revealed differences between *Per1* $^{-/-}$ and WT ($p = 0.0154$) as well as between *Per2* $^{-/-}$ and WT ($p = 0.0098$). This is confirmed by a one-way ANOVA with genotype as factor and the area under the glucose curve as a measure of the blood glucose level. Again, there was a significant difference in the ANOVA ($F(2) = 5.539$, $p = 0.0068$), and *post hoc* comparison revealed a significantly smaller area under the glucose curve for *Per2* $^{-/-}$ ($p = 0.0091$) and a nearly significant smaller area under the glucose curve for *Per1* $^{-/-}$ ($p = 0.0808$, n.s.) than for wildtype (Figure 27).

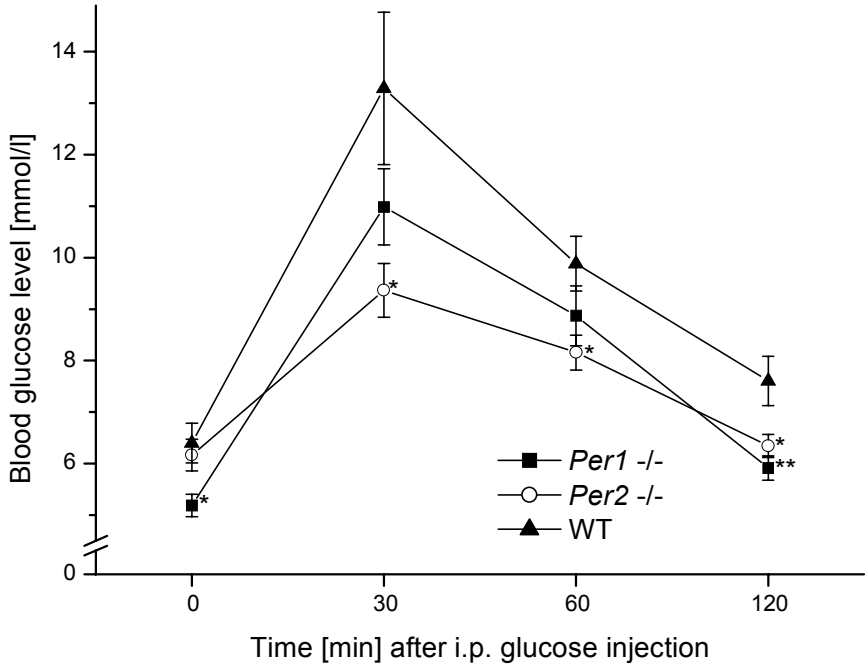

Figure 26. Glucose tolerance after overnight fasting in all three genotypes. The animals were injected with glucose solution (2 g/kg body weight) at minute zero (0). Blood was sampled directly before 30, 60 and 120 min following the injection. Group size: *Per1* $^{-/-}$ n = 17, *Per2* $^{-/-}$ n = 17, WT n = 20. Data are given as means ± sem. * $p < 0.05$, ** $p < 0.01$

In all three genotypes, the blood glucose level was significantly elevated ($F(3,144) = 48.576$, $p < 0.0001$) following treatment, but had readjusted to the basal level 120 min after the injection of glucose (*Post hoc* comparison 0 *vs.* 120, $p = 0.1176$, n.s.). One-way ANOVA was used to look at the four individual points in time. The three genotypes showed significant differences in all of these (0 min: $F(2,48) = 3.78$, $p = 0.0299$; 30 min: $F(2,48) = 3.956$, $p = 0.0275$; 60 min: $F(2,48) = 3.543$, $p = 0.0367$; 120 min: $F(2,48) = 6.814$, $p = 0.0025$). *Post hoc* comparison revealed that value of *Per1* $^{-/-}$ was significantly lower than that of WT at the initial ($p = 0.0350$) and 120 min ($p = 0.0044$) times, whereas *Per2* $^{-/-}$ was significantly lower at all but the initial times (30 min: $p = 0.0327$; 60 min: $p = 0.0494$, 120 min: $p = 0.0358$).

In principle, the test revealed a lowered basal blood glucose level and an increased glucose tolerance in the transgenic animals than in the wildtype. This indicates that the transgenic animals are able to clear a glucose load more easily than wildtype animals.

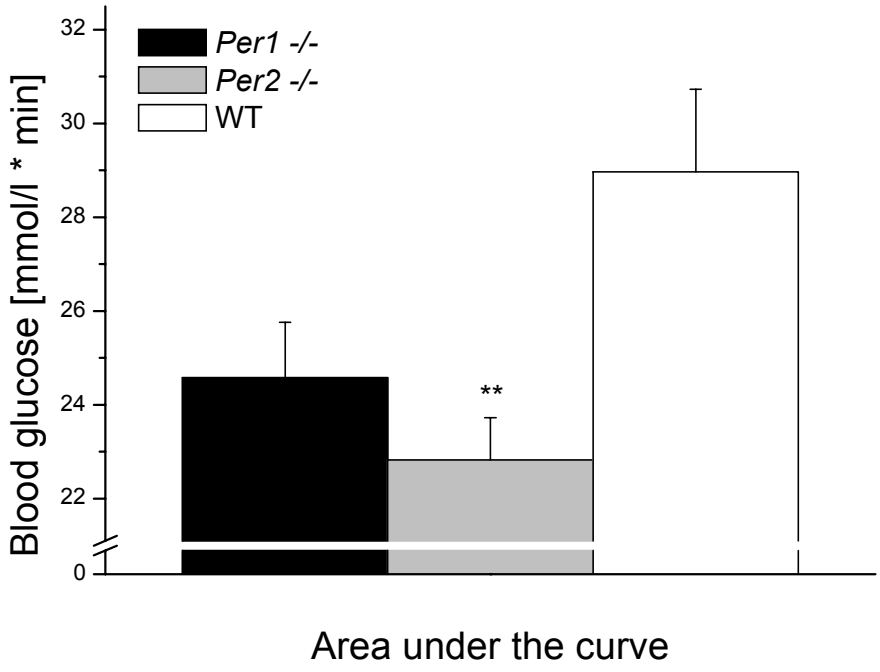

Figure 27. Results of glucose tolerance test given as the area under the curve for all four times. Group size: *Per1* $^{-/-}$ n = 17, *Per2* $^{-/-}$ n = 17, WT n = 19 (One WT had to be excluded because of a missing value). Data are given as means ± sem. ** $p < 0.01$.

3.7 Stress-induced hyperthermia

In stress-induced hyperthermia, the rise in rectal body temperature (T_b) was measured following a stressor. As the pre-tests showed (see also: van der Heyden *et al.*, 1997), rectal temperature recording itself is a potent stressor. Thus, in this study the mice were stressed only by the handling procedure and the rectal insertion of the temperature probe. Due to missing values, test group size differed among the genotypes. In total, 38 animals were included in the analysis (*Per1* $^{-/-}$ n = 15, *Per2* $^{-/-}$ n = 11, WT n = 12).

The differences in basal T_b observed in the telemetric recordings (see 5.3) of the data obtained from this experiment were evaluated in two ways: (1) the course of T_b during the test, and (2) the changes in T_b calculated relative to the initial T_b.

Figure 28 shows the mean group T_b for all genotypes under comparison. Although not statistically significant ($F(2) = 2.674$, $p = 0.0831$, n.s.), the *Per1*$^{-/-}$ (36.5 ± 0.2 °C) animals seem to have a slightly lower basal T_b than *Per2*$^{-/-}$ (37.0 ± 0.1 °C) and WT (37.1 ± 0.2 °C) animals. This is reflected by the fact that the repeated measures ANOVA becomes significant for the effect of the variable "genotype" ($F(2,36) = 3.628$, $p = 0.0367$) if calculated with T_b. This significant result, however, cannot be found when the repeated measures ANOVA is applied to the T_b changes ($F(2,36) = 1.627$, $p = 0.2107$).

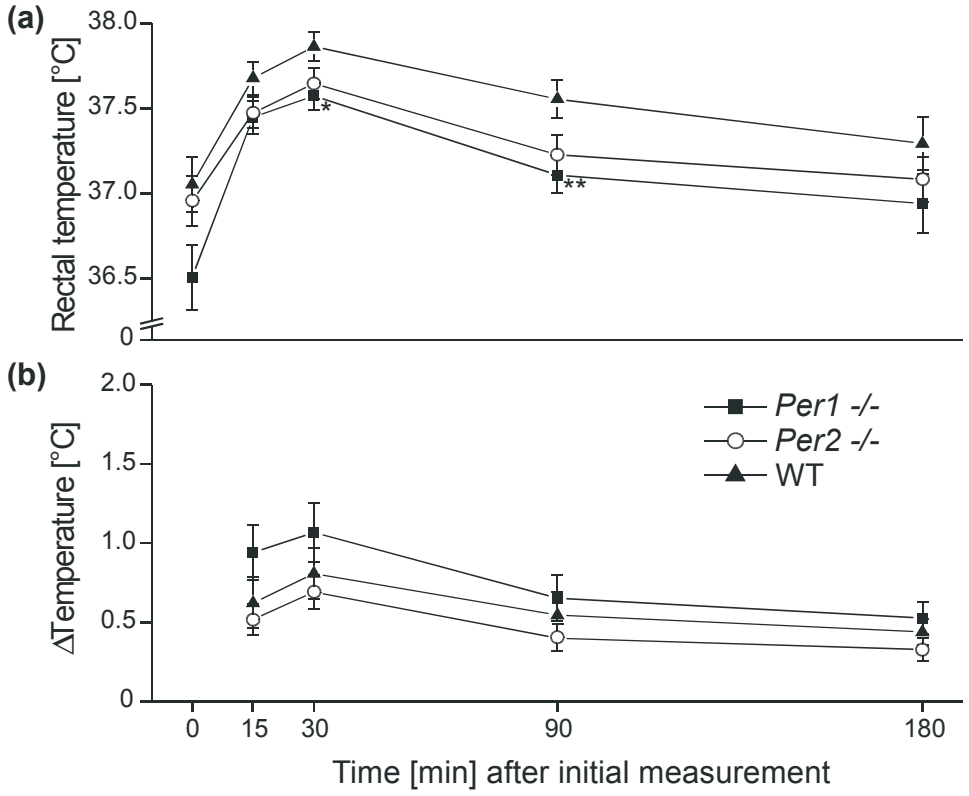

Figure 28. Rectal temperature recordings for the stress-induced hyperthermia test (a) as absolute values, and (b) as difference to initial measurement. Data are shown as mean ± sem. Group size was *Per1*$^{-/-}$ n = 15, *Per2*$^{-/-}$ n = 11, and WT n = 12.

These findings were confirmed by the analysis of the area under the temperature curves (Figure 29). The one-way ANOVA detected a significant difference between the genotypes

(F(2) = 3.708, p = 0.0346), namely, a smaller area under the T_b curve of the *Per1* $^{-/-}$ group compared to WT(p = 0.0355), but no such significances were found when computed with the T_b changes (F(2) = 1.454, p = 0.2474).

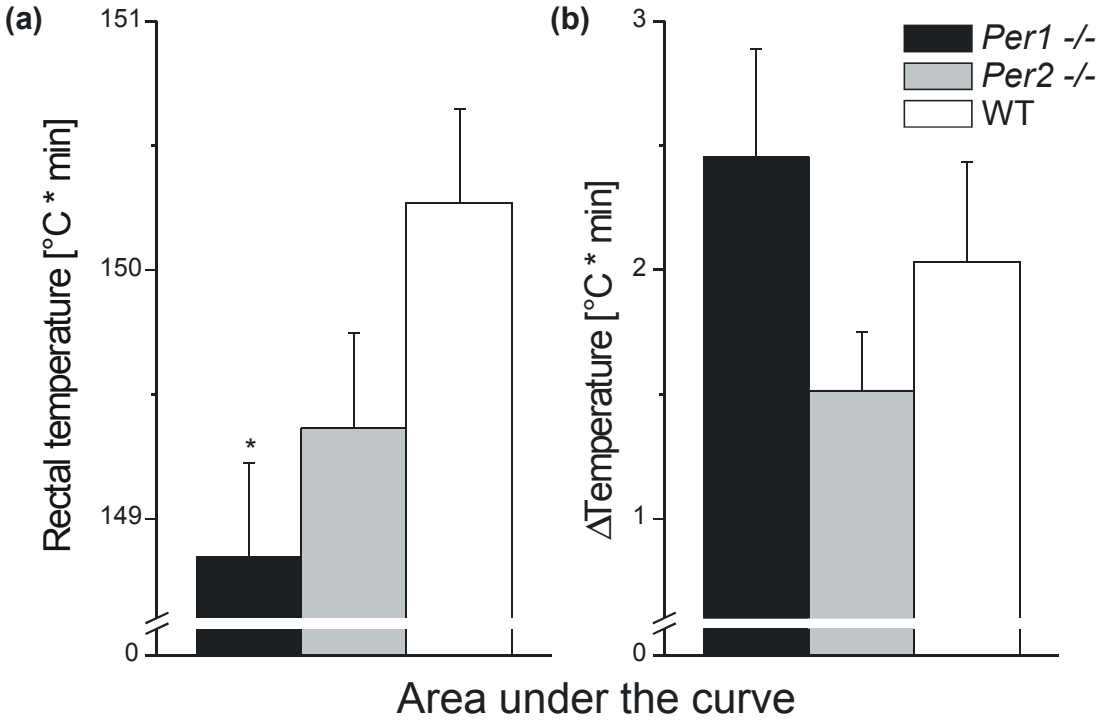

Figure 29. Area under the temperature curve for *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals. (a) Calculated with T_b, and (b) calculated with the T_b changes relative to the basal T_b.

3.8 LPS stimulation

Two parameters were determined after the stimulation with LPS: the body temperature reaction in thermoneutral environment, which was measured by intraperitoneally implanted transmitters, and the response of the cytokines TNF-α and IL1-β, which were measured by ELISA.

Body Temperature response

A total of 11 animals (*Per1* $^{-/-}$ n = 3, *Per2* $^{-/-}$ n = 4, WT n = 4) which had been implanted with temperature transmitters were injected i.p. with lipopolysaccharide (LPS) solution. Figure 30 shows the course of T_b shortly before and after the LPS injection. The *Per2* $^{-/-}$ and WT showed an increase in T_b after LPS injection. In contrast, the *Per1* $^{-/-}$ mice showed a

decrease in T_b. These effects lasted about six hours. Due to the large variation in the results and the low sample size, these data were tested for statistically significant differences.

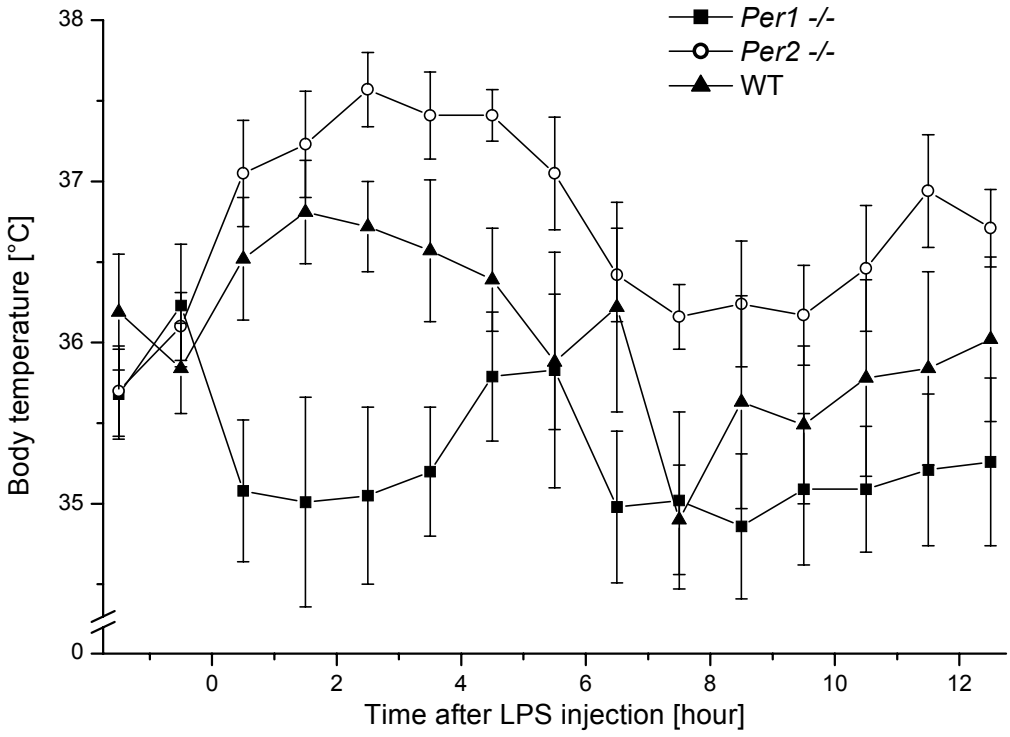

Figure 30. Time course of T_b shortly before and after the *i.p.* LPS injection. Group size is *Per1*$^{-/-}$ n = 3, *Per2*$^{-/-}$ n = 4, WT n = 4. Data is given as mean ± sem.

