Circadian genes *Period 1* and *Period 2* in the nucleus accumbens regulate anxiety-related behavior

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Abstract

It has been suggested for some time that circadian rhythm abnormalities underlie the development of multiple psychiatric disorders. However, it is unclear how disruptions in individual circadian genes might regulate mood and anxiety. Here we found that mice lacking functional *mPeriod 1* (*mPer1*) or *mPeriod 2* (*mPer2*) individually did not have consistent behavioral abnormalities in measures of anxiety-related behavior. However, mice deficient in both *mPer1* and *mPer2* had an increase in levels of anxiety-like behavior in multiple measures. Moreover, we found that *mPer1* and *mPer2* expression was reduced in the nucleus accumbens (NAc) after exposure to chronic social defeat stress, a paradigm that led to increased anxiety-related behavior. Following social defeat, chronic treatment with fluoxetine normalized *Per* gene expression towards wild-type levels. Knockdown of both *mPer1* and *mPer2* expression via RNA interference specifically in the NAc led to a similar increase in anxiety-like behavior as seen in the mutant animals. Taken together, these results implicate the *Per* genes in the NAc in response to stress and the development of anxiety.

Keywords

circadian rhythms; gene expression; mouse; psychiatric disease; stress; striatum

Introduction

Circadian rhythms are prominent in virtually every species on this planet and nearly all bodily and cognitive functions in humans follow a 24-h cycle (Ko & Takahashi, 2006). Several studies have found that disruptions in normal rhythms in the sleep/wake cycle can lead to a variety of health problems such as jet lag, shift workers' syndrome, and even increase the risk for cancer and heart disease (Moser *et al.*, 2006). It has become increasingly clear that circadian rhythms also contribute to differences in mood state, reward and motivation. Abnormal rhythms are strongly associated with psychiatric diseases like seasonal affective disorder, bipolar disorder, major depression, and drug addiction (McClung, 2007; Falcon & McClung, 2009). Moreover, many of the treatments used for these illnesses are known to modulate the circadian clock. Nonetheless, the exact role of

rhythm disruptions in these diseases still remains elusive, as does the contribution of specific circadian genes in individual brain regions in the regulation of mood.

The circadian clock in mammals is regulated by a core transcriptional/translational loop that cycles over the course of 24 h (Ko & Takahashi, 2006). The suprachiasmatic nucleus (SCN) is the location of the central pacemaker in the brain; however, most circadian genes are widely expressed throughout the brain and in other organs. The central components are the circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1) proteins that dimerize and induce the expression of the *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. The CRY and PER proteins can inhibit the activity of CLOCK and BMAL1, thus creating a negative feedback loop. Several other proteins, such as Rev-erbα and β, retinoid-related orphan receptor beta (RORβ), casein kinase 1 epsilon and glycogen synthase kinase 3 beta (GSK3β), are also involved in regulating the timing of these rhythms. It was shown previously that the three *Period* genes have differential functions in the regulation of circadian rhythms (Shearman *et al.*, 2000; Bae *et al.*, 2001). Mice with a mutation in either *mPeriod 1* (*mPer1*) or *mPeriod 2* (*mPer2*) have disrupted free-running rhythms with lower amplitude in constant darkness. However, most mice retain a significant period of approximately 22–23 h for several days in constant darkness before losing all rhythmicity (Bae *et al.*, 2001). Animals with mutations in both *mPer1* and *mPer2* have a much more dramatic and immediate loss of rhythmicity in constant darkness, suggesting that PER1 and PER2 can compensate for one another to some extent when one protein is lost to help maintain circadian rhythms (Bae *et al.*, 2001). In contrast, mice with a mutation in *mPeriod 3* (*mPer3*) have surprisingly few disruptions in circadian locomotor activity. Indeed, mice with double mutations in *mPer3* along with *mPer1* or *mPer2* do not have an increase in circadian rhythm disruption over the single *mPer1* or *mPer2* mutations alone, suggesting that this gene has a minimal role in core circadian clock function even though it displays rhythmic expression levels in the SCN (Shearman *et al.*, 2000; Bae *et al.*, 2001). However, recent studies suggest a more tissue-specific role for *mPer3* in the circadian timekeeping of peripheral oscillators throughout the body, like the liver, lung, and esophagus, among others (Pendergast *et al.*, 2012).

Several human genetic studies have identified single nucleotide polymorphisms and haplotypes in individual circadian genes that associate with various psychiatric disorders. For example, variations in *Clock*, *Bmal1*, *GSK3*β, *Per3*, *ROR*β and *Rev-erb*α have all been linked to a bipolar disorder diagnosis or various aspects of bipolar disorder (Benedetti *et al.*, 2004; Nievergelt *et al.*, 2006; Benedetti *et al.*, 2007; Kishi *et al.*, 2008; Kripke *et al.*, 2009; McGrath *et al.*, 2009). Moreover, polymorphisms in *Per2* are associated with vulnerability to major depression and seasonal depression, as well as alcoholism (Spanagel *et al.*, 2005; Partonen *et al.*, 2007; Lavebratt *et al.*, 2010), and levels of *Clock*, *Per1* and *Bmal1* mRNA are elevated in blood leukocytes of people with a history of depression (Gouin *et al.*, 2010). Recently, single nucleotide polymorphisms in *Bmal2* (a functional homologue to *Bmal1*) have been identified that associate with social phobia anxiety-related disorders (Sipila, 2010).

Work in animal models has found that mice with a mutation in the *Clock* gene (*Clock*Δ19) (King *et al.*, 1997; Vitaterna *et al.*, 2006) have a complete behavioral profile that is strikingly similar to human bipolar patients in the manic state, including hyperactivity, lowered levels of anxiety-like behavior or increased "risk-taking" behavior, lowered levels of depression-like behavior, and an increase in the reward value for a variety of stimuli (McClung *et al.*, 2005; Roybal *et al.*, 2007). A few studies have similarly begun to examine the influence of the *Per* genes on mood and reward-related behavior in mice (Abarca *et al.*, 2002; Spanagel *et al.*, 2005; Halbout *et al.*, 2011). Here we wanted to examine the influence of the *Per* genes

on anxiety-related behavior and start to understand the mechanisms by which these genes are involved in the response to stress.

Materials and methods

Mice

The homozygous *mPer1^{ldc}*, *mPer2^{ldc}*, and *mPer1;mPer2^{ldc}* mice used in this study were generously provided by David Weaver and colleagues at UMass Medical School (Shearman *et al.*, 2000; Bae *et al.*, 2001) and bred and genotyped at UT Southwestern Medical Center at Dallas. All mutant mice were on a 129sv background. Individual wild-type (WT) littermates generated from heterozygote breeding were used as controls for all single gene knockouts and combinations of these WT animals from the single mutation crosses were compared with the double knockout animals (which were maintained as homozygotes for both mutations). No behavioral differences were found between WT animals from the *mPer1* or *mPer2* crosses (data not shown). C57BL/6J mice used in RNA interference were purchased from Jackson Laboratory and group housed for at least 1 week prior to stereotaxic surgery. Mice used in social defeat experiments are described in detail below. All animals were maintained in a 12:12 light/dark cycle (lights on at 07:00 h) with food and water freely available. Experimental mice used in behavioral analysis were adult males (8–12 weeks old) and behavioral tests of locomotor activity, anxiety, and depression-related behavior were conducted between ZT 3 and ZT 6 (social defeat experiments are detailed below). All procedures were approved by our Institutional Animal Care and Use Committee at UT Southwestern.

Behavioral tests

We utilized a battery of behavioral tests to phenotype mice working from the least to most stressful as previously characterized (Tarantino & Bucan, 2000; Paylor *et al.*, 2006). Importantly, supporting research indicates that there is little to no effect on behavioral performance between inter-test intervals of 1 week compared with 1–2 days allowing for rapid and semi-high-throughput screening of behavior (Paylor *et al.*, 2006). An inter-test interval of 1–2 days was adopted for this study in line with our previously published results (Roybal *et al.*, 2007; Mukherjee *et al.*, 2010).

Locomotor response to novelty—Mice were individually placed in automated locomotor activity chambers equipped with infrared photobeams (San Diego Instruments, San Diego, CA, USA) and measurements began immediately. The activity of the animal was continuously measured and the data were collected in 5-min blocks over a period of 2 h.

Elevated plus maze—Mice were placed in the center of an elevated plus maze (EPM) (arms are 30×5 cm, with 25-cm-high walls on the closed arms) under low light levels and their behavior was monitored for 5 min. The time spent on the closed and open arms, as well as the number of explorations of open and closed arms, were determined by video tracking software (Ethovision 3.0, Noldus, Leesburg, Virginia, USA). The apparatus was cleaned and allowed to dry between mice.

Dark/light test—The dark/light apparatus consisted of two-chambered boxes (25×26 cm for each side) (Med Associates, St Albans, Vermont, USA). One side was kept dark (room light entry limited) and the other side was brightly lit by a fluorescent bulb across the top. Mice were first placed in the dark side for 2 min, then the door between the compartments was opened and they were allowed to freely explore either the light or dark side for 10 min. Anxiety-like behavior was measured as the activity in the lit side during the final 10 min.

Open field test—Mice were placed in the periphery of a novel open field environment (44 × 44 cm, 30-cm-high walls) in a dimly lit room and allowed to explore for 5 min as described previously (Roybal et al., 2007). The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision 3.0, Noldus) to determine the time, distance moved and number of entries into two areas: the periphery (5 cm from the walls) and center (14 × 14 cm). The open field arenas were wiped and allowed to dry between mice.

Social defeat and avoidance testing

Social defeat and avoidance testing were performed according to published protocols (Berton *et al.*, 2006; Tsankova *et al.*, 2006; Krishnan *et al.*, 2007). Briefly, CD1 retired breeder mice (purchased from Jackson Laboratory) were screened for consistent attack latencies (<30 s on three consecutive screening sessions with a C57BL/6J intruder). C57BL/6J mice were purchased from Jackson Laboratory and group housed in a 12-h light/dark cycle with food and water *ad libitum* for at least 1 week prior to the defeat protocol in our facility. During each defeat episode, intruder mice were allowed to interact for 10 min with an unfamiliar aggressive CD1 mouse, during which they were attacked and displayed subordinate posturing. Non-defeated controls were housed in identical cages opposite each other and were rotated similarly. Immediately after the tenth defeat all mice were singly housed. Social defeat was always performed in the few hours before the onset of the dark phase (17:30–18:30 h). Social interaction tests were performed 24 h after the last defeat and then again after the last day of antidepressant treatment (fluoxetine pellets implanted s.c. for 28 days). On these days the time spent in the interaction zone during the first (target absent) and second (target present) trials was measured and the interaction ratio was calculated as $100 \times$ (interaction time, target present)/(interaction time, target absent). Previous work has found that depression-related behavior persists for 28 days after the social defeat protocol and that antidepressant treatment completely reverses the social interaction deficit seen in susceptible animals (Berton *et al.*, 2006; Tsankova *et al.*, 2006). Animals were killed 24 h after the last fluoxetine or vehicle treatment and nucleus accumbens (NAc) dissections were taken by punch dissection between ZT 4 and ZT 6 as described previously (McClung & Nestler, 2003).

Quantitative polymerase chain reaction

The cDNA was mixed with buffer, primers, SYBR green, and hot-start Taq polymerase in a prepared master mix (Applied Biosystems, Foster City, CA, USA). The polymerase chain reactions followed by a dissociation reaction to determine the specificity of the amplified product were run on a Real-Time polymerase chain reaction machine (7300 Real-Time PCR machine, Applied Biosystems). The amount of gene expression was quantified using the $\Delta\Delta Ct$ method as previously described (LaPlant *et al.*, 2009). The following primer sets were used to measure *Per* expression: mPer1 forward, CTCTGTGCTGAAGCAAGACCG; mPer1 reverse, TCATCAGAGTGGCCAGGATCTT; mPer2 forward, GAGTGTGTGCAGCGGCTTAG; and mPer2 reverse, GTAGGGTGTCATGCGGAAGG.