Cytokine response

The results of the TNF-α ELISA are given in Figure 31. In all three genotypes, the stimulated samples show elevated values compared to the basal values. Due to the large variation, these differences and thus those between the genotypes are not statistically significant. Nevertheless, it seemed that the *Per2*$^{-/-}$ mice exhibited somewhat elevated TNF-α levels compare to WT and *Per1*$^{-/-}$ mice.

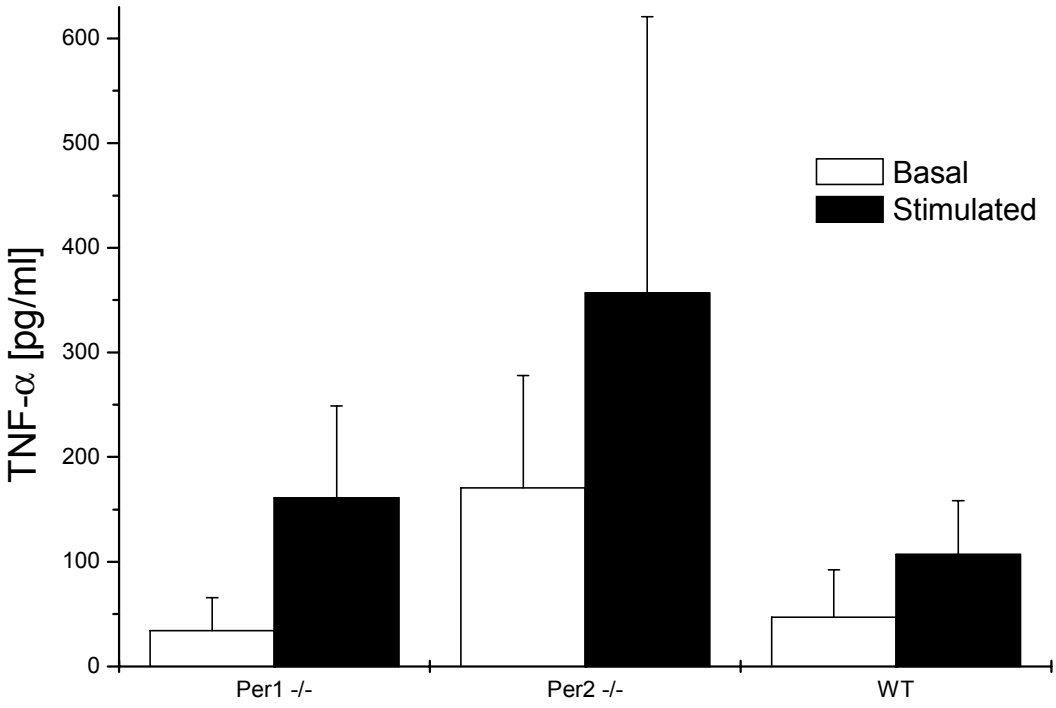

Figure 31. Results of the TNF-α ELISA of *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals before (basal) and after (stimulated) LPS injection. Group size *Per1* $^{-/-}$ n = 4, *Per2* $^{-/-}$ n = 4, WT n = 3. Data are given as mean ± sem.

The second experiment was done without implanted temperature transmitters. The basal levels of IL1-β were not detectable in the ELISA. The stimulated levels, however, could be measured and the results are given in Figure 32. Although the *Per2* $^{-/-}$ (7.5 ± 3.8 pg/ml) animals seemed to show a decreased reaction following the LPS stimulus compared to the *Per1* $^{-/-}$ (68.5 ± 30.0 pg/ml) and WT (58.8 ± 14.2 pg/ml) animals, there were no significant differences between the genotypes ($F(2,14) = 2.539$, $p = 0.1146$, n.s.).

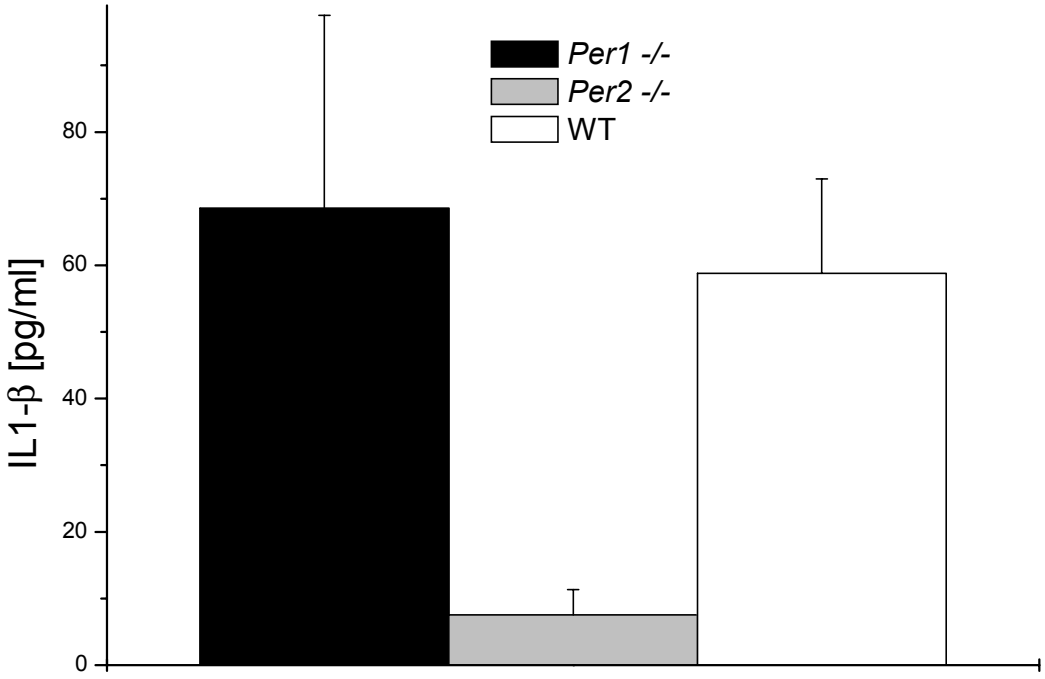

Figure 32. Results of the IL1-β ELISA of *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals before (basal) and after (stimulated) LPS injection. Group size *Per1* $^{-/-}$ n = 6, *Per2* $^{-/-}$ n = 5, WT n = 6. Data are given as mean ± sem.

3.9 Phagocytosis assay

A quantitative phagocytosis assay was used for the evaluation of the phagocytosis properties of the macrophages. The blood was collected from eleven *Per1*$^{-/-}$, seven *Per2*$^{-/-}$, and seven WT mice, but samples were excluded if the percentage of phagocyting cells was below 15%. Thus, sample size was reduced to 8, 6, and 5 for *Per1*$^{-/-}$, *Per2*$^{-/-}$, and WT, respectively. A typical example of the results for one sample is given in Figure 33.

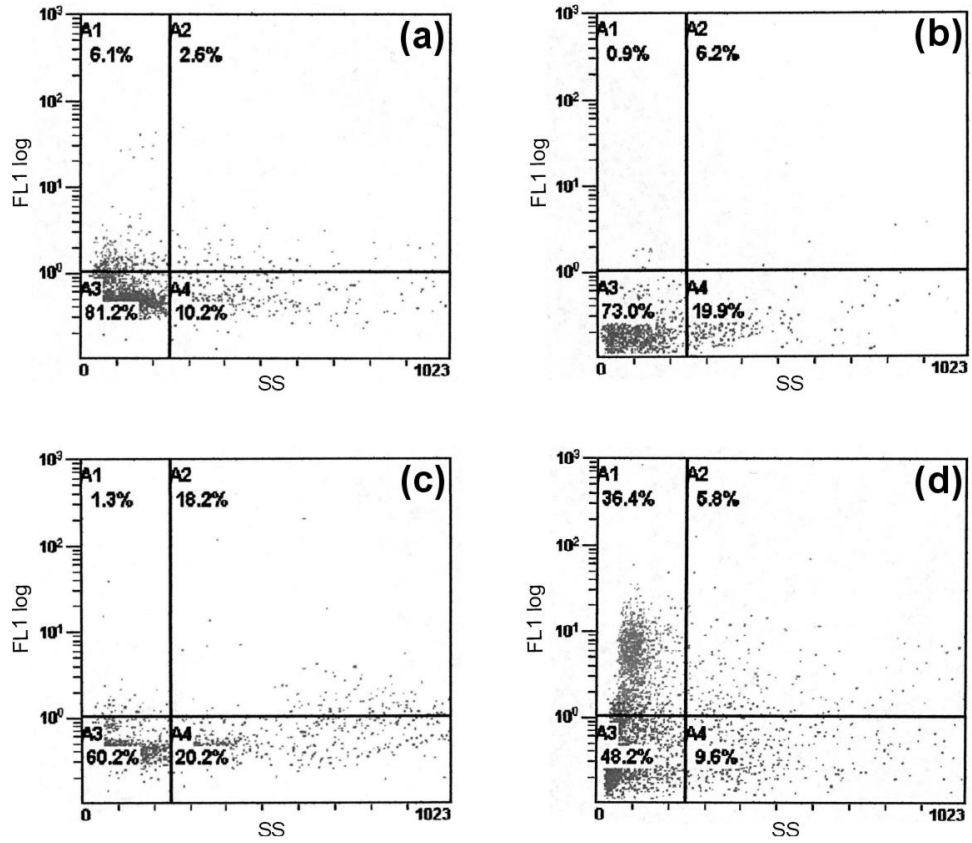

Figure 33. Results of flow cytometry depicted as dot plots of sidewards scatter (SS) *vs.* fluorescence 1 (FL1) after incubation (a + c) on ice, and (b + d) at 37 °C. (a) + (b) Typical example of an excluded sample. (c) + (d) Typical example of an included sample. See text for details.

Numerically, the results show the highest rate of phagocyting cells in *Per1*$^{-/-}$ (33.9 ± 3.6%), followed by WT (29.8 ± 2.4%) and *Per2*$^{-/-}$ (27.7 ± 3.8%, Figure 34). Statistical comparison, however, did not confirm this trend ($F(2,15) = 1.078$, $p = 0.3652$, n.s.). The number of particles that were ingested by the cells was not assessed because there were no

clear clusters of cells with a peak at a specific intensity in FL1. If judged by visual inspection, there did not seem to be any differences.

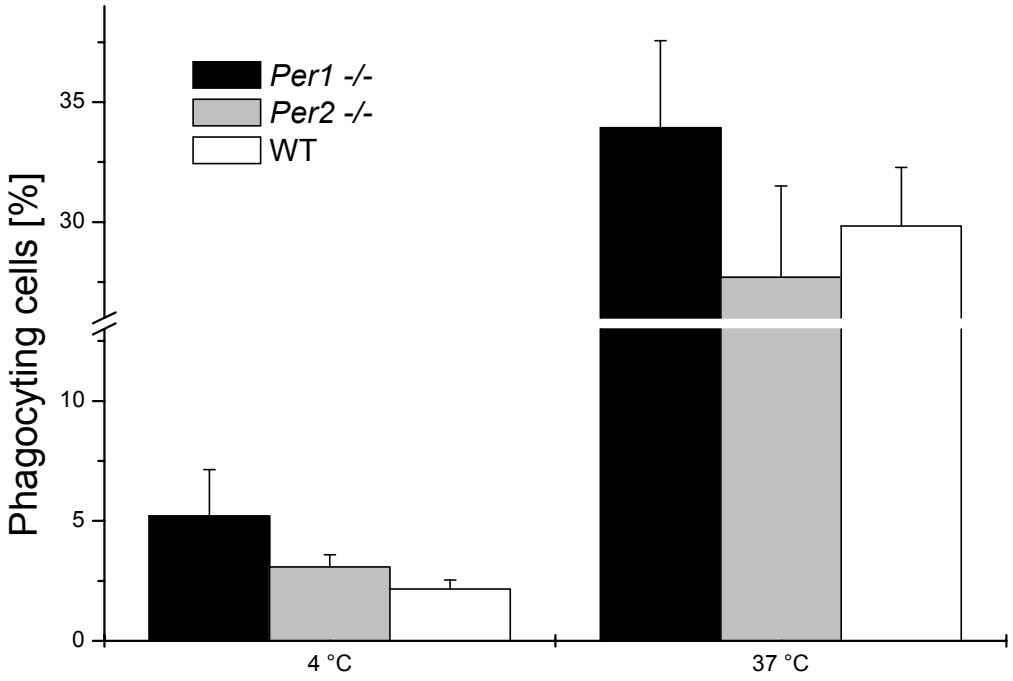

Figure 34. Result of the quantitative phagocytosis assay. Data are given as mean ± sem. Group size: *Per1* $^{-/-}$ n = 8, *Per1* $^{-/-}$ n = 6, WT n = 5. For details see text.

3.10 Hormonal rhythms in feces

The animals were held in metabolic cages for the sampling of hormonal rhythms in urine and feces. A total of 24 animals (n = 8 per genotype) were used, and the sampling interval was set to 3 hours.

Figure 35 shows the amount of feces per 3 hours over a 48-hour period. All genotypes under comparison showed a clear diurnal pattern in this parameter. Following the rhythm of food intake, the peak of excretion was at the end of the dark phase and the trough was during the light phase. The total amount of feces was highest in WT animals (1.6 ± 0.2 g, n = 8) and quite similar in *Per1* $^{-/-}$ (1.3 ± 0.1 g, n = 8) and *Per2* $^{-/-}$ (1.4 ± 0.1 g, n = 8) animals. These differences were not significant in a one-way ANOVA ($F(2,21) = 1.255$, $p = 0.3057$, n.s.). For

the analysis of the time course of feces amount, the 48-hour data were folded once in order to get more reliable values. In the repeated measures ANOVA with the factors *genotype* and *ZT*, the effect of *genotype* was not significant ($F(2,19) = 2.493$, $p = 0.1068$, n.s.). Nevertheless, there seemed to be a trend to more defecation in WT animals at ZT4 and ZT7, i.e. late at night. When these two times were compared separately by one-way ANOVA, the effect of genotype was significant for ZT4 ($F(2,19) = 4.076$, $p = 0.0315$), but not for ZT7 ($F(2,19) = 3.440$, $p = 0.0510$, n.s.).