Construction of mPer1;mPer2 small hairpin RNA

A small hairpin RNA (shRNA) was constructed against *mPer1* and *mPer2* mRNA by selecting a conserved 24-base sequence (5′-ATCCCTCCTGACAAGAGGATCTTC-3′) in the coding region. For the scrambled shRNA, a random sequence of 24 bases (5′-CGGAATTTAGTTACGGGGATCCAC-3′) that had no sequence similarities with any known genes/mRNA was used. An antisense sequence of the selected mRNA region followed by an miR23 loop of 10 nucleotides (CTTCCTGTCA) was added at the 5′ end of the above sequences. These shRNAs were designed as synthetic duplexes with overhang

ends identical to those created by Sap I and Xba I restriction enzyme digestion. The annealed oligonucleotides were cloned into the adeno-associated virus (AAV) plasmid expressing enhanced green fluorescent protein (Stratagene, La Jolla, CA, USA). Plasmids were sent to the University of North Carolina Viral Vector Core for production (Chapel Hill, NC, USA).

Stereotaxic surgery

Stereotaxic surgery was performed as described in Mukherjee et al. (2010). Mice were anesthetized with a mixture of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline (0.9% NaCl). Bilateral stereotaxic injections of 1 μL of purified high-titer AAV encoding scrambled or AAV-mPer1;mPer2 shRNA was injected into the NAc (from bregma: angle 10°; AP, +1.5 mm; Lat, +1.5 mm; DV, −4.4 mm) using a 33-gauge hamilton syringe (Hamilton, Reno, NV, USA). The injection speed was 0.1 μL/min, and the needle was kept in place for an additional 5 min before it was slowly withdrawn. Mice recovered for 2 weeks in their home cage prior to behavioral testing to allow for full virus expression.

Immunohistochemical localization of adeno-associated virus expression

Mice were anesthetized with 50 mg/kg sodium pentobarbital in saline, and transcardially perfused with 4% paraformaldehyde in $1\times$ phosphate-buffered saline (1 mM KH_2PO_4, 10 M Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The brains were allowed to post-fix in 4% paraformaldehyde for 24 h and then placed in $1\times$ phosphate-buffered saline/30% glycerol sucrose protection for an additional 24 h before being stored in $1\times$ phosphate-buffered saline/0.05% sodium azide. Brain sections (30 μm) were obtained with a microtome (Leica, Wetzlar, Germany, USA) and immunohistochemical staining against GFP (AbCam, Cambridge, MA, USA) was carried out using standard procedures. Secondary antibodies (anti-rabbit conjugated with Alexa 488) were purchased from Molecular Probes (Carlsbad, CA, USA). Brain sections were mounted using Vectashield (Vector Labs, Burlingame, CA, USA) with DAPI counterstaining and observed with an epifluorescence microscope with a $10\times$ objective. Animals were excluded from our study if their infection spread was not localized to the NAc with spillover to adjacent areas or throughout the injection tract, or if there was a significant disproportionate amount of infection between both sides. Exclusion by these criteria accounted for approximately 10% of animals.

Statistical analysis

All data are expressed as mean ± SEM. Significance for two group comparisons in behavioral assays was determined by one-way ANOVA and post-hoc analysis. Behavioral results from the shRNA experiments and qPCR analysis were analyzed by Student's t-test. In all experiments $P<0.05$ is considered significant.

Results

mPeriod 1 and *mPeriod 2* are involved in regulating anxiety-related behavior

To determine if mice deficient in *mPer1*, *mPer2*, or both have any anxiety-related behavioral abnormalities, we utilized the EPM and dark/light tests. These tests have been validated extensively as a measure of anxiety-related behavior and for their sensitivity to anxiolytic and anxiogenic drugs (Belzung & Griebel, 2001). Compared with WT animals, we found a significant decrease in the percentage of time spent in the light side of a dark/light chamber in the *mPer1;mPer2* double mutant mice (Fig. 1A) (one-way ANOVA, $F_{3,51} = 2.771$, $P = 0.05$; post-hoc Bonferroni test, $P < 0.05$). Likewise, in Fig. 1B, when we examined activity on the light side of the dark/light chamber there was a sizeable decrease in activity in mice

lacking both *mPer1* and *mPer2* (one-way ANOVA, $F_{3,51} = 3.579$, P = 0.02; post-hoc Bonferroni test, P < 0.05). Importantly, when we examined total activity for the 10-min testing period, there was no difference between the genotypes (Supplementary Fig. 1A). Mice deficient in *mPer2* alone were similar to WT animals in the dark/light test, whereas mice deficient in *mPer1* showed a non-significant trend towards a decrease in the percent time and activity in the light. In the EPM, analysis of percent time spent in the open arms of the EPM was statistically significant between groups as measured by one-way ANOVA in Fig. 1C ($F_{3,47} = 3.279$, P = 0.029), whereas Bonferroni post-hoc tests revealed a significant decrease in time spent in the open arms for both *mPer2* single and *mPer1;mPer2* double mutant mice (P < 0.05). Analysis of the number of open arm entries nearly met significance by one-way ANOVA (Fig. 1D) ($F_{3,47} = 2.692$, P = 0.0568) with a trend towards a decrease in open arm entry number for *mPer1* (6.125 ± 1.695), *mPer2* (6.50 ± 1.142), and *mPer1;mPer2* double mutants (7.222 ± 1.051) as compared with WT mice (10.10 ± 1.044). There were no differences between genotypes in the total crosses, percent time in the closed arm, or time in the center of the EPM, indicating no general change in activity (Supplementary Fig. 1B, D and F). Additional analysis in the open field test revealed a strong trend toward a decrease in the frequency of times that the *mPer1;mPer2* mutant mice spent in the center of the field when analyzed by one-way ANOVA (WT mean, 4.9 ± 0.90; *mPer1;mPer2* mean, 2.10 ±0.83; *mPer1* mean, 2.88 ± 0.81; *mPer2* mean, 4.8 ±0.99; $F_{3,67}=2.551$, P=0.0629) with no change in total activity, in support of the overall conclusion that *mPer1;mPer2* mutant mice displayed increased anxiety-like behavior. Importantly, as the single *mPer1* and *mPer2* deficient lines showed greater inconsistency in these measures, this suggests that each protein is likely able to compensate to some extent for the loss of the other.

Mutations in the *Period* genes do not alter the locomotor response to novelty

Mice with a mutation in the *Clock* gene are hyperactive in response to a novel environment (Roybal *et al.*, 2007). Thus, we wanted to determine if mice with a mutation in either *mPer1*, *mPer2*, or *mPer1;mPer2* had any differences in novelty-induced activity. Mice were placed in an unfamiliar chamber for 2 h and beam breaks were counted in 5-min bins. We found that loss of *Period* gene function did not lead to significant changes in total locomotor activity between all genotypes tested ($F_{3,43} = 2.181$, P = 0.1041) (Fig. 2). These results suggest that *mPer1* and *mPer2* are not involved in the regulation of the locomotor response to novelty. These results also confirm that the decreased exploratory behavior seen in the anxiety-related tests was not due to an overall decrease in locomotor activity.

Chronic social defeat and antidepressant treatment alter *Period* gene expression in the nucleus accumbens

As mice with mutations in both *Per* genes have significant behavioral phenotypes in anxiety-related measures, we wanted to know if chronic stress or anxioloytic/antidepressant treatment would alter *Per* gene expression in the NAc. We decided to employ a social defeat paradigm in which mice are subjected to attacks by an aggressive mouse once per day for 10 days. They are also housed in an environment where they can see and smell the aggressor at all times during the 10-day protocol. Previous studies have found that, in the majority of mice, this paradigm leads to a profound increase in social avoidance behavior and anxiety-related behavior that is extremely long lasting, but can be reversed with chronic antidepressant treatment (Berton *et al.*, 2006; Tsankova *et al.*, 2006). Indeed, with this experiment, we found a highly significant decrease in social interaction following defeat that was rescued by 28 days of fluoxetine treatment (Supplementary Fig. 2). We chose to examine changes in expression in the NAc, which is highly involved in both depression and anxiety-related behavior because previous studies have found that social defeat leads to specific gene expression changes in this region (Nestler & Carlezon, 2006; Krishnan *et al.*,

2007). Furthermore, *Per* gene expression is strong in striatal regions, and can be altered in these regions by other behavioral paradigms such as those that employ chronic exposure to drugs of abuse (Nikaido *et al.*, 2001; Iijima *et al.*, 2002). When we measured *Per* gene expression we found that mice that had been exposed to social defeat stress for 10 days had a significant decrease in both *mPer1* ($T_{13} = 2.387$, P = 0.0329) and *mPer2* ($T_{13} = 2.33$, P = 0.0366) expression (Fig. 3). Furthermore, mice that received fluoxetine treatment following defeat had levels of both *mPer1* and *mPer2* that were not different from control mice, suggesting that antidepressant treatment normalizes *Per* gene expression (Fig. 3). Interestingly, control mice that did not experience the social defeat but did have chronic fluoxetine treatment had no significant changes in *mPer1* or *mPer2* (Fig. 3). These results demonstrate that chronic stress leads to a long-lasting decrease in *Per* gene expression in the NAc that is partially reversed by antidepressant treatment.

Knockdown of *mPeriod 1* and *mPeriod 2* in the nucleus accumbens contributes to anxiety-related behavior

To determine if a knockdown of *mPer1* and *mPer2* gene expression specifically in the NAc would be sufficient to increase anxiety, we employed an AAV virus with a short hairpin RNA that targeted a common sequence in both *mPer1* and *mPer2*. This leads to a decrease in the expression of both genes via RNA interference mechanisms. In mouse embryonic fibroblast cells, this AAV-shRNA led to large decreases in *mPer1* and *mPer2* expression (7.2-fold and 13.3-fold, respectively, data not shown) when compared with expression of an AAV-scrambled control shRNA that does not match the sequence of any known gene. Infection with the AAV-*mPer1/mPer2* shRNA virus in the NAc of intact mice led to a more modest threefold decrease in *mPer1* ($T_4 = 4.959$, P = 0.0077) and a 2.4-fold decrease in *mPer2* ($T_5 = 3.704$, P = 0.0139) compared with controls (Fig. 4B). To ensure that this decrease was specific to *mPer1* and *mPer2*, we measured levels of *mPer3* and found no change in expression (data not shown). After waiting 2 weeks for full viral expression in the NAc, we subjected AAV-*mPer1/mPer2* shRNA-infected mice and mice expressing the AAV-scrambled control shRNA to the EPM and open field. Even with this modest decrease in *mPer1* and *mPer2* expression in the NAc, we saw significant differences in anxiety-related behavior. In the EPM, there was a significant decrease in the number of open arm entries in the *mPer1/mPer2* shRNA-infected mice ($t_{19} = 1.845$, P = 0.04) (Fig. 4C). Total crosses, closed arm entry frequency and center time were similar between groups (Supplementary Fig. 1C, E and G). In addition, there was a significant decrease in percent time spent in the center of an open field in the *mPer1/mPer2* shRNA-infected mice ($t_{19} = 1.708$, P = 0.05) (Fig. 4D). These data suggest that *mPer1* and *mPer2* expression in the NAc is important in the regulation of anxiety-related behavior.

Discussion

Our results show that the *Per* genes are involved in the regulation of anxiety-related behavior. Mice lacking both *mPer1* and *mPer2* have a robust increase in anxiety-related behavior, whereas the single gene mutants display inconsistent results across tests with mice lacking a functional *mPer1* or *mPer2* gene alone having greater anxiety-like behavior in a more restricted subset of measures. A recent study found that *mPer1* mutant mice show enhanced alcohol consumption following social defeat stress relative to WT mice (Dong *et al.*, 2011). Moreover, an SNP in the *hPer1* promoter that leads to lowered cortisol-induced *Per1* transcription was associated with psychosocial adversity and drinking in adolescents (Dong *et al.*, 2011). In line with our results, these results suggest that lowered *Per* gene expression is associated with a heightened and anxiogenic response to stress.