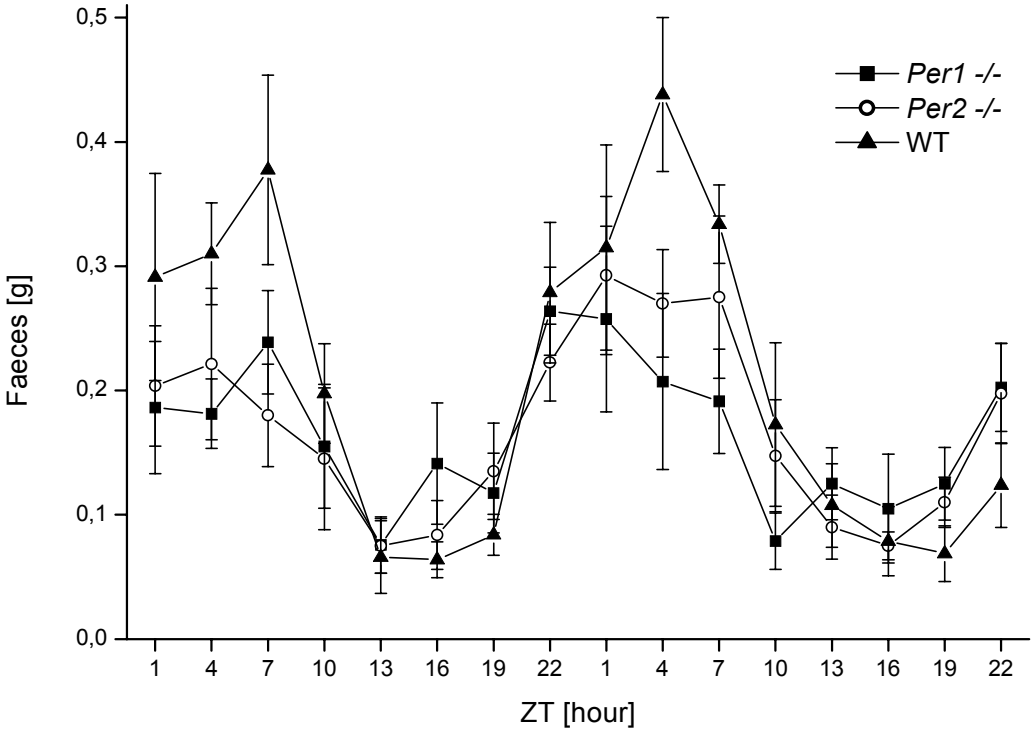

Figure 35. Amount of feces for n = 8 animals of each genotype over a 48-hour period with 3-hourly sampling intervals given as mean ± sem.

The analysis of fecal corticosterone metabolites revealed a clear diurnal pattern in the concentration of these metabolites in the *Per2* $^{-/-}$ and WT. This rhythm is characterised by high levels at night and low levels during the day. In contrast, the *Per1* $^{-/-}$ animals showed a constantly high level of corticosterone metabolites even throughout the day. This finding is reflected by the results of the repeated measures ANOVA for the respective genotypes.

Whereas the *Per2* $^{-/-}$ ($F(7,49) = 5.134$, $p = 0.0002$) and the WT ($F(7,49) = 4.261$, $p = 0.0010$) show significant differences between the early day and the early night in the *post hoc* comparison (*Per2* $^{-/-}$ ZT6 *vs.* ZT18 $p = 0.0398$; WT ZT3 *vs.* ZT15 $p = 0.0097$), the *Per1* $^{-/-}$ mice did not even show differences in the repeated measures ANOVA ($F(7,49) = 0.346$, $p = 0.9283$). In order to illustrate the amplitude of the daily variations, Figure 36 compares the trough and peak for each genotype.

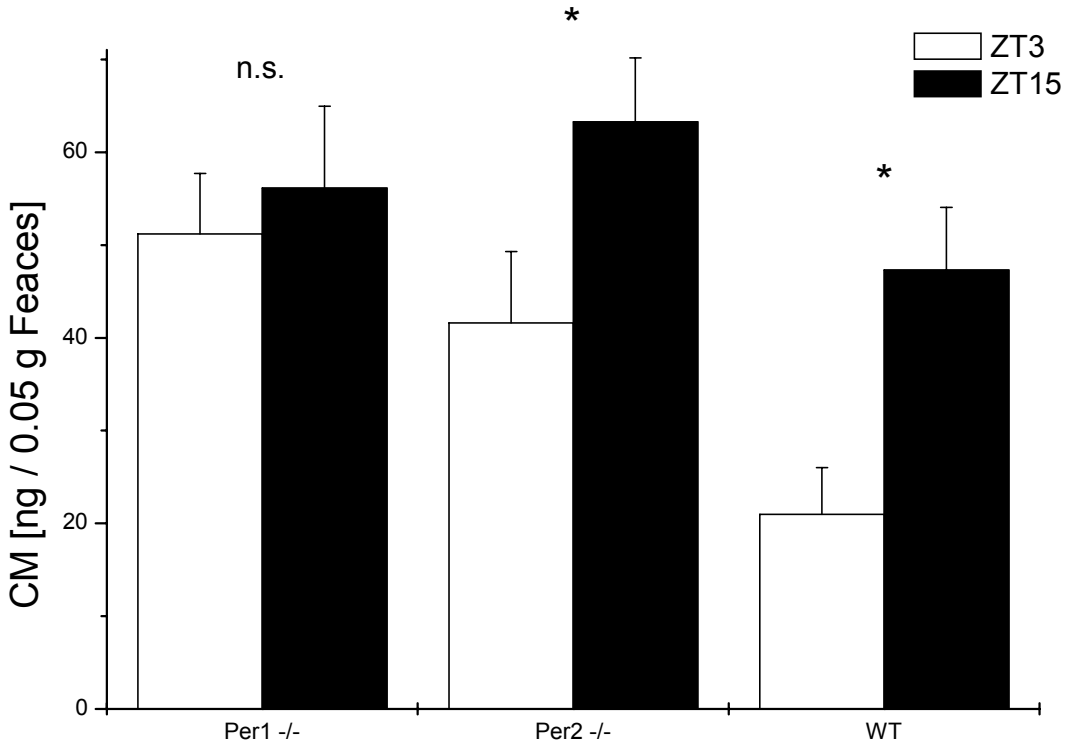

Figure 36. Corticosterone metabolites (CM) in feces of the *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT animals at the beginning of the day (ZT3) and the beginning of the night (ZT15) illustrating the absent daily variation in *Per1* $^{-/-}$ animals. Group size n = 8 per genotype. Data are given as mean ± sem.

The mean group values were folded once and plotted as 24-hour values in Figure 37. The values for each individual and each time are depicted in Appendix B. The data for the time course revealed statistical differences between the genotypes in the repeated measures ANOVA ($F(2,20) = 3.791$, $p = 0.0402$). In subsequent *post hoc* testing only *Per1* $^{-/-}$ and WT

animals were significantly different (p = 0.0444). Additionally, there seemed to be a trend towards higher values in the *Per2* $^{-/-}$ than in the WT animals (p = 0.0871).

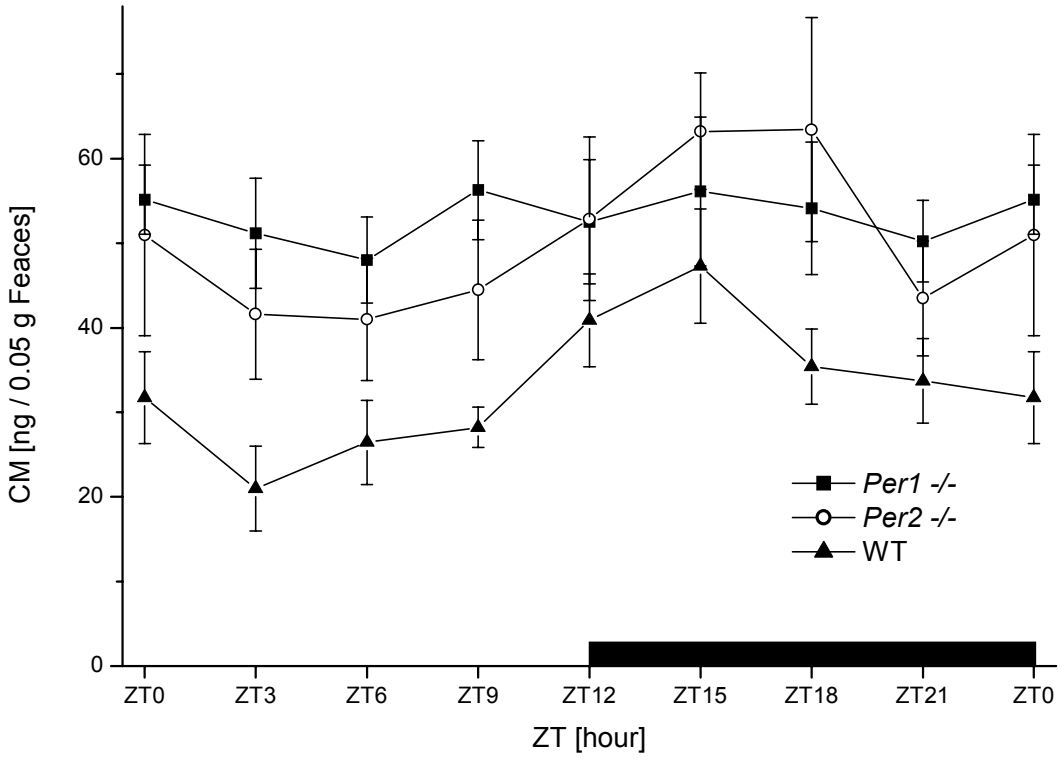

Figure 37. Time course of corticisterone metabolites in feces of *Per1* $^{-/-}$, *Per2* $^{-/-}$, and WT mice. Group size n = 8 per genotype. The black bar indicates the dark phase. Data are given as mean ± sem. Note that the values for ZT0 are plotted twice.

3.11 Drinking rhythms

The drinking rhythm of the mice was assessed by a lickometer developed by the author, the Lick-O-Mat. Drinking activity was shown to be a valuable parameter as output of the clock.

Lick-O-Mat validation experiment

In order to evaluate the Lick-O-Mat, we compared the data derived from PIDs with those simultaneously recorded by the Lick-O-Mat. The comparison was made under LD 12:12 as well as under constant conditions (DD). Figure 38 shows representative actograms of

one animal for both recording methods. Obviously, both methods are equally suitable for the determination of the daily patterns. A periodogram analysis revealed equal values for both methods. The free-running period of activity measured by PIDs was identical to the drinking activity measured by the Lick-O-Mat in DD. This indicates that both read-outs reflect the endogenous circadian rhythm with the same accuracy.

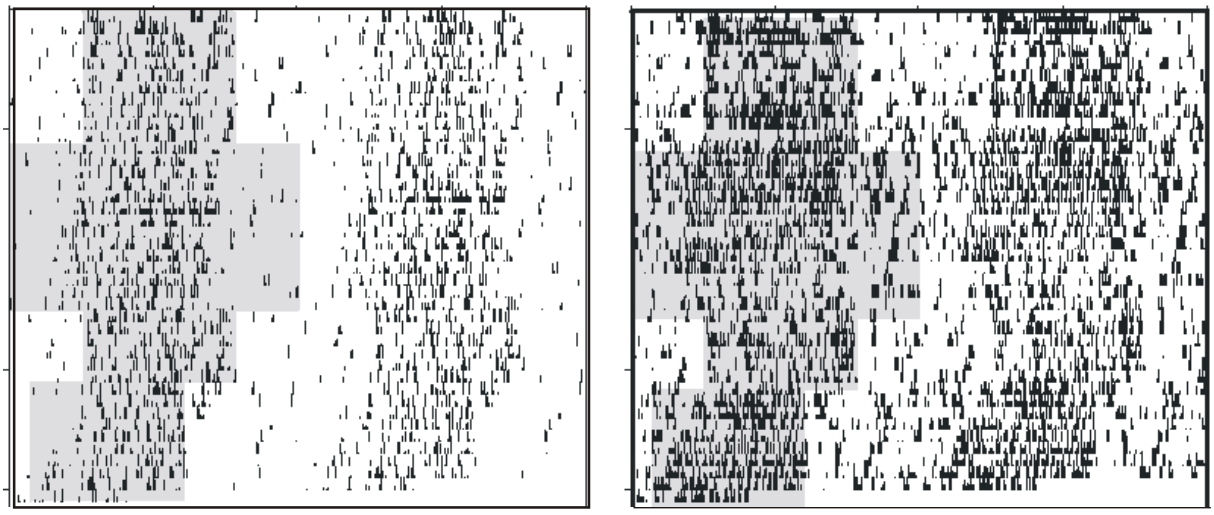

Figure 34. Representative double-plotted actogram of one B6 mouse over 40 days recorded with the Lick-O-Mat (*left*) and by a passive infrared detector (*right*). The dark phase is indicated by the shaded areas, and is shown on the left side of the double plot only to allow unbiased inspection of the actogram on the other side.

Ultradian rhythms in drinking activity

The drinking rhythm of *Per1*$^{-/-}$, *Per2*$^{-/-}$ and WT animals was investigated under varying light conditions. Both the actograms and the lickograms show an extraordinary amount of ultradian rhythms. The occurrence of ultradian rhythms has been previously shown for the *Per2*^{Brd} mice (Figure 3 of Zheng *et al.*, 1999). Here, the *Per1*$^{-/-}$ mice exhibited a strong ultradian rhythm, while the WT mice showed free-running behaviour (Figure 39). Unfortunately, there was a breakdown in the light control system during the first 13 days, so that the light regime was erratic, mainly constant darkness with some light events in it.

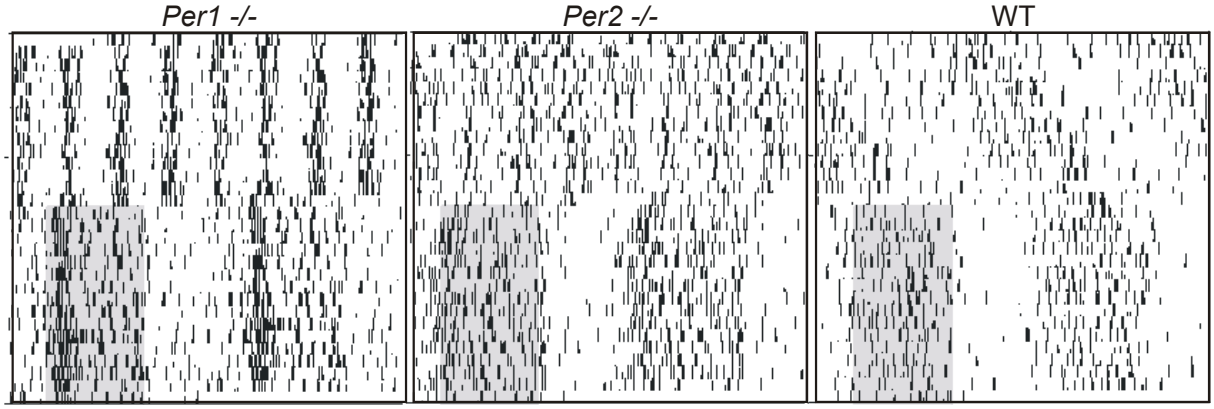

Figure 35. Representative double-plotted lickograms of one animal of each genotype over 30 days, the first 13 with a erratic LD cycle. and the the latter 17 with LD 12:12. The shaded area indicates the dark phase, and is shown on the left side of the double plot only to allow unbiased inspection of the actogram on the other side.

3.12 Constant light conditions for double-mutant animals

The two double mutants, the *Per1*$^{-/-}$ *Per2*$^{-/-}$ and the *Per2*$^{-/-}$ / *Cry1*$^{-/-}$, were tested for their free-running behaviour. Both became immediately arrhythmic in constant darkness but exhibited stable rhythmic behaviour under higher light intensities such as 200 and 300 lx. Whereas the τ of the WT mice became longer with higher light intensities (DD = 0 lx 24.2 ± 0.05 h; 5 lx 24.6 ± 0.02 h; 200 lx 25.0 ± 0.2 h) and became slightly disrupted in 300 lx, the double mutant mice exhibited a shortening of τ. It was shortest in the *Per2*$^{-/-}$ / *Cry1*$^{-/-}$ mice, with 20.5 ± 0.1 h in 200 lx and 19.7 ± 0.1 h in 300 lx (n = 9). The results for the *Per1*$^{-/-}$ / *Per2*$^{-/-}$ mice, (LL 300 lx: 22.15 ± 0.9 h, n = 2), were not as extreme as those for the *Per2*$^{-/-}$ / *Cry1*$^{-/-}$ mice. At, and correspond to those reported for *Per2*$^{-/-}$ mice (Steinlechner *et al.*, 2002). Unfortunately, the number of *Per1*$^{-/-}$ / *Per2*$^{-/-}$ animals tested is too low to carry out statistical comparison. Obviously, there was a highly significant difference between the *Per2*$^{-/-}$ / *Cry1* $^{-/-}$ and WT animals (200 lx, MWU, z = -2.793, p = 0.0052).