It is interesting that the *mPer1;mPer2* mutant mice have an increase in anxiety-related behavior, whereas mice with a mutation in the *Clock* gene have a decrease in anxiety-related

behavior (Roybal et al., 2007). The *Clock* mutant mice are also hyperactive in response to novelty, whereas the *Per* gene mutants are normal in these measures. These results show that disruption in any of the core circadian genes does not result in a common behavioral profile. CLOCK and PER proteins have opposing activity in the circadian loop with PER proteins acting as inhibitors of CLOCK. Therefore, perhaps an opposing behavioral phenotype in these measures is not surprising if it is dependent upon CLOCK activity. Interestingly, SCN lesions lead to an antidepressant effect in measures of behavioral despair, but no change in anxiety-related behavior (Engelmann *et al.*, 1998; Tataroglu *et al.*, 2004; Tuma *et al.*, 2005). This suggests that the anxiogenic effects seen in the m*Per1;mPer2* mutant mice are due to a lack of *Per* gene function in other brain regions outside the SCN.

It is recognized that anxiety and depression occur more frequently in females compared with males in the human population (Palanza, 2001). A few laboratories have investigated gender-specific differences in stress responses and anxiety-related behaviors with mixed results. In one set of studies, female rats displayed heightened anxiety-like behavior in the Vogel conflict test, whereas in the EPM male rodents were the more anxious of the two (Johnston & File, 1991). In a more recent study examining NPY knockout mice, although the genetic manipulation increased anxiety-like responses in both sexes, the effect was more pronounced in males compared with females (Karl *et al.*, 2008). In addition, working with female rodents requires consideration of the estrous cycle, adding an additional experimental variable with indices of anxiety-related behavior affected by cycle phase (Mora *et al.*, 1996). In the present investigation, male mice were exclusively utilized in the behavioral assays for a number of reasons including those outlined above. We used tests of anxiety-related behavior that have been well-validated in male rodents. The chronic social defeat stress paradigm, for instance, is applicable only for male rodents with valid defeat stress models lacking for female rodents. Male and female rodents respond very differently to similar stressors, thus the same tests may not be suitable for the two populations. For instance, although male rodents have greater levels of anxiety-like behavior after exposure to a single restraint stress but adapt upon repeated exposure, female rodents display the opposite phenotype (Kennett *et al.*, 1986). It is recognized that these studies will need to be extended to female mice in the future to understand the biological mechanisms underlying gender differences in anxiety-related behavior. Intriguingly, previous studies have implicated the circadian proteins CLOCK and PER2 in regulation of the female reproduction and estrous cycle (Pilorz & Steinlechner, 2008; Shimizu *et al.*, 2011). Furthermore, it is known that sex hormones affect circadian rhythms (Iwahana *et al.*, 2008), thus there may be some dynamic feedback between the two systems in the brain.

We decided to examine changes in expression in the NAc, which is highly involved in regulating behaviors in response to emotional stimuli because previous studies have found that social defeat leads to specific gene expression changes in this region (Nestler & Carlezon, 2006; Krishnan *et al.*, 2007). Furthermore, *Per* gene expression is strong in striatal regions, and can be altered in these regions by other behavioral paradigms such as those that employ chronic exposure to drugs of abuse (Nikaido *et al.*, 2001; Iijima *et al.*, 2002). There is a growing body of literature implicating the NAc in the response to stress and in particular the development of anxiety (Barrot *et al.*, 2002; Krishnan & Nestler, 2008; Chen *et al.*, 2012). Importantly, the NAc is situated to integrate information from a large number of brain structures involved in processing stress and anxiety-related responses, including the prefrontal cortex, hypothalamus, hippocampus, and basolateral amygdala. Moreover, most measures of anxiety-like behavior in rodents evoke the conflict between trepidation and the desire to explore a novel environment, which is highly NAc dependent. A recent fMRI study conducted in human subjects demonstrated that the NAc is activated and inactivated during active and passive avoidance, respectively, and the degree of activation/deactivation correlates with individual levels of anxiety (Levita *et al.*, 2012). These data demonstrate that

the NAc is important for both positively and negatively influenced motivated behavior. In the present investigation, chronic social defeat stress led to a decrease in *Per* gene expression in the NAc at 28 days after the last defeat. Moreover, treatment with fluoxetine resulted in levels of both *Per* genes that were not different from control mice. Similar results were seen following treatment with a tricyclic antidepressant, imipramine (data not shown). Interestingly, a recent study (Koresh *et al.*, 2012) found that expression of *mPer1* and *mPer2* was elevated in the hippocampus, frontal cortex and SCN at 8 days after exposure to predator scent stress in animals with an "extreme" (i.e. PTSD-like) behavioral response. Immediate treatment with agomelatine reversed these changes. Conversely, PER2 levels were decreased in the SCN but not hippocampus or prefrontal cortex in a time-specific manner in a study that employed chronic restraint as the stressor (Kinoshita *et al.*, 2012). Night-time administration of the mood-stabilizer lithium normalized the stress-induced change in PER2 (Kinoshita *et al.*, 2012). This suggests that there are widespread disruptions in *Per* gene expression throughout the brain following stress, and whether the changes are upwards or downwards might be specific to the region of the brain, type of stressor, amount of time following stress, and the time of day at which changes are measured.

Per gene expression appears to be particularly important in the NAc, as we find that local knockdown in this region is sufficient to increase anxiety-related behavior. Indeed, a relatively modest two- to threefold decrease in expression level for *mPer1* and *mPer2* was sufficient to produce a significant behavioral effect. It has been reported that the levels of the *mPer1* transcript in the striatum fluctuate naturally by approximately threefold in a 24-h period (Uz *et al.*, 2003). Research indicates that both the abundance and circadian oscillation pattern of the PERs are critically important for the circadian clock mechanism both *in vitro* and *in vivo* (Lee *et al.*, 2011; Chen *et al.*, 2012), thus it is perhaps not surprising that such a modest change could result in a significant outcome. The interpretation of constitutive shRNA knockdown data for a circadian rhythm-related gene is complicated because it cannot be said whether the effects are due to a change in transcript levels, rhythms or some combination of the two.