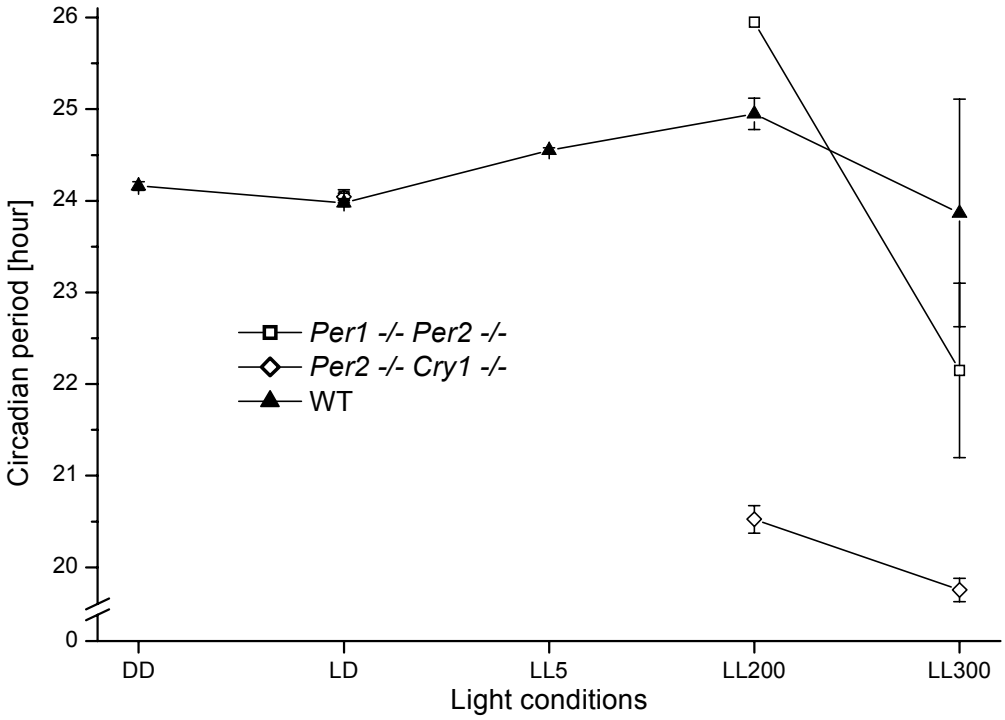

Figure 36. Free-running (circadian) period of double-mutant mice compared to WT. Note that all double-mutant mice exhibited arrhythmic behaviour in constant darkness (DD) and constant light of low intensity (LL5). Group size: *Per1*$^{-/-}$ / *Per2*$^{-/-}$ n = 3; *Per2*$^{-/-}$ / *Cry1*$^{-/-}$ n = 9; WT n = 4. Data are plotted as mean ± sem. Abbreviations: LD 12 h light per day; LLx constant light of x lx intensity.

3.13 Non-24-hours T-cyle

After an initial period of at least 14 days of entrainment to LD 11.25:11.25, the free-running rhythm of wheel-running was evaluated for the following 30 days. In this experiment, group size was 6, 6, and 3 animals for *Per1* $^{-/-}$, *Per2* $^{-/-}$ and WT, respectively.

Free-running period

The period (τ) of each animal was determined three times: (1) during the presence of the zeitgeber, (2) during the first 5 days of free running, and (3) 13 days after the transfer to constant darkness. The results for each genotype and condition are given in Table 3. Due to technical problems after the first week in DD, only a few days were available for rhythm

analysis in DD. Thus, the rhythmicity was classified by visual inspection of the double-plotted actogram by two independent persons, and the τ length was not calculated.

Table 3. The length of the free-running period (τ) under the presence of the zeitgeber (LD), directly after the beginning of constant darkness (DD1), and 15 days after the onset of constant darkness (DD2). Group size was *Per1* $^{-/-}$ n = 6, *Per2* $^{-/-}$ n = 6, and WT n = 3. For details see text.

Genotype	Period [hours]		
	LD	DD1	DD2
Per1 $^{-/-}$	21.7 ± 1.1	23.7 ± 0.2	rhythmic
Per2 $^{-/-}$	22.4 ± 0.1	21.9 ± 0.1	arrhythmic
WT	22.6 ± 0.1	22.8 ± 0.4	rhythmic

These results show that a preceding T-cycle of 22.25 hours did not prevent the *Per2* $^{-/-}$ animals from becoming arrhythmic in DD, as proposed by Dernbach (2003), although the animals did remain remarkably stable with a τ near 22.5 h (21.9 ± 0.1 h) during the first week in DD.

Phase angle difference

In addition to the period length of the rhythm, the phase relative to the zeitgeber (= lights off) was calculated. Here, there were major differences among the three genotypes. Whereas the *Per1* $^{-/-}$ and WT animals exhibited a negative phase angle of -3.0 ± 0.7 h and -1.4 ± 1.4 h, respectively, the *Per2* $^{-/-}$ mice show a leading phase of 1.4 ± 0.4 h (Figure 41a). In order to verify these results, the data of Dernbach (2003) were analysed for the phase angle differences. Although his data were recorded with PIDs instead of running wheels. the outcome was similar to this study. Again, the *Per1* $^{-/-}$ (-3.5 ± 1.2 h, n = 8) and WT (-0.6 ± 0.4 h, n = 4) had a negative phase angle and the *Per2* $^{-/-}$ (1.36 ± 0.4 h, n = 8) a positive phase angle (Figure 41b). Taken all together, these data revealed significant differences among the genotypes in a one-way ANOVA ($F(2,28) = 19.536$, $p < 0.0001$), and *post hoc* comparison indicated that this significance was due to the differences between *Per1* $^{-/-}$ and

Per2 $^{-/-}$ ($p < 0.0001$) as well as to those between WT and *Per2* $^{-/-}$ ($p = 0.0478$), but not between *Per1* $^{-/-}$ and WT ($p = 0.686$).

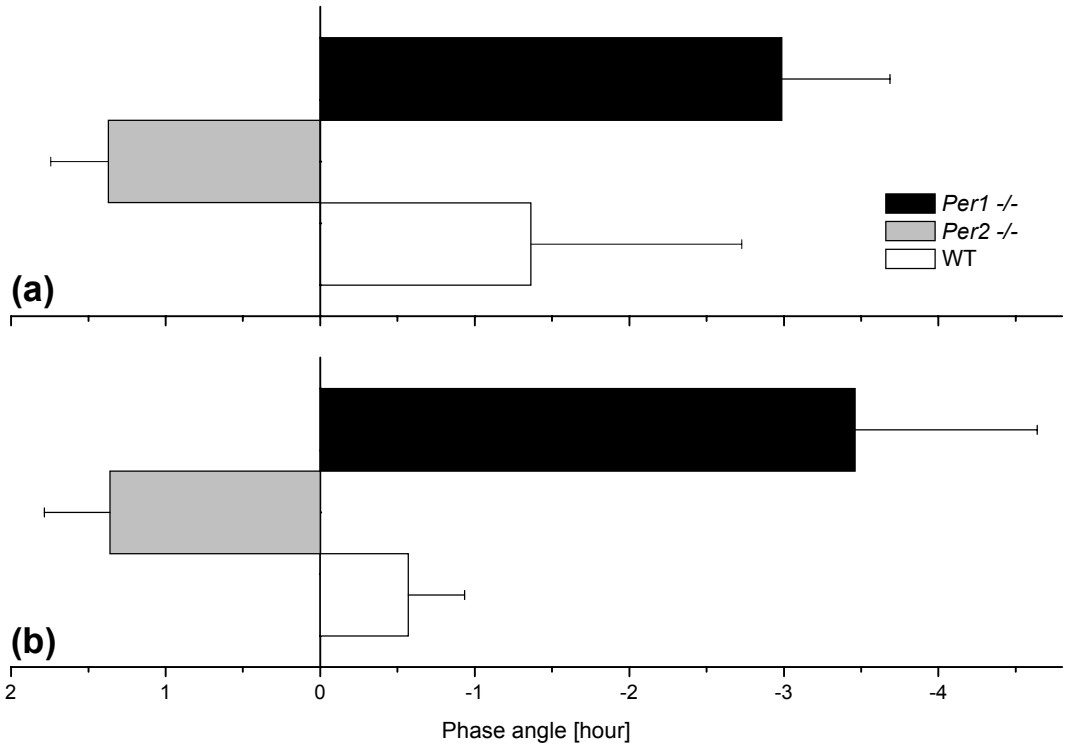

Figure 37. Phase angle difference of the activity onset relative to the light offset. (a) Data of this study. Group size: *Per1* $^{-/-}$ n = 5, *Per2* $^{-/-}$ n = 6, WT n = 2 (b) Re-analysed data from Dernbach (2003). Group size: *Per1* $^{-/-}$ n = 8, *Per2* $^{-/-}$ n = 8, WT n = 4. Data are given as mean ± sem.

Further results

Three of six of the *Per1* $^{-/-}$ animals exhibited an unexpected activity pattern during the T-cycle with 22.5 hours day. Figure 42 shows an example of such an activity pattern. It seems that the mouse could not entrain to the zeitgeber and exhibited a high degree of masking.

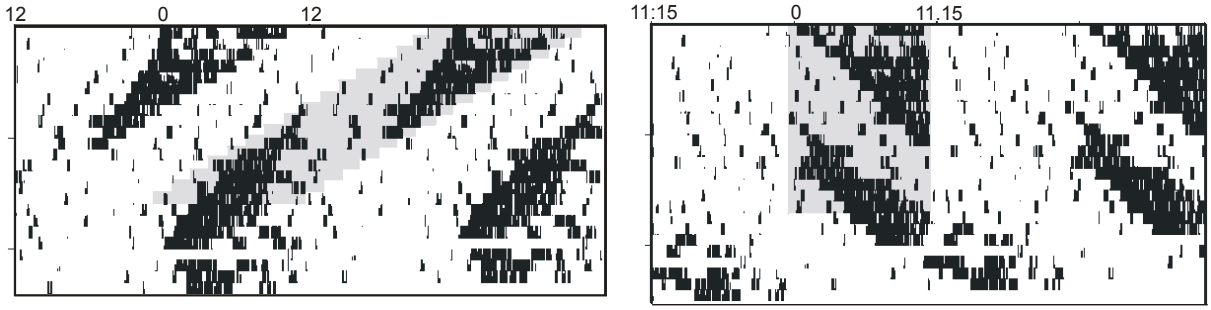

Figure 38. Actograms of one *Per1* $^{-/-}$ animal for 25 days, plotted as "normal" 48 hours double plot (*left*), and as a 45-hour T-cycle adjusted double plot (*right*). Shaded area indicates the dark phase.

3.14 Temperature as zeitgeber

Temperature cycles and activity

A temperature cycle with an amplitude of 8 °C was used as a zeitgeber. The general result is that WT animals were able to entrain to such a zeitgeber whereas both *Per1*$^{-/-}$ and *Per2* mutants seemed to have difficulty entraining.

Figure 43 shows representative actograms of the first experiment, i.e. a cold/warm 12:12 ambient temperature cycle with an amplitude of 8 °C starting at the first day in constant darkness. The *Per1* $^{-/-}$ animals showed free running with a period shorter than 24 hours (23.3 ± 0.2, $n = 5$), and the *Per2* $^{-/-}$ animals became arrhythmic (6 of 6). The WT animals, however, showed a period of 24 hours (24.06 ± 0.04, $n = 6$). Some of the WT animals exhibited a stable positive phase angle in relation to the zeitgeber. In summary, we found that animals lacking either *Per1* or *Per2* could not be entrained to the presented temperature cycle, while the WT were able to entrain.

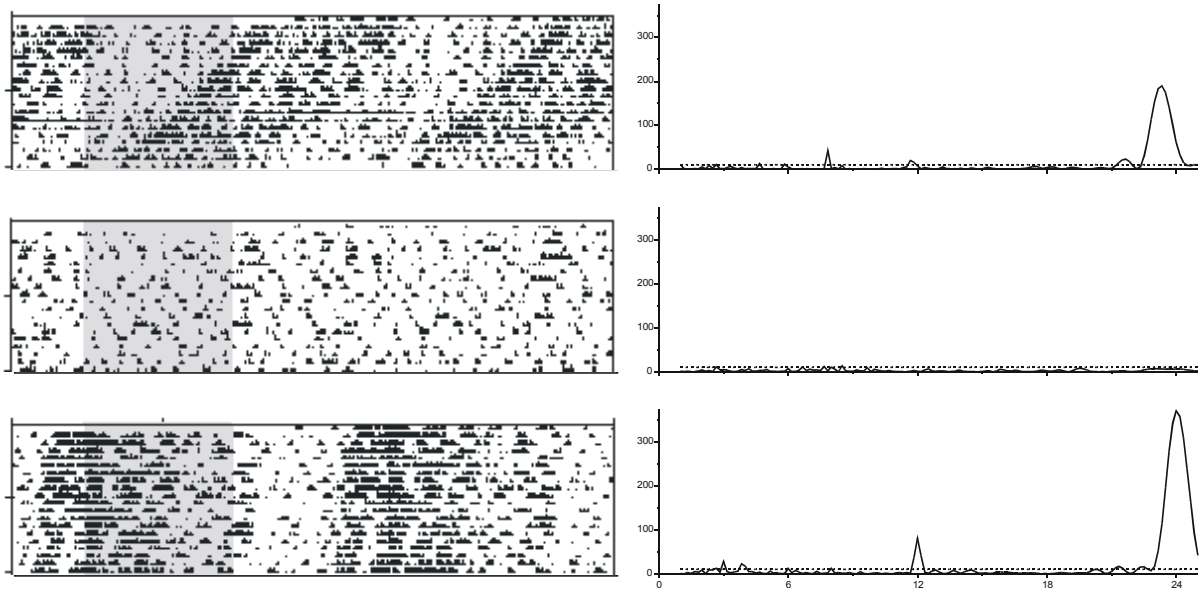

Figure 39. Representative double-plotted actograms (left) and periodograms (right) of total activity for one animal per genoytpe in DD with a temperature cycle. Top *Per1* $^{-/-}$, middle *Per2* $^{-/-}$, and bottom WT. Shaded area indicates low temperature. It is shown on the left side of the double plot only to allow unbiased inspection of the actogram on the other side. Periodograms are plotted as time in hours against normalised power.

The results of second experiment were very similar to those of the first. However, the behavioural rhythms were more variable in the second. In principle, three different patterns occurred. As observed in the first experiment, all of the WT animals (4 of 4) entrained to the zeitgeber whereas most of the *Per* mutant animals, i.e. 5 of 6 *Per1* $^{-/-}$ and 5 of 6 *Per2* $^{-/-}$ failed to do so. Figure 44 depicts typical examples of the different wheel running patterns that occurred in this experiment. Some of the mutants seemed to be entrained or to show relative co-ordination (no example shown), and at least the *Per2* $^{-/-}$ animals exhibited a high amount of masking. For example, the actogram of the *Per2* $^{-/-}$ mouse plotted in Figure 44 (middle panel) showed a free-running component with a τ shorter than 24 h, but this component became weaker after a few days under constant conditions. In addition, there was a second component which seemed to be locked to the onset of the cold phase. At the beginning of the second constant conditions period, however, the animals were clearly arrhythmic, indicating that the second component was only a masking effect.

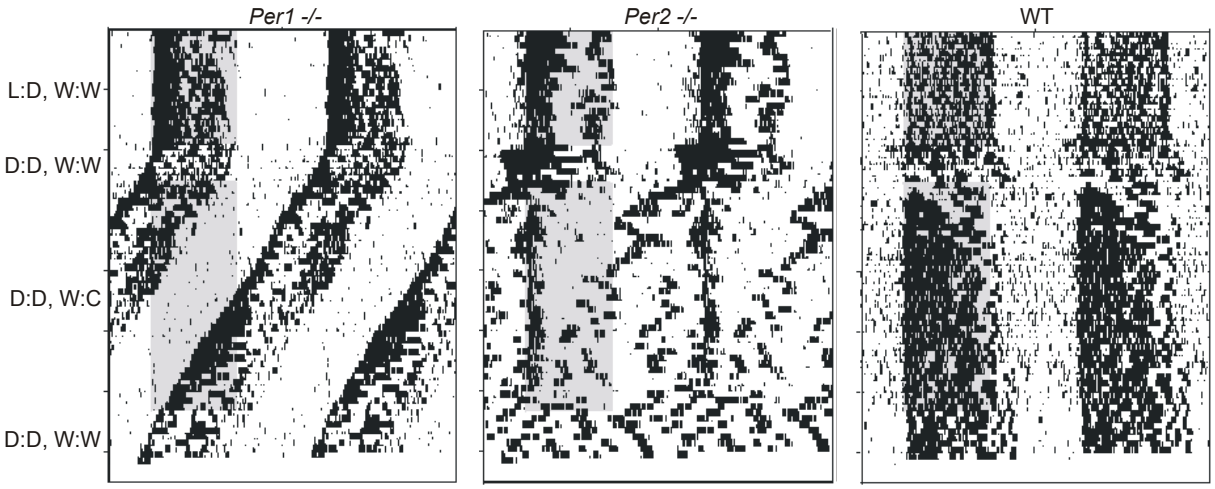

Figure 40. Double-plotted actograms of different types of wheel-running pattern in the second experiment. Left panel: free-running *Per1* $^{-/-}$, middle panel: arrhythmic *Per2* $^{-/-}$ with high degree of masking; right panel: entrained WT. Shaded areas indicate dark phase in initial LD cycle, and cold phase under CW cycle. It is shown on the left side of the double plot only to allow unbiased inspection of the actogram on the other side. Abbreviations: LD light/dark clcle ; WC cold/warm cylce.

Temperature pulse and *Per1* expression

After a temperature pulse of 30 °C for 30 min at ZT17, i.e. a time when *Per1* expression is rather low, the level of *Per1* mRNA was measured by in situ hybridisation. Compared to the respective control animals no elevated *Per1* expression were found in the animals which received the temperature pulse. In contrast, it seemed as if the control animals which received no heat pulse had higher levels of *Per1* expression. The results of this experiment are not quantified due to the low number of animals and the obvious results of the visual inspection (Figure 45).

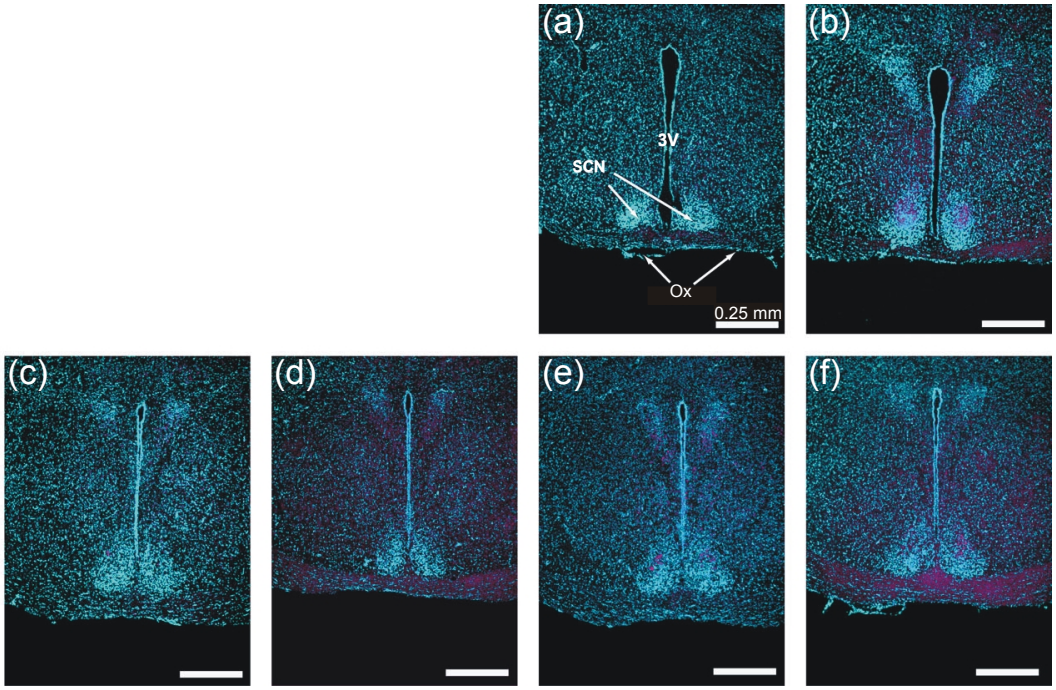

Figure 41. In situ hybridisation for *Per1* expression after heat pulse of the treated animals (a) & (b) and the untreated control group (c) – (f). Abbreviations: 3V third ventricle; SCN suprachiasmatic nucleus; Ox Optic chiasm.

3.15 Feedback lighting

The results of the feedback lighting experiment have to be considered as preliminary because only one animal of every genotype was tested. This was due to the time-consuming recording of data. Only one animal could be tested at a time.

However, the actograms of the mice look like typical actograms of mice in LL (Figure 46). Actually, however, only about 80% of a 24 hour period were lights on.

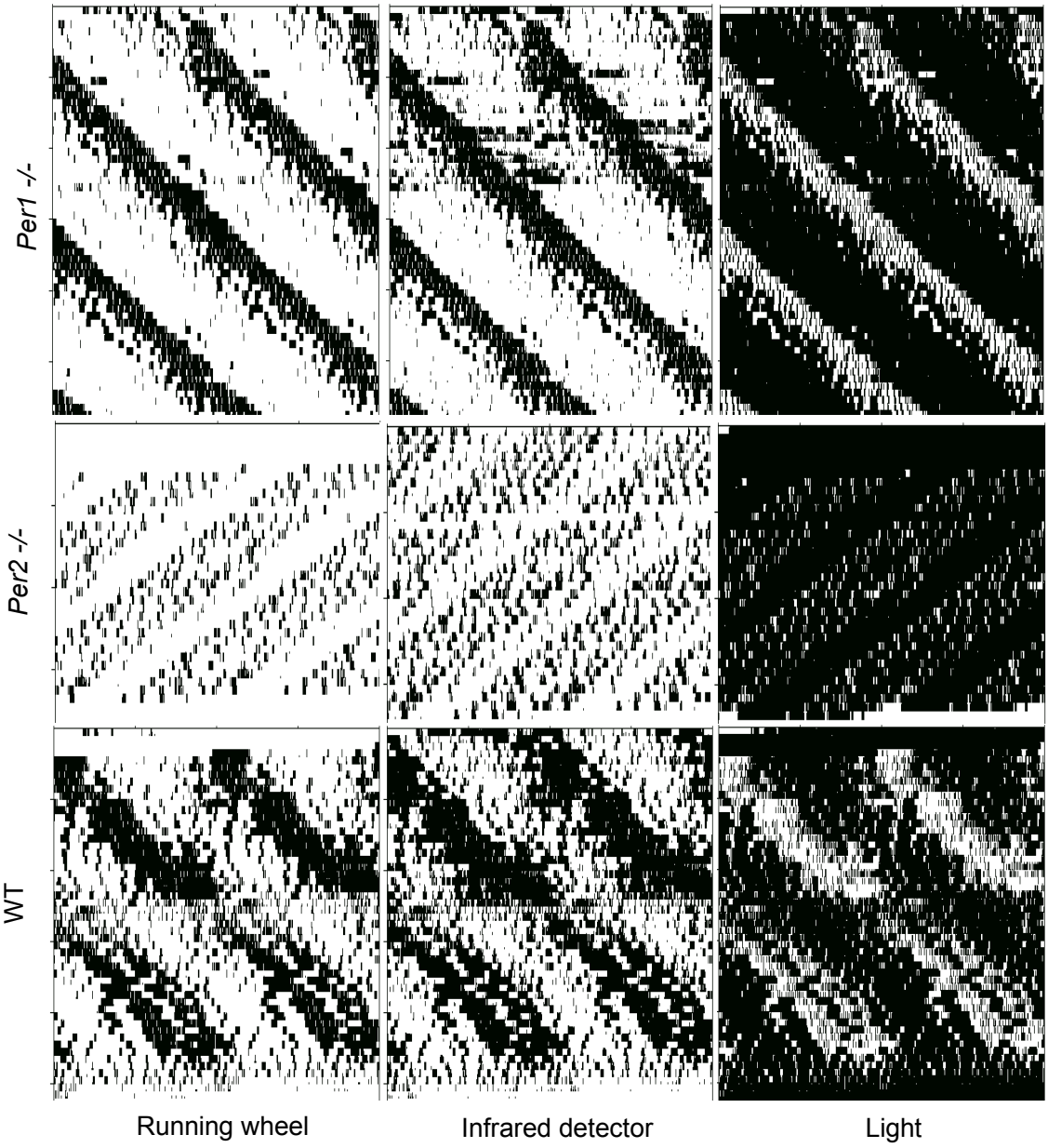

Figure 42. Double-plotted actograms of animals in feedback lighting conditions of one *Per1* $^{-/-}$ (top row), one *Per2* $^{-/-}$ (middle row), and one WT (bottom row) mouse. Left column, running wheel data; middle column, passive infrared detectors; right column. light detector (black indicates lights on).

4. Discussion

4.1 The animal model

In modern biomedical research it is strongly recommended that the genetic background of the animal model be highly defined (Banburry Conference on Genetic Background in Mice, 1997; Gerlai, 1996; Wolfer *et al.*, 2002). The mPer1 and mPer2 deficient mice used in this study were bred on a segregating background of C57Bl6/J and 129S7 mice (see Materials & Methods, and Zheng *et al.*, 2001; Zheng *et al.*, 1999). This has to be considered carefully, due to the fact that there are significant differences between those strains in several parameters, e.g. learning and fear conditioning (Bovet *et al.*, 1969; Contet *et al.*, 2001; Stiedl *et al.*, 1999), anxiety (Bouwknecht and Paylor, 2002; Trullas and Skolnick, 1993; van Gaalen and Steckler, 2000), emotionality (Võikar *et al.*, 2001). There is also variation in activity, neuroendocrine and biochemical parameters (Brodkin *et al.*, 1998), and in the circadian characteristics (Marston *et al.*, 2001). Differences can even be observed within single 129 (Simpson *et al.*, 1997). In addition, our animals were bred homozygous. In consequence of this we were not able to use litter mates as control animals. Alternatively, we bred B6:129S7F2 as wildtype controls.

There have been reports in circadian biology of an influence of the background, and for example, the activity pattern differs between strains (Hofstetter *et al.*, 2003; Kopp, 2001). Furthermore, the time to entrain to a zeitgeber differs among inbred strains (Kopp *et al.*, 2000) as does the free-running period in DD (Schwartz and Zimmerman, 1990). Even the phenotype of mice with a homozygous knock-out of the *Per1* gene seems to be affected either by the construction of the transgenic allele itself or by the genetic background of the animals. It has for example been demonstrated that a high percentage (9 out of 11) of $Per1^{ldc}$ mice become arrhythmic in DD (Bae *et al.*, 2001), while $Per1^{psc}$ (Cermakian *et al.*, 2001) and $Per1^{Brd}$ (Zheng *et al.*, 2001) mice stay rhythmic, with a shorter free-running period than their

respective wildtype controls. Moreover, there have been additional findings concerning light-induced phase shifting that show further discrepancies among these different transgenic lines. The *Per1*^{psc} (Cermakian *et al.*, 2001) and Per*1*^{ldc} (Bae *et al.*, 2001) mice can phase shift upon a late night light pulse, whereas the *Per1*^{Brd} (Zheng *et al.*, 2001) mice do not respond to light pulses at ZT22. These contradictory findings remain unresolved. When the *Per1*^{psc} mice were checked for the effect of the genetic background by comparing N2 (50% 129 and 50% B6) with N5 (6% 129 and 94% B6) (Cermakian *et al.*, 2001) no differences could be found between the two experimental groups, which suggests that the effect of background is not crucially important for the circadian phenotype of these transgenic mice.

4.2 General health

Despite the skin problems and the anal prolapse in the mutant animals, no obvious abnormalities were observed. In addition, these phenotypes did not have a high penetrance. A microbiological cause of these phenomena could be excluded with high probability because of the negative microbiological results and the occurrence of these phenomena in different laboratories in Germany and in Switzerland.

It could not be ruled out that the cause of the skin problems could be the genetic background of the animals, which is a segregating mixture of B6 and 129S7, because a similar phenomenon is known in B6 mice, the so-called B6 dermatitis (Sundberg *et al.*, 1994). Thus, the varying percentage of B6 genes could have been responsible for the occurrence of the skin problems, explaining the low penetrance of the skin problems. It would remain unclear, however, why none of the WT animals suffered from this problem although they were derived from the same background strains. Another way of explaining the skin problems could be stereotypic behaviours such as scratching with the hind paws. Such behavioural alterations are a possible side effect observed in transgenic animals (Crawley, 2000).

Finally, there is one hypotheses related to the observed elevated daytime corticosterone levels. In Siberian hamsters, it has been shown that an acute stressor (restrained stress) can change leukocyte distribution in a way that the leukocytes exit the blood and enter primary immune defence areas such as the skin in order to prevent infections following injuries (Bilbo *et al.*, 2002). In the same study, elevated glucocorticoid levels were observed under short-day conditions (LD 8:16), indicating that short days are chronic stress for the hamsters. This chronic stress also leads to elevated numbers of immune cells in the skin. As the *Per1*$^{-/-}$ animals show high daytime levels of corticosterone, this might induce such a migration into the skin causing the itching followed by scratching and finally leading to skin irritations.

In contrast, the anal prolapse remains enigmatic. A possible cause of such a phenomenon could be an inflammatory bowel disease, but this was ruled out by histological examinations of the complete intestine.

4.3 Body weight

The genotypes differ significantly in the parameter body weight. The *Per1*$^{-/-}$ animals are lighter than the WT animals while the *Per2*$^{-/-}$ mutants tend to be heavier than the wildtypes. Dernbach (2003 p. 66) did not describe any such differences between the genotypes. This might be due to the lower numbers of animals he measured or to the use of animals of a different age. An additional effect might have been the housing and light conditions, for he shows differences in the metabolic rates under different light regimes.

It has been shown that elevated levels of corticosterone have a negative influence on body weight (Bartolomucci *et al.*, in press; Touma *et al.*, in press). Since the *Per1*$^{-/-}$ animals exhibited higher corticosterone levels in feces at least during the day, this seems to provide some explanation for their lower body weight.

It remains unknown whether the differences in the body weight might be directly caused by the null mutation of the *Per1* gene or whether this is an indirect effect mediated by

alterations in the clock as a whole. As stated for the use of transgenic animals in research for body weight regulation mechanisms, it might be a fruitful approach to use conditional or tissue specific knock-outs of the *Per1* gene (Inui, 2000). Unfortunately, those animals are not available.

4.4 Body temperature

All genotypes showed a clear diurnal variation in T_b recordings with higher night and lower daytime values (see also: Connolly and Becker-Lynch, 1981; Decoursey *et al.*, 1998; Tankersley *et al.*, 2002; Weinert and Waterhouse, 1998). The *Per1*$^{-/-}$ animals showed a lower T_b compared to WT, whereas the Per2 $^{-/-}$ mice could not be distinguished from WT. This differences – of about 0.5 °C – was detectable at all times of the day, but was most prominent after the onset of the light phase and thus shortly after the end of the activity phase of the animals. Due to the low number of animals, the difference at the beginning of the light phase was statistically significant. Additional data re-analysed from Steinlechner *et al.* (2002) showed a different temperature pattern. Apart from the few hours after the onset of lights, the *Per1*$^{-/-}$ do not exhibit a lower T_b in that study. In the beginning of the resting phase, however, they exhibit a significantly lower T_b. The differences between the data from Steinlechner *et al.* (2002) and the present study may be mainly due to the following factors: (1) The animals of Steinlechner *et al.* (2002) were held at 21 °C and not at 28 °C, i.e. the thermoneutral zone for mice (Fraifeld and Kaplanski, 1998; Gordon, 1993; Williams *et al.*, 2002). Thus, the animals had to thermoregulate. (2) In contrast to this study, the animals of Steinlechner *et al.* (2002) had access to a running wheel. The use of a running wheel heightens the amplitude of T_b (Golombek *et al.*, 1993), which is in accordance with our data. The revolutions of the running wheels were not counted the study of Steinlechner *et al.* (2002). Therefore, it is not known whether the animals differed in the amount of activity. Data from other studies, however, suggest that there are no differences in this respect (Albrecht *et al.*, 1997b; Albrecht

et al., 2001; Oster, 2003). As a consequence of the lower T_a and the use of a running wheel, the T_b of the *Per1*$^{-/-}$ was indistinguishable from that of the controls during most of the 24-hours cycle (Steinlechner, 2002). At the beginning of the (inactive) light phase, however, T_b of the *Per1*$^{-/-}$ mice dropped below those of the controls, as it was also the case in this study. As the mice are known to exhibit an activity peak at the end of their active phase (Kopp, 2001), the animals are heated up just before the lights go on. Subsequently, they passively cool down until - under thermoneutral conditions – they reach their T_b set point, which they maintain, or – at a lower T_a – they start to thermoregulate. Thus, the data of both the present and the previous study support the hypothesis that the *Per1*$^{-/-}$ animals have a lowered or less sensitive setpoint for T_b than WT animals.