High rates of comorbidity between substance use and anxiety disorders have been well documented (Compton, 2007). Thus, the anxiogenic phenotype of the *mPer1;mPer2* mutants and the *mPer1;mPer2* shRNA knockdown in the NAc is particularly interesting in light of the reward-related phenotypes that have previously been reported in these mice. Interestingly, in 75% of cases in which anxiety disorders are comorbid with substance use disorders, the anxiety-related diagnosis pre-dates the substance use problem (Kushner *et al.*, 2008). Although these data do not point to causality, they may suggest directionality with anxiety recognized as a risk factor for substance use disorders. It has been shown that *mPer1* mutants fail to sensitize to the locomotor stimulating effects of cocaine or develop expression of cocaine conditioned place preference (Abarca *et al.*, 2002), although more recent data show that these deficits do not extend to operant self-administration and reinstatement of cocaine-seeking behavior (Halbout *et al.*, 2011). Conversely, *mPer2* mutants show a hypersensitized response to cocaine with normal expression of conditioned place preference (Abarca *et al.*, 2002). These studies also extend to other drugs of abuse. The *mPer2* mutants display enhanced voluntary consumption of alcohol (Spanagel *et al.*, 2005), whereas *mPer1* expression is involved in morphine reward via extracellular signal-related kinase signaling mechanisms (Liu *et al.*, 2007). Taken together, these data point to the involvement of the circadian PER proteins in reward-related behavior in addition to this newly defined role in anxiety-related behavior. As drug abuse and dependence are highly sensitive to stress and mood state, in the future it will be interesting to determine how these phenotypes interact in the *mPer1* and *mPer2* mutant animals.

In summary, *mPer1* and *mPer2* in the NAc appear to be centrally involved in the response to stress and development of anxiety-related behavior. Our results also suggest that an increase in these proteins in the NAc could be beneficial in the reversal of anxiety-related behavior following chronic stress. Thus, the *Per* genes represent new potential therapeutic targets for the treatment of anxiety disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AAV	adeno-associated virus
BMAL1	brain and muscle ARNT-like protein 1
CLOCK	circadian locomotor output cycles kaput
Cry	*Cryptochrome*
EPM	elevated plus maze
GSK3β	glycogen synthase kinase 3 beta
mPer1	*mPeriod 1*
mPer2	*mPeriod 2*
mPer3	*mPeriod 3*
NAc	nucleus accumbens
Per	*Period*
RORβ	retinoid-related orphan receptor beta
SCN	suprachiasmatic nucleus
shRNA	small hairpin RNA
WT	wild-type

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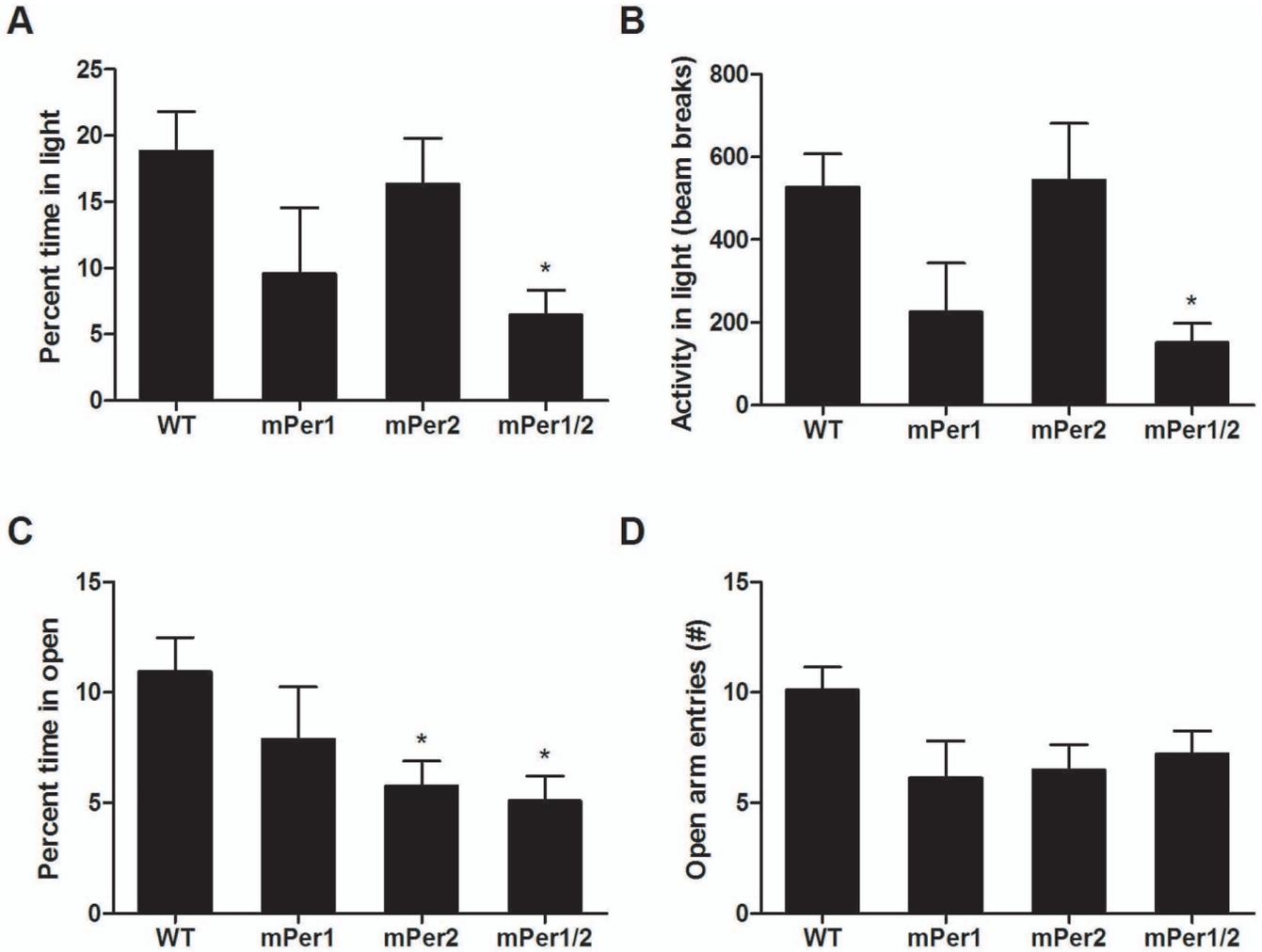
Fig. 1.