As stated in Animals, Materials and Methods, the animals used for this study are not extensively backcrossed to C57BL6/J. It would be quite feasible that the genetic background might have an influence on T_b, because the different inbred strains of mice show differences in T_b. For example, C57BL6/J mice have a lower rectal T_b during the night phase than BALB/cIbg (Connolly and Becker-Lynch, 1981), but Bouwknecht *et al.* (2002) found no significant difference in rectal T_b of C57BL6/J and 129S8/EvTac, which is closely related to the ES cell donor for the transgenic mice used in this study (129S7). These contradictory findings can be explained by the time of measurement. Connolly *et al.* (1981) exclusively found differences in the dark phase, a phenomenon which may be explained by the different activity levels of the animals shown for C3H/HeJ and C57BL6/J mice (Tankersley *et al.*, 2002). In contrast, Bouwknecht *et al.* (2002) measured in the early morning (2 – 3 h after lights on), when there is no activity at all in any of the strains. The T_b of the *Per1* $^{-/-}$ animals, however, was lowered during the entire 24-hours cycle, and therefore cannot be explained simply by differences in the level of activity.

It has been proposed that bradicardia and hypotension correlate with hypothermia (Swoap, 2001). Therefore, it would be interesting to know whether the heart rate and blood

pressure are lowered, as is suggested by the observed hypothermia. Unfortunately, there are no such data available at the moment.

4.5 Behavioural phenotyping

Behavioural phenotyping revealed no gross abnormalities in any of the two types of transgenic mice. Nevertheless, there were some differences concerning stress-related parameters in the *Per1*$^{-/-}$ and body weight-related parameters in the *Per2*$^{-/-}$ mice.

The food and water intake was altered in the mutant animals as compared to WT. Whereas the *Per2*$^{-/-}$ mice had significantly higher values in food and water intake per mouse and per g body weight compared to wildtype, the situation for the *Per1*$^{-/-}$ mice was more complicated. The *Per1*$^{-/-}$ could not be discriminated from the WT mice with respect to food and water intake per mouse. However, if the food and water consumption were calculated per g body weight the *Per1*$^{-/-}$ mice showed values comparable to *Per2*$^{-/-}$ and, hence, values significantly higher than those of the WT mice. The time course of fecal excretion did not differ significantly between the genotypes, indicating a feeding pattern that was in accordance with the values reported in the literature (Heinrichs, 2001; Kurokawa *et al.*, 2000). Thus, it is unlikely that the differences in food intake are due to a disturbed temporal pattern.

The latency of the *Per2*$^{-/-}$ in the pole test was higher than in WT mice. Normally, this would indicate that the striatal dopamine level is lower in the *Per2*$^{-/-}$ mice (Fernagut *et al.*, 2003; Karl *et al.*, 2003c; Matsuura *et al.*, 1997), but in this particular case, the body weight differences seem to play a role. This is also indicated by the results of the open field, in which the *Per2*$^{-/-}$ mice travelled a shorter distance, and in the wire hang test, where they lost their grip after a few seconds.

The differences observed in the *Per1*$^{-/-}$ mice are mainly stress-related. as is discussed in the following sections. Most of these, e.g. a shorter latency to reach the periphery in the open

field were not significant, but showed a tendency towards a more "emotional" (Ader *et al.*, 1967) *Per1*$^{-/-}$ mouse than WT.

4.6 Hot plate

The hot plate task is commonly used to test the nociception capabilities of rodents (Karl *et al.*, 2003c). This test also measures a spinal reflex which requires higher brain regions, i.e. a reaction to a painful thermal stimulus (Crawley, 1999). Two results were obtained. First, latency was higher in the *Per1*$^{-/-}$ and *Per2*$^{-/-}$ mice than in WT mice. Second, there was no significant difference, at least for the *Per1*$^{-/-}$ mice, if the animals were tested after habituation to the hot plate.

On the one hand, these results suggest that either the heat perception or the nociception of *Per2*$^{-/-}$ mice is altered. Although none of the possible explanations for this suggestion could be tested here, they should at least be discussed. As the *Per2*$^{-/-}$ mice tend to be heavier than the WT animals, the simplest explanation might be that they have more fat or a thicker skin at the ventral part of the paws and thus a somewhat greater heat resistance. This would be ruled out either by using a tail flick test as an additional standard test for nociception (Crawley, 1999, 2000; Crawley and Paylor, 1997; Karl *et al.*, 2003c) or by making a histological comparison of the of the paws. The latter could also clarify if there are differences in the heat receptors of the paws.

On the other hand, the results for the *Per1*$^{-/-}$ mice indicate a stress-related phenomenon, i.e. stress-induced analgesia (Valverde *et al.*, 2000). The elevated latency in the baseline test might have been due to an elevated stress level, which has an analgesic effect and attenuates nociception.

4.7 Glucose tolerance test

The glucose tolerance test revealed that glucose tolerance of the *Per2*$^{-/-}$ animals was higher than that of WT and a similar tendency in the *Per1* $^{-/-}$ animals. Both the basal blood

glucose level and glucose tolerance exhibit a daily rhythm which is under the control of the SCN (La Fleur *et al.*, 1999; La Fleur *et al.*, 2001; Ruiter *et al.*, 2003; Yamamoto *et al.*, 1987). La Fleur *et al.* (2001) conclude that the SCN anticipates the activity period and prepares the animal by increasing basal glucose levels and the tolerance of body tissues to glucose shortly before the onset of activity. The data of the present study were sampled at ZT1 only. According to the cited literature, one should expect lower basal values and a reduced glucose tolerance at ZT1 than at ZT11, for example, and this was the case in the WT mice of this study. The initial values were lower in *Per1*$^{-/-}$ than in WT mice, which might be simply due to the difference in metabolic rates. After 14 h of fasting, the smaller *Per1*$^{-/-}$ mice might be more physically depleted than the WT and *Per2*$^{-/-}$ mice, and thus have a lower basal blood glucose level. In contrast, the increased glucose tolerance of the *Per2*$^{-/-}$ might be best explained by an effect of the *Per2* gene on the signal which mediates the SCN information to the body. The glucose rhythm may be disturbed in the *Per2* deficient animals. This hypotheses should be investigated by a repeated glucose tolerance test at multiple times of the day. In addition, the insulin levels should be measured in order to determine the insulin secretion pattern. Together, these tests should make it possible to determine whether the clock is involved or if *Per2* has a function in glucose homeostasis outside the clock. In the latter case it would be useful to compare the physiological parameters of the *Per2*$^{-/-}$ with the various of animal models that have been reported for an increased glucose tolerance (e.g. Ahren *et al.*, 2000; Cooney *et al.*, 2004; Dryden *et al.*, 1996; Karl *et al.*, 2003b). This should lead to further clues to explain the *Per2*$^{-/-}$ mouse phenotype.

4.8 Stress-induced hyperthermia

The stress-induced hyperthermia test assays the anxiety-related behaviour of mice. As it measures a general physiological response, the influence of strain-specific traits is lower than in other anxiety-related behavioural tests, e.g. the light-dark transition (Bouwknecht and

Paylor, 2002). In the latter, the mice are placed in a box whose floor is divided into two compartments, one-third dark and two-thirds brightly lit (700 lx). According to the test paradigm, the time spent in the dark compartment is correlated with the animal's anxiety (Crawley and Goodwin, 1980). Here, the influence of strain-specific traits, such as the activity pattern (Connolly and Becker-Lynch, 1981; Klante *et al.*, 1999), would be expected to influence greatly the results of the light-dark transition.

The values obtained in the stress-induced hyperthermia experiment were comparable to those reported in the literature (van der Heyden *et al.*, 1997). Although no significant effects could be detected, there was a clear trend towards a higher temperature rise in the *Per1*$^{-/-}$ than in WT mice. If there was any response at all in the *Per2*$^{-/-}$ mice, it was weakly attenuated. A higher rise in T_b indicates a higher stress reaction of the animal to the stressor, i.e. a higher corticosterone level (Groenink *et al.*, 1996). As the *Per1*$^{-/-}$ mice indeed had higher corticosterone levels during the day (see below), the higher amplitude in these animals' test response was to be expected.

4.9 LPS stimulation

The results of the cytokine response to the LPS stimulation were not as clear as expected. This might be mainly due to the experimental procedures and the low number of samples tested. However, the reaction of T_b to the LPS injection is an interesting preliminary result and deserves further investigation. Whereas the *Per2*$^{-/-}$ and WT mice show an increase in T_b after LPS injection, i.e. a fever reaction as described in the literature (Alheim *et al.*, 1997; Fraifeld and Kaplanski, 1998), the opposite is the case in the *Per1*$^{-/-}$ mice. They show a clear decrease in T_b, which is another indication that the control of T_b might be disrupted in some way.

As stated above, the pattern of the cytokine response was not very clear. The TNF-α response indicated a higher cytokine level in the *Per2*$^{-/-}$ animals, while the IL1-β level was

below those of the other genotypes. As IL-1β is induced by TNF- α (Ostberg *et al.*, 2000) this must be interpreted as an artefact. Taken together, the results of the LPS stimulation experiments did not reveal any consistent interaction between the lack of *Per1* or *Per2* and an altered cytokine response, although this has been suggested by previous work in which the *Per1* gene expression could be induced *in vitro* by IL-6 (Motzkus *et al.*, 2002), and *in vivo* by LPS injection (Takahashi *et al.*, 2001). Obviously, the connection between the induction of *Per1* and an inflammatory stressor are more complex than can be detected by simply measuring cytokine levels. However, this appears to be a fruitful approach.

4.10 Phagocytosis assay

The phagocytosis assay was performed with macrophages from whole blood. It could be shown that the macrophages of all three genotypes are able to ingest *E. coli*, and there were no significant differences between the genotypes. However, there was a trend towards a higher phagocytotic activity in the *Per1*$^{-/-}$ than in the *Per2*$^{-/-}$ and WT mice.

If stressed by forced swimming, Balb/c mice exhibit a higher amount of phagocytotic activity of peritoneal macrophages than non-stressed controls (Barriga *et al.*, 2001). In fact, stressed mice exhibit not only a higher level of plasma corticosterone but the diurnal rhythm seems to be disrupted, as well. Peritoneal macrophages obtained from such mice show a higher phagocytation rate at all times of the day than non-stressed controls (Barriga *et al.*, 2001). Interestingly, the *Per1*$^{-/-}$ mice have elevated corticosterone levels with an absent diurnal rhythm (see below) and they tend to have a higher phagocytic activity. Similarly, it has been found in an *in vitro* study that corticosterone can enhance the phagocytotic capacities of macrophages (Barriga *et al.*, 2002). However, it must be mentioned that macrophages obtained from stressed mice did show such increased phagocytation in the study of Barriga *et al.* (2002). This was explained by the fact that the macrophages presumably had already been activated by the stress applied to the mice. In addition, it has been found that forced

swimming can induce the expression of *Per1*$^{-/-}$ in certain areas of the brain such as the PVN (but not in the SCN, Takahashi *et al.*, 2001). In WT mice *Per1* has its peak expression around midday (Albrecht *et al.*, 2001; Shearman *et al.*, 1997) and corticosterone levels are low during the day (Kalsbeek *et al.*, 1996; Saba *et al.*, 1963). Thus, *Per1* seems to have an attenuating effect on the corticosterone level, thereby enhancing the phagocytotic response of the macrophages.

4.11 Hormonal rhythms in feces

The activity of the hypothalamic pituitary adrenocortical (HPA) axis was measured by a recently established non-invasive technique (Touma *et al.*, 2004). The level of corticosterone, which is the major glucocorticoid in mice (Nelson, 2000, p. 81; Spackman and Riley, 1978) was assessed by measuring corticosterone metabolites in feces. The results showed a clearly diurnal pattern of corticosterone metabolites in WT and *Per2*$^{-/-}$ mice, with a peak phase at the beginning of the night and a through the beginning of the day. This pattern has been shown in various night active species. The *Per2*$^{-/-}$ tended to have higher levels than the other animals, but the difference was not significant. In contrast, the *Per1*$^{-/-}$ animals exhibited constantly high levels of corticosterone metabolites both during the day and during the night. The daily rhythm seems to be disrupted. Interestingly, preliminary results suggest that the *Per2*$^{-/-}$ / *Cry1*$^{-/-}$ mice exhibit significantly elevated plasma corticosterone levels (H. Oster, pers. comm.). Thus, the lack of *Cry1* seems to have effects similar to those of *Per1* deficiency. As stated above, not only *Per1* but also *Cry1* might have an influence on the corticosterone rhythm. Both genes are part of the morning oscillator, which suggests that the corticosterone rhythm might to some degree be dependent on the morning oscillator.

Similar phenomena are found in psychologically depressed human subjects, whose cortisol glucocorticoid levels are higher than those of healthy controls. In addition, the diurnal corticosterone rhythm of depressed individuals is attenuated in its amplitude, and the trough

phase is markedly shortened (Deuschle *et al.*, 1997; Heuser *et al.*, 1998). In addition, these subjects fail to respond properly to dexamethasone and do not suppress the cortisol production (Nelson, 2000, p. 642). Thus, it seems appropriate in *Per1*$^{-/-}$ mice to measure plasma corticosterone levels in a dexamethasone suppression test and to carry out an adrenocorticotropin (ACTH)-challenge test (Raber *et al.*, 2000; Touma *et al.*, in press). In addition, these animals should be submitted to standard tests for depression (Crawley, 1999; Crawley and Paylor, 1997; Solberg *et al.*, 1999). In this context one conflicting finding from the literature must be mentioned. During the daytime, depressive humans exhibit an elevated T_b in comparison to healthy subjects (Rausch *et al.*, 2003). In contrast, the *Per1*-deficient mice exhibited a lowered T_b. These contradictory results might be due to the recording methods. As discussed above (see section Body Temperature), the data from Steinlechner *et al.* (2002) showed a higher temperature during the active phase in the *Per1*$^{-/-}$ than in WT mice. This suggests that the results of Rausch *et al.* (2003) may be due to differences in the amount of activity (Weinert and Waterhouse, 1998).

Furthermore, an increased corticosterone level seems to enhance the reinforcing effects of drug abuse due to an increased dopamine utilisation in the mesocorticolimbic pathway (Deutch and Roth, 1990). This reinforcing effect has been shown for cocaine in rats (DeVries *et al.*, 1998; Goeders, 1997). Furthermore, stressed mice drink more alcohol than their respective controls (Sillaber *et al.*, 2002). Interestingly, *Per2*$^{-/-}$ mice show hypersensitivity to cocaine, whereas the *Per1*$^{-/-}$ animals show reduced sensitivity compared to wildtype controls (Abarca *et al.*, 2002)

4.12 Lick-O-Mat

A simple and inexpensive new lickometer was designed and tested for this study, the Lick-O-Mat. First experiments showed that the findings obtained from this device are useful

for the determination of the circadian clock output, and can be compared with the actograms derived from passive infrared detectors (PIDs).

There have been a number of apparatuses available for recording drinking and feeding behaviour (e.g. Badiani *et al.*, 1995; Kurokawa *et al.*, 2000; Weijnen, 1989, 1998). In general, there are two ways to measure the contacts of the tongue to the tip of the drinking bottle either by an electrical circuit, or by an optical sensor. Both methods have their advantages (reviewed in Weijnen, 1989). However, the Lick-O-Mat is less expensive than commercially available devices; its costs per cage are less than 5 €. In addition, it can easily be attached to any standard cage simply by attaching some cables to the drinking bottle. This is not the case in most of the other systems (e.g. Kurokawa *et al.*, 2000). The major disadvantage of the Lick-O-Mat 1.0 – and of all commercial available devices as well - is that either a wire cage must be used or wire mesh must be put inside the cage in order to close the signal circuit. This will be overcome in the next generation of this device by adapting circuits used in alarm devices.

There should be numerous possible applications in the field of circadian biology for the Lick-O-Mat. It can be used even if the cages are too small for running wheels or if the animals are too old for running wheels as is the case with aged rats (Biemans *et al.*, 2003). In individually ventilated cages (IVC) such as those made by Biozone, for example, no PIDs can be mounted on top of the cage. Thus, the Lick-O-Mat can help bring further insights into behavioural rhythms by serving as the hands of the clock, as it were, in such cases. Possibly the most interesting field of application will be in the search for a possible food oscillator (see Introduction), the "other circadian system" (Stephan, 2002) which has recently come into the focus of circadian research (Challet *et al.*, 2003; Damiola *et al.*, 2000; Dudley *et al.*, 2003; Pitts *et al.*, 2003; Stephan, 2003). It appears to be critically important not only to measure the running wheel activity but also to have a direct parameter of feeding activity. Given that the drinking (= licking) activity in mice is highly correlated with feeding activity when they fed on standard pellet diet (Badiani *et al.*, 1995; Heinrichs, 2001; Kurokawa *et al.*, 2000),

recording of licking activity should provide an exact measure of the feeding time pattern. Thus, the Lick-O-Mat should be a very useful and affordable tool for the further investigation of feeding pattern, and help cast light onto the feedback mechanisms of food on the master clock.

4.13 Constant light conditions for double-mutant animals

Both the *Per1*$^{-/-}$ / *Per2*$^{-/-}$ as well as the *Per2*$^{-/-}$ / *Cry1*$^{-/-}$ mice became rhythmic in constant high- intensity light (LL_{hi}). Due to the small sample size, the free-running period (τ) of the *Per1*$^{-/-}$ / *Per2*$^{-/-}$ mice could not be determined properly. However, the τ in LL_{hi} seemed to be shorter than 24 h, and quite similar to the τ reported for *Per2*$^{-/-}$ mice in LL_{hi} (Steinlechner *et al.*, 2002). Under these conditions, the τ of the *Per2*$^{-/-}$ / *Cry1*$^{-/-}$ mice is remarkably short, about 20 h. These values correspond to those previously published in the additional materials of Oster *et al.* (2003). Although the light intensity was about 400 lx higher in the experiment of Oster *et al.* (2003), τ was not shorter there than in this study, as would be indicated by Aschoff's rule (Aschoff, 1951; Steinlechner *et al.*, 2002). Thus, a τ of 20 h may represent a minimal period length for the circadian system of these mice.

In terms of the two-oscillator model (Daan *et al.*, 2001), the double mutants can be said to lack both of these oscillators as follows: morning (M: *Per1* and *Cry1*) and evening (E: *Per2* and *Cry2*). Thus, the animals should be arrhythmic, as they are in DD. In LL, however, the situation is more complex, because the two double mutants exhibit markedly different τs. Here, the two-oscillator model fails to explain these differences. Under LL conditions it seems to be important which component of the M is intact, either *Per1* or *Cry1*. Hence, one possible explanation for the observed differences would be that these two genes differ in their reaction to light, i.e. their acceleration by light. Another explanation might be sought by postulating a hierarchy of *Per* and *Cry* genes (Oster *et al.*, 2002). Based on various results from *in vivo* transgenic mice studies, Oster *et al.* (2002) argue that *Per2* and *Cry1* should have

a higher repressor potential than *Per1* and *Cry2*, respectively. Based on the hypothesis that circadian rhythms are generated by limit cycles of feedback transcriptions (Leloup *et al.*, 1999), the repressor potential has to be inside a certain range or the limit cycle is driven to equilibrium, i.e. arrhythmicity. In DD, this is the case in the two double mutants as well as in *Per2*$^{-/-}$ (Zheng *et al.*, 1999), *Cry1*$^{-/-}$, *Cry2*$^{-/-}$, and *Cry1*$^{-/-}$ / *Cry2*$^{-/-}$(van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999) mice. In LL_{hi}, however, both the *Per2*$^{-/-}$ (Steinlechner *et al.*, 2002) and the double mutants are rhythmic, indicating that the limit cycle is forced out of equilibrium by the light input. One should bear in mind, however, that this study did not provide data on clock gene expression and that the following explanations are therefore based on the behavioural data.

According to the above model, the *Per2*$^{-/-}$ / *Cry1*$^{-/-}$ animals lack the best repressor genes, which suggests that the limit cycle of feedback-transcription oscillates with the highest possible frequency. Thus, these mice should exhibit the shortest τ. In another approach, Oster (2003) made a prediction about the actual τ in mutant mice. He predicts a τ of 22.3 h, which is remarkably close to our data. On the other hand, the *Per2*$^{-/-}$ / *Per1*$^{-/-}$ mice should exhibit a τ of nearly 24 h. This could not be confirmed because our experimental values were much lower. This might have been due to the influence of the *Per1* mutation, because Oster (2003) stated that "the only mutation the model completely fails to predict is the *mPer1*." In addition, Dernbach (unpublished data) reported a τ of between 28 h and 30 h for the *Per1*$^{-/-}$ / *Per2*$^{-/-}$ animals in LL_{hi}. Interestingly, most of those animals became arrhythmic after a prolonged time under those conditions. Thus, the case of the *Per1*$^{-/-}$ / *Per2*$^{-/-}$ is still open and needs further investigation.

4.14 Non-24-hour T-cycles

A 22.5-hour cycle with LD 11.25:11.25 was tested in order to evaluate a hypothesis of Dernbach (2003) in which he proposes a stable, free-running rhythm of *Per2*$^{-/-}$ animals as an

aftereffect of a previous 22.5-hour T-cycle. The hypotheses of Dernbach (2003) could not be confirmed in our experiment. Most of *Per2*$^{-/-}$ animals became arrhythmic.

In order to investigate the entrainment capabilities of the mice, Dernbach (2003) tested T-cycles with period lengths varying between 17 and 30 hours. According to one of the predictions of the M and E model (Daan *et al.*, 2001), there should be no aftereffects in *Per1*$^{-/-}$ and *Per2*$^{-/-}$-deficient mice and a positive linear correlation in the τ length of the WT animals. The data of Dernbach (2003), however, contradict this prediction. In principle, he observed no aftereffects of the zeitgeber period on τ in the *Per1*$^{-/-}$ and WT mice, but observed a shortening of τ after shorter T-cycles in *Per2*$^{-/-}$ mice. In addition, the rhythm of the *Per2*$^{-/-}$ mice seemed to be more stable after T-cycles shorter than 24 h. The experiments of the present study suggested, in combination with re-analysed data of Dernbach (2003), measuring the phase angle to the zeitgeber instead of τ, and we found a difference in phase angle in the genotypes. Whereas *Per1*$^{-/-}$ and WT mice had a negative phase angle of the activity onset to the light offset, the *Per2*$^{-/-}$ animals exhibited a positive phase angle. As a positive phase angle indicates an endogenous τ shorter than the period of the zeitgeber and *vice versa* for a negative phase angle (Roenneberg and Merrow, 2003), the intrinsic τ of *Per2*$^{-/-}$ mice should be shorter than 22.5 hours, whereas the τ of *Per1* $^{-/-}$ and WT mice is somewhat longer than 22.5 hours.

4.15 Temperature as zeitgeber

The influence of temperature as zeitgeber on the circadian system of the *Per1*$^{-/-}$ and *Per2*$^{-/-}$ was investigated. While WT mice were able to entrain to a temperature cycle with an amplitude of 8 °C, both *Per1* $^{-/-}$ and *Per2*$^{-/-}$ mice failed to entrain to this zeitgeber. The activity pattern of the transgenic mice was like that of constant darkness, i.e. the *Per1*$^{-/-}$ showed free-running and the *Per2*$^{-/-}$ arrhythmic behaviour. Thus, the *Per* genes seem to be essential for either the perception of the temperature signal or the transduction, at least on a behavioural level.

Temperature as a zeitgeber has been extensively examined in prokaryotes (Lin *et al.*, 1999), *Neurospora* (Liu *et al.*, 1998), higher plants (Beator and Kloppstech, 1996), and invertebrates (Lewis, 1999; Zimmerman *et al.*, 1968). The ability to compensate the clock for the ambient temperature ($Q_{10} = 1$, see Introduction), and to entrain to temperature cycles has been found even in *in vitro* systems such as isolated fibroblasts (Tsuchiya *et al.*, 2003) or SCN slices (Ruby *et al.*, 1999) and chicken pineals (Barrett and Takahashi, 1995). In mammals, it could be demonstrated that palm squirrels (Rajaratnam and Redman, 1998), squirrel monkeys (Aschoff and Tokura, 1986), and marmosets (Palkova *et al.*, 1999) are able to entrain to a temperature cycle. Nevertheless, the results were never as clear as with the photic zeitgeber. Palkova *et al.* (1999) suggest that the capability to entrain to a temperature cycle might be dependent on the amplitude of the daily variation in what they call the body/brain temperature. According to their argumentation, an animal with a higher amplitude in T_b is more likely to entrain to an ambient temperature cycle, and one with a lower amplitude is less likely to. In this study, at least for the data obtained from Steinlechner *et al.* (2002), this did not seem to be the case. All animals exhibited nearly the same daily T_b amplitude. In rats, massive hypothermia can change the endogenous circadian period length (Gibbs, 1981). Whether this is due to a loss in temperature compensation as argued by the authors is questionable. Since it is known that stress, i.e. forced swimming, can induce *Per1* gene expression in the brain (Takahashi *et al.*, 2001), and even mild restraint stress has an impact on the clock (Weibel *et al.*, 2002), stress might be another possible explanation for the phenomenon described by Gibbs *et al.* (1981).

The mechanisms of entrainment to a temperature zeitgeber as observed in WT mice but not in the *Per*-deficient mice are unknown. The lack of entrainment suggests that the *Per* genes might play a role in these mechanisms. If *Per1* is involved, it should be inducible by a heat pulse, because a 3 °C heat pulse can shift the electrophysiological activity of the SCN *in vitro* (Ruby *et al.*, 1999). In addition, Brown *et al.* (2002) found a sustained oscillation of

peripheral clocks exposed to a temperature cycle. In the present experiment, *Per1* expression could not be induced by a heat pulse of 10 °C at ZT18 *in vivo*. Obviously, absence of proof is not proof of absence, and this cannot be regarded as a final result. However, it seems that the connection between temperature perception and core clock is more complex and indirect than previously thought.

Nevertheless, it seems to be important to look for *Per* gene, and clock output gene (e.g. DBP, VIP) expression patterns in the central and also in the peripheral oscillators such as the skin during an entrainment to a temperature zeitgeber. This could bring new insights in the non-photic mechanisms of clock resetting.

4.16 Feedback lighting

A simple feedback lighting system was established which is fully adjustable by a software programmed for this study. The preliminary results obtained with this system suggest that all three genotypes under comparison showed LL behaviour when maintained under LD_{FB}.

Two previous studies used a hard-wired system for feedback lighting (Ferraro and McCormack, 1984; Ferraro *et al.*, 1984). The software-based system used here, however, has one major advantage over those previously described, i.e. its adjustability. Not only a delayed feedback of a certain number of running-wheel revolutions can be varied, but the pattern or temporal distribution can be used to create criteria for lights on or off, as well. Thus, the use of this system in well-defined experiments might give further insight into masking by light or the nature of resetting processes. For example, it is still an open question as to whether resetting is a parametric or a non-parametric process (Boulos *et al.*, 2002; Pittendrigh and Daan, 1976a; Refinetti, 2001). In addition, it could be useful to gain further understanding of Aschoff's rule. If an LD_{FB} acts on the clock like an LL, according to Aschoff's rule, different light intensities would be predicted to produce different free-running periods. Thus, further

light could be shed on the question of whether Aschoff's rule is a function of the absolute number of photons per 24 h or of the number of photons at a given point in time within a specific phase of the circadian cycle (Ferraro and McCormack, 1984).

With constant light conditions, especially LL of high intensities, one also has to consider the possibility of retinal damage in night-active rodents (Li *et al.*, 2001). As demonstrated by Ferraro (1984), one major advantage of such a LD_{FB} system could be the prevention of retinal damage.

4.17 Putting it all together

It has been shown that the *Per1* $^{-/-}$ mice exhibit a increased glucose tolerance, a disrupted corticosterone rhythm, a prolonged latency to react on a nociception stimulus on the hot plate, and an elevated temperature rise in the stress-induced hyperthermia experiment. In addition, the behavioural data from the open field suggests that the *Per1*$^{-/-}$ are more emotional than the WT controls.

Interestingly, the *Per1*$^{-/-}$ and the *Per2*$^{-/-}$ mice feed more per g body weight than WT mice. As the *Per1*$^{-/-}$ animals are lighter and the *Per2*$^{-/-}$ animals slightly heavier than the WT. In addition, the O_2 consumption of both mutants is elevated under LD 12:12, indicating a higher metabolic rate than in WT (Dernbach, 2003), which seems to be contradictory. While this is expected for the *Per1*$^{-/-}$ mice, the opposite would have been much more plausible for the *Per2*$^{-/-}$.

There might also be other consequences of elevated corticosterone levels than those discussed above. This might result in a decreased life span of the mice, as suggested by data on rats (Cavigelli and McClintock, 2003). If the levels of corticosterone are permanently elevated in the *Per1* $^{-/-}$ mice, learning and memory capabilities should also be lower. The effect of hypersecretion of corticosterone is detrimental to the hippocampus, which has the highest density of glucocorticoid receptors of any body tissue (McEwen, 1972; McEwen *et*

al., 1986). It has been reported that the *Per1*$^{-/-}$ mice do not show any abnormalities in their learning abilities (Abarca *et al.*, 2002). As these results were obtained by fear conditioning, which is a high stressor for the animals, it is not certain whether such extreme stress – possibly interpreted by the mouse as a life-threatening situation – might have a detrimental effect on learning and memory of the *Per1*$^{-/-}$ mice (Amit and Galina, 1986). In addition, the age of the animals seems to be of critical importance. Thus, it would be very interesting to test the *Per1*$^{-/-}$ mice in a milder learning paradigm such as the radial maze (Karl *et al.*, 2003c).

In recent years, the existence of peripheral oscillators has been discovered (Balsalobre, 2002). It has been proposed that glucocorticoids are one of the pathways by which the SCN synchronises the peripheral oscillators (Balsalobre *et al.*, 2000; Le Minh *et al.*, 2001). This is critically important for organising the metabolism of the whole body.

The findings of the present study may be thus useful for further investigations of this topic, which probably will come into the main focus of molecular chronobiology during the next years (Oster, 2003). It would be interesting to know whether the peripheral oscillators in *Per1*$^{-/-}$ mice are still synchronised to the SCN. If not, that might explain some of the phenotypic alterations in the *Per1*$^{-/-}$ mice. Similarly, chronic circadian desynchronisation caused by a weekly changing light regime can have severe effects on cardiac function and life span in golden hamsters (Penev *et al.*, 1998). Perhaps these two phenomena are both due to an internal desynchronisation of the peripheral clocks. The occurrence of ultradian rhythms in the *Per*-deficient mice (see Drinking Rhythms, and Zheng *et al.*, 1999) point in the same direction. Ultradian rhythms may be determined by peripheral oscillators, e.g. in the liver, and represent feeding rhythms. This phenomenon can also be observed in the premature circadian system, when ultradian rhythms are most prominent (Löhr and Siegmund, 1999).

In contrast to the well-defined picture that could be drawn for the *Per1*-deficient animals, that of the *Per2*$^{-/-}$ mice seem to be more complicated. It could be found that the null mutation of *Per2* has consequences on the physiology and behavioural response of the

animals: the glucose tolerance was increased, and the pain threshold was elevated in the hot plate nociception task.

In conclusion, the phenotyping of the *Per1*^{Brd} and *Per2*^{Brd} mice revealed differences to the WT mice in several domains both on a behavioural as well as on a physiological level. In order to find out whether these are due the influence of genes inherited from background strains or the *Per* genes themselves, it seems to be critically important to carry out a thorough phenotyping of the other available transgenic animal models for the *Per* genes. If the effects found in the *PerX*^{Brd} mice are reproducible in *PerX*^{ldc} and *PerX*^{psc} mice, this should provide good evidence that the *Per* genes are responsible. The results of the present study underline the importance of proper mutant phenotyping.