mPer1;mPer2 mutant mice display increased anxiety-related behavior. (A) *mPer1;mPer2* mutant mice spent less time in the lighted compartment of the dark/light box. *P < 0.05, one-way ANOVA followed by Bonferroni post-hoc test (n = 25, WT; n = 7, *mPer1*; n = 12, *mPer2*; n = 11, *mPer1/2*). (B) *mPer1;mPer2* mutant mice had less locomotor activity in the light as measured by beam breaks in the lit compartment of a dark/light box compared with WT mice. *P < 0.05, one-way ANOVA followed by Bonferroni post-hoc test (n = 20, WT; n = 8, *mPer1*; n = 14, *mPer2*; n = 9, *mPer1/2*). (C) *mPer2* and *mPer1;mPer2* mutant mice spent less time in the open arms of the EPM. *P < 0.05, one-way ANOVA followed by Bonferroni post-hoc test (n = 25, WT; n = 7, *mPer1*; n = 12, *mPer2*; n = 11, *mPer1/2*). (D) Open arm entry frequency in the EPM (n = 20, WT; n = 8, *mPer1*; n = 14, *mPer2*; n = 9, *mPer1/2*).
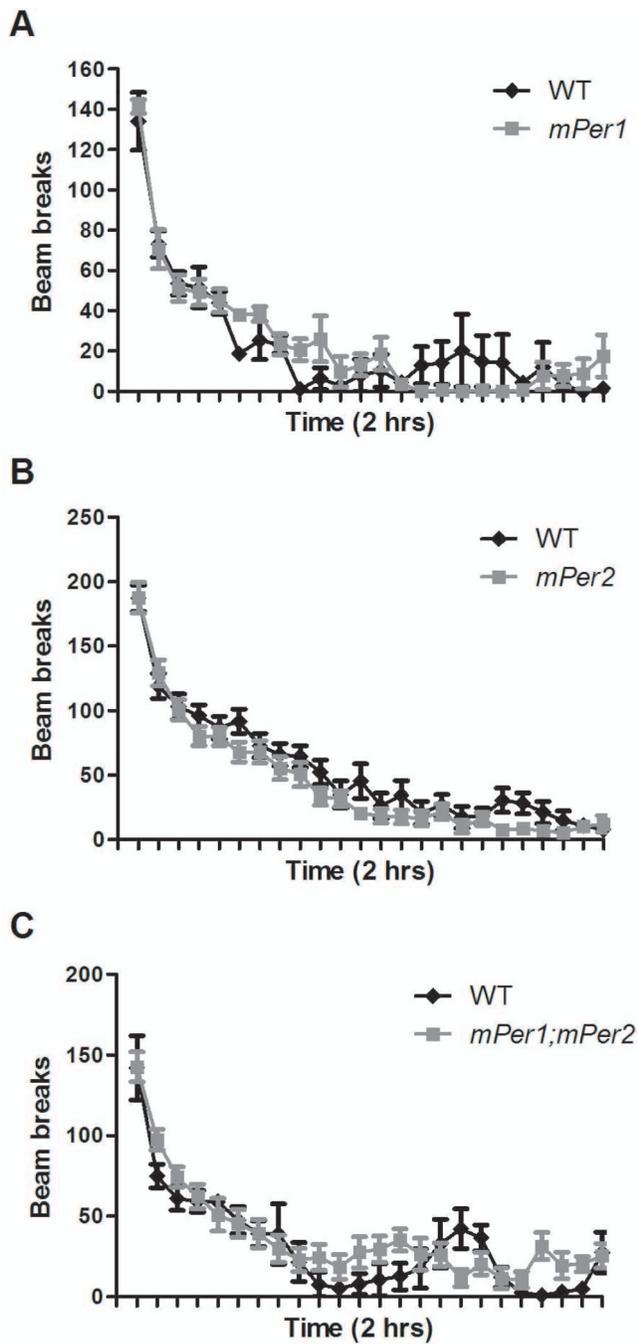
Fig. 2.
Mice with mutations in the *Per* genes have a normal locomotor response to novelty.
Locomotor activity of (A) *mPer1* (n = 5), (B) *mPer2* (n = 14), and (C) *mPer1;mPer2* (n = 9)
mutant mice was not significantly different from WT littermates (n = 20) by two-way
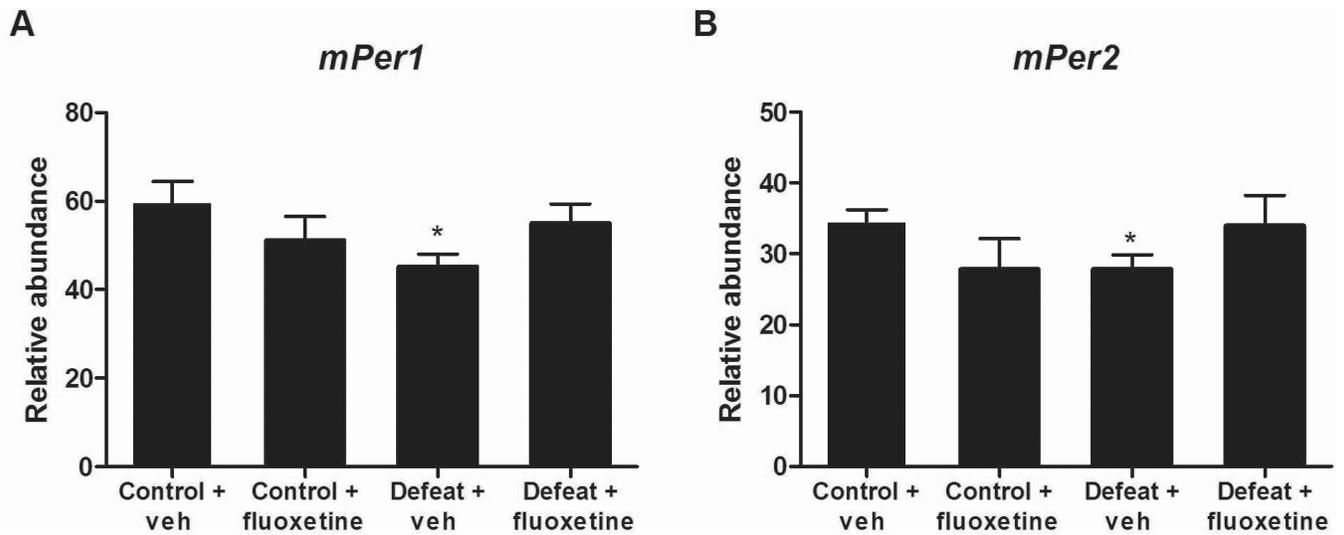ANOVA.