Furthermore, first evidence was presented that the *Per* genes are not only involved in the photic entrainment of the clock, but also in the processing of a non-photic zeitgeber, i.e. temperature. If investigated on a molecular level, this might bring further insight into the clock function or even reveal new functions of the *Per* genes.

5. References

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6. Appendices

Appendix A – List of abbreviations

α	significance level
%	per cent
τ	free-running period length
°C	degrees Celsius
3V	3rd ventricle
5-HT	serotonine
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
aPVN	autonomic paraventricular nucleus
AVP	arginine vasopressin
B6	C57BL6/J
BLAST	basic local alignment search tool
Bmal1	brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1
cAMP	cyclic adenosine monophosphate
CCG	clock controlled gene
cDNA	copy DNA
cGMP	cyclic guanosine monophosphate
CK1ε	casein kinase 1 ε
Clock	circadian locomotor output cycles kaput
CRE	cAMP responsive elements
CREB	cAMP responsive element binding protein
CRH	corticotrophin-releasing hormone
Cry	*Cryptochrome*
Csnk1ε	caseine kinase 1-epsilon
CT	circadian time
Dbp	D-albumin-binding protein
DD	constant darkness
DMH	dorsomedial hypothalamic nucleus
E	evening oscillator
ELISA	enzyme-linked immuno-sorbent assay
ENU	N-ethyl-N-nitrosourea
ES cells	embryonic stem cells

et al.	and co-workers
FASPS	familial advanced sleep phase syndrome
g	grams
GABA	γ-amino butyric acid
GH	gonadotropic hormone
GHT	geniculohypothalamic tract
GnRH	gonadotrophin-releasing hormone
GRP	gastrin-releasing peptide
h	hours
Hprt	hypoxanthine phosphoribosyltransferase gene
HT	hypothalamus
IGL	intergeniculate leaflet
IL1-β	interleukine1-beta
kb	kilobases
l	liters
LD	light/dark cycle
LGN	lateral geniculate nucleus
LL	constant light
LL_{FB}	feedback lighting conditions
LL_{hi}	constant light of high intensity
LPS	lipopolysaccheride
lx	lux (light intensity)
m	meters
M	mole per liter
M	morning oscillator
min	minutes
MOP3	member of PAS superfamily 3
MPN	medial preoptic nucleus
mRNA	messenger ribonucleic acid
MWU	Mann-Whitney U-test
NCAM	neuronal cell adhesion molecule
NPAS2	neuronal PAS domain protein 2
NPY	neuropeptide Y
Ox	optic chiasm

p	error probability
PACAP	adenylyl cyclase activating peptide
PAS	period / aryl hydrocarbon receptor nuclear translocator / singleminded
PBS	phosphate-buffered saline
Per	*Period*
Per1$^{-/-}$	B6.129S7-*Per1*^{tm1Brd}
Per2$^{-/-}$	B6.129S7-*Per2*^{tm1Brd}
PFA	paraformaldehyde
PID	passiv infrared detector
PRC	phase response curve
PVN	paraventricular nuclei of the hypothalamus
RevErbα	nuclear receptor subfamily 1, group D, member 1.
RHT	retinohypothalamic tract
s	seconds
SCN	suprachiasmatic nuclei
sPVN	sub-paraventricular nucleus
SS	somatostatin
T_a	ambient temperature
T_b	body temperature
Tim	*Timeless*
TK	*Herpes simplex* virus thymidine kinase gene
TNF-α	tumor necrosis factor alpha
Tris	Tris-(hydroxymethy)-aminomethane
TTL	transcriptional/ translational feedback loop
V	Volt
VIP	vasoactive intestinal peptide
vs.	versus
WC	warm/cold cycle
WT	wildtype
ZT	*zeitgeber* time
Ω	Ohm

Appendix B – Corticosterone metabolites in feces

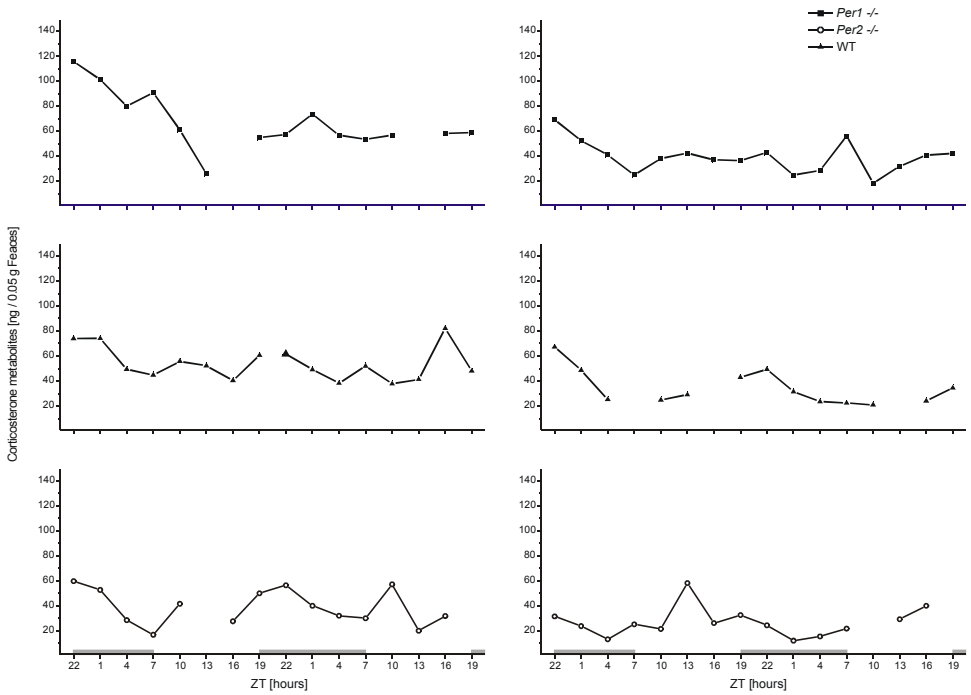


Figure 43. Concentration of corticosterone metabolites in feces for each animal of the first set.

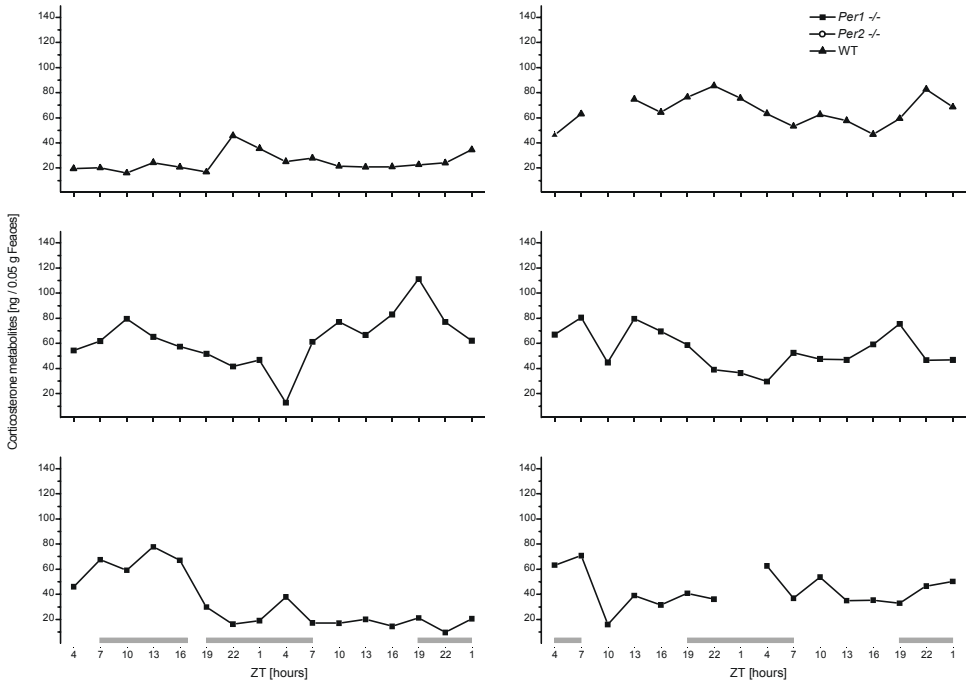


Figure 44. Concentration of corticosterone metabolites in feces for each animal of the second set.

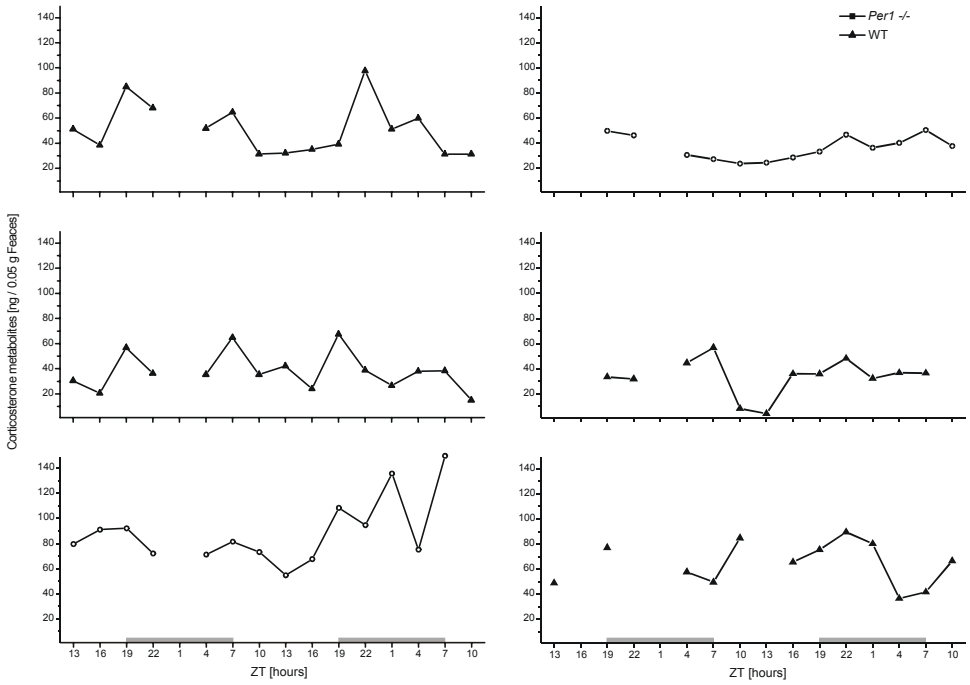


Figure 45. Concentration of corticosterone metabolites in feces for each animal of the third set.

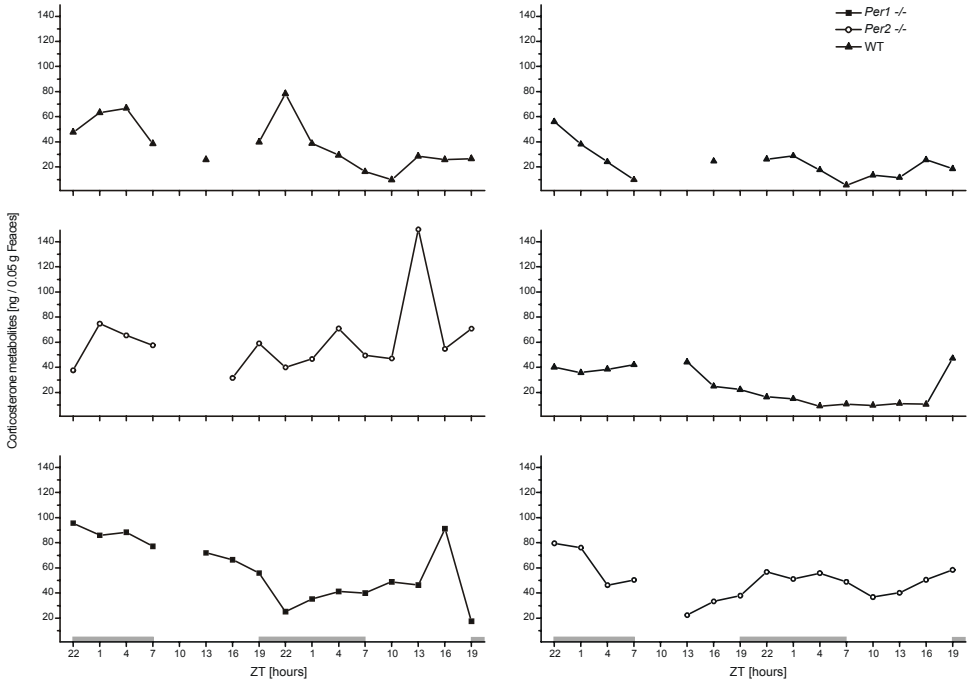


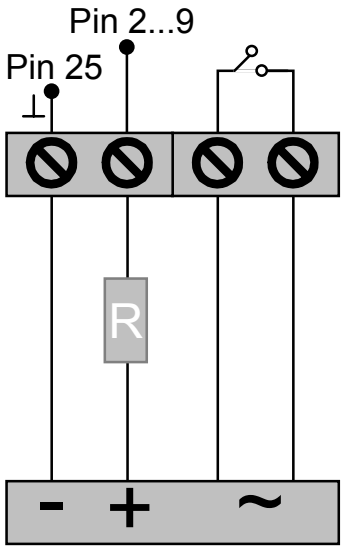
Figure 46. Concentration of corticosterone metabolites in feces for each animal of the fourth set.

Appendix C – The steady state relay card

Parts list

1	standard breadboard
1	steady state relay (S202S12, Sharp, Germany)
1	resistor (100 kΩ)
1	sub-D-25 connector
2	cable clamps
3 m	shielded cable (25 x 0.14 mm)

Circuit diagram

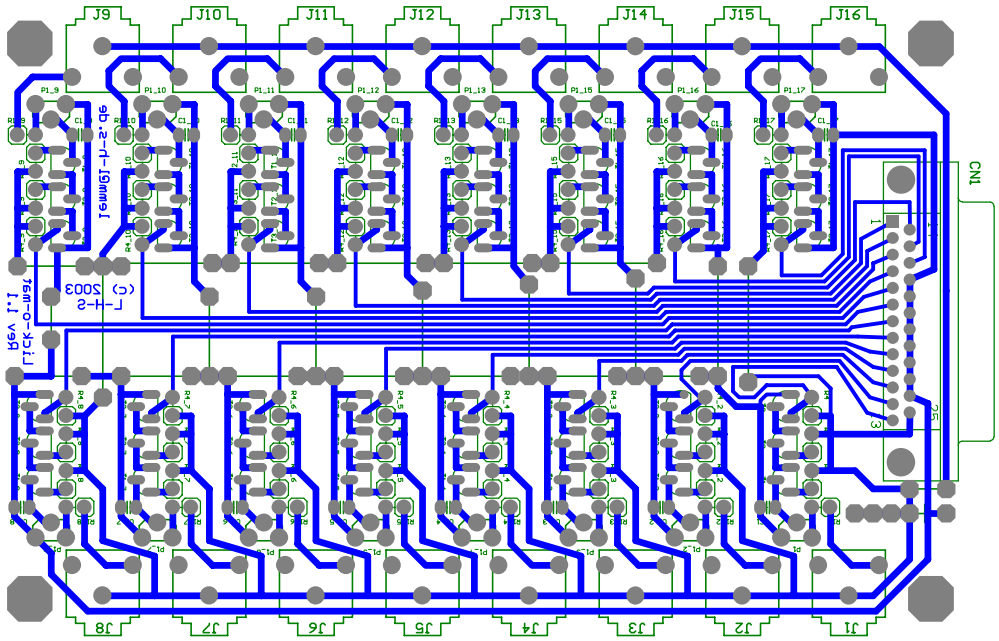


Appendix D – The Lick-O-Mat

Parts list

1	eurocard (10 x 15 cm)
32 m	shielded cable (2 x 0.14 mm)
5 m	shielded cable (25 x 0.14 mm)
16	ceramic capacitor
16	stereo input jack (3.5 mm)
16	stereo jack (3.5 mm)
16	pre-set potentiometer (1MΩ)
1	sub-D 25 strip
48	transistors (BC 547B)
16	resistors (100kΩ)
48	resistors (10kΩ)

Circuit diagram



7. Curriculum vitae

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Dallmann R. 2000. Die Gesangsvariabilität beim Silbergibbon (*Hylobates moloch*). GfP Rundbrief, 25.

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