Fig. 3.
Chronic stress leads to a decrease in *mPer1* and *mPer2* expression in the NAc. WT mice were subjected to 10 days of social defeat stress followed by 28 days of vehicle (veh) (n = 8) or fluoxetine (n = 6) treatment (pellets implanted s.c.). Control mice were only subjected to the 28 days of vehicle (n = 7) or fluoxetine pellets (n = 6), but no stress. *mPer1* and *mPer2* mRNA expression was significantly decreased in the NAc following chronic stress (*p<0.05) (A and B). Fluoxetine treatment following social defeat stress resulted in expression levels that were not different from control animals.
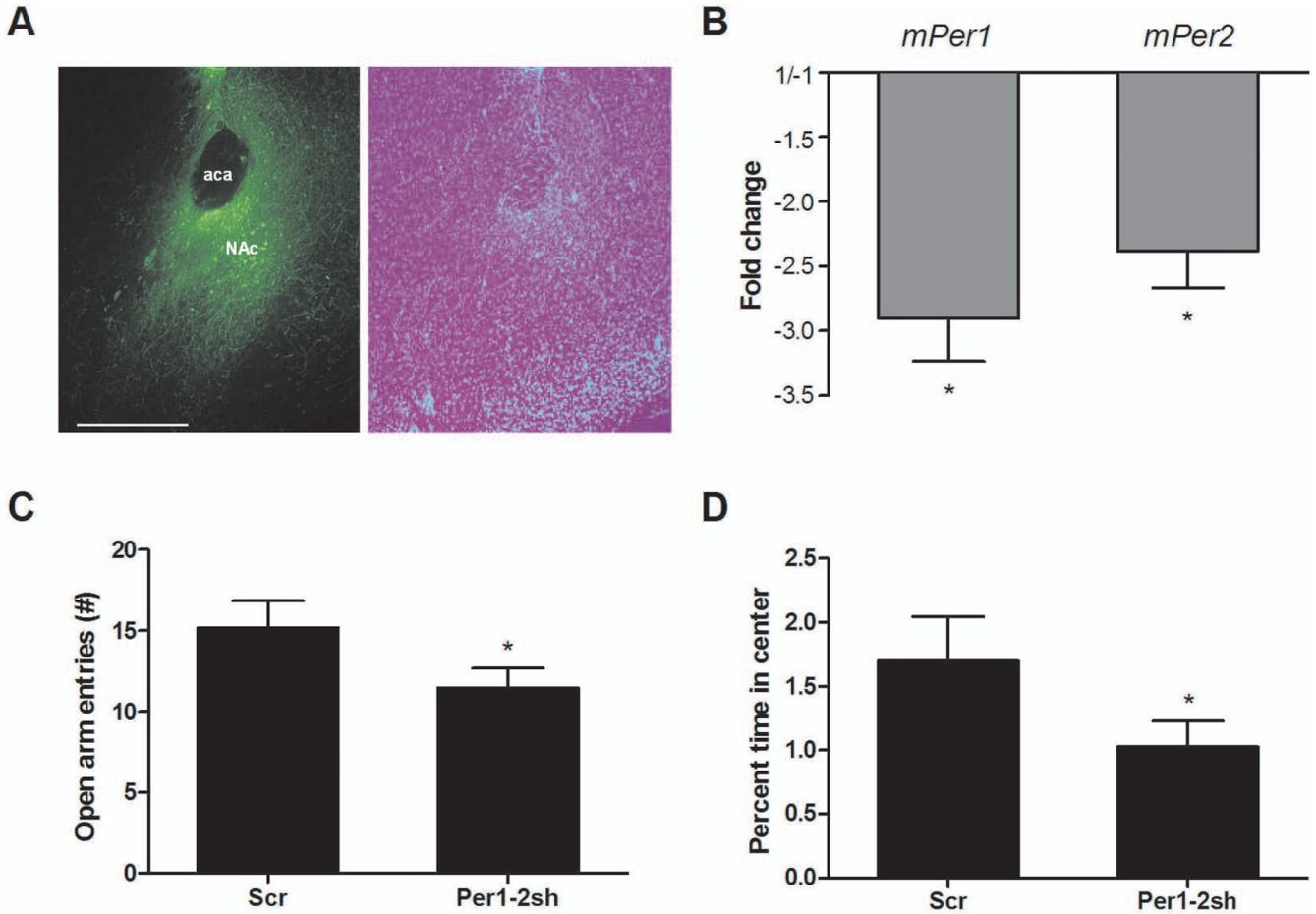
Fig. 4.
Knockdown of *mPer1* and *mPer2* in the NAc increases anxiety. Mice were infused with the AAV-mPer1;mPer2 shRNA (sh) or AAV-scrambled control shRNA (Scr) into the NAc and behavior was measured 2 weeks later. (A) Representative image showing viral expression in the NAc and DAPI staining of the same slice. Scale bar, 500 μm. (B) AAV-mPer1;mPer2 infusion leads to a significant knockdown of *mPer1* and *mPer2* expression in the NAc (n = 6, Scr-infected mice; n = 5–6 mPer1;mPer2 shRNA-infected mice with two biological replicates of each). (C)AAV-mPer1;mPer2 shRNA NAc-infected mice (n = 10) had fewer open arm entries in the EPM than controls (n = 11) (P < 0.05, *t*-test). (D) AAV-mPer1;mPer2 shRNA NAc-infected mice spent less time in the center of the open field (*P<0.05, *t*-test).