European Neuropsychopharmacology (2010) **20**, 509–517

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Development of morphine-induced tolerance and withdrawal: Involvement of the clock gene *mPer2*

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Received 11 September 2009; received in revised form 22 January 2010; accepted 11 March 2010

KEYWORDS
Clock genes;
Per2 gene;
Morphine;
Tolerance;
Withdrawal;
Mice

Abstract

The present study has been designed to assess specifically the involvement of the clock gene *mPer2* in morphine-induced tolerance and withdrawal. At first, we checked the absence of initial differences in the expression of several gene transcripts involved in the development of morphine dependence in $Per2^{Brdm1}$ mutant mice and in their respective wild-type (WT) control littermates. Morphine-induced tolerance as well as precipitated withdrawal was then assessed in these mice. The $Per2^{Brdm1}$ mutant mice clearly developed less tolerance and showed attenuated withdrawal signs compared to WT. These results show that mPER2 is involved in morphine-induced tolerance and withdrawal.
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1. Introduction

Numerous physiological and behavioural functions show an endogenous daily pattern of expression. And these circadian rhythmic patterns are controlled by the biological clock which molecular clock-work comprises the so-called clock genes (Ko and Takahashi, 2006). Several recent studies using mutated *Drosophila* flies or transgenic mouse models, or human genetic association studies, have revealed that the activity of several clock genes also influences the efficiency of psychoactive drugs such as cocaine and alcohol (Abarca et al., 2002; Falcon and McClung, 2009; Perreau-Lenz and Spanagel, 2008; Perreau-Lenz et al., 2009; Spanagel et al., 2005). Likewise, opiate effects show a clear relationship with the circadian clock as well. Thus, in the early 1970, it was already reported that opiate-induced mortality depends on the circadian cycle (Argyle, 1973; Lenox and Frazier, 1972; Reinberg et al., 1975; Sinnett and Morris, 1977). Clinical reports further revealed a circadian rhythmicity of admissions following events of opiate intoxication and overdose. In fact, overdose presentations to an emergency department underlie a significant daily rhythm with a typical early evening peak (acrophase) at about 7:00 PM (Raymond et al., 1992). On the other hand, rest and sleep

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doi:10.1016/j.euroneuro.2010.03.006

disturbances are habitual complains among opiate dependent patients (Oyefeso et al., 1997). And, one method to identify spontaneous morphine withdrawal in rodents is the disruption of their circadian locomotor activity cycle (Caille et al., 2002; Stinus et al., 1998). Furthermore, Yoshida et al. (2003) have also demonstrated the existence of a 24 h cycle in the analgesic effects of morphine and a circadian influence on tolerance. What is more, Cutler et al. (1999) have shown that morphine withdrawal is associated to an enhanced activity in a large population of neurons of the suprachiasmatic nucleus of the hypothalamus, which one contains the master-clock (Aton and Herzog, 2005).

In addition, the different clock genes seem to be also involved in the neurobiological changes observed during chronic morphine treatment. Indeed, the expression of the clock gene *mPer1* has been shown to be altered in morphine-dependent and morphine-withdrawn mice (Wang et al., 2006). Furthermore, it has been recently shown that the expression of several clock genes is differently affected by spontaneous morphine-induced withdrawal in the mesolimbic brain regions (Li et al., 2009). In particular, the rhythmic expression of the clock gene *rPer2* seems to be either shifted (the expression peaks at a different time) in the VTA and NAc shell, or blunted in the NAc core, following spontaneous withdrawal from chronic morphine treatment. In addition, Ammon et al. (2003) and Ammon-Treiber and Hollt (2005) have revealed that the clock gene *rPer2* was among the 3 genes up-regulated in the rat frontal cortex following naloxone-precipitated withdrawal.

Altogether, these studies indicate a close involvement of the biological clock and the expression of clock genes in the neurobiological mechanisms underlying the development and expression of morphine tolerance and dependence. The present study has been therefore designed in order to further our knowledge in this respect, and to assess the involvement of the *mPer2* gene, specifically, in morphine-induced tolerance and withdrawal responses. At first, we thus examined, in different mesocorticolimbic areas, the expression of several gene transcripts involved in drug dependence in $Per2^{Brdm1}$ mutant mice and in their respective wild-type (WT) littermates. We then assessed and compared morphine-induced tolerance as well as precipitated withdrawal in these mice.

2. Experimental procedures

2.1. Animals

In the present study, we used 12 to 16-week-old male $Per2^{Brdm1}$ mutant mice, described in Zheng et al. (1999), and their respective control littermates, stemming from a heterozygous breeding. All mice were housed individually, kept under 12 h light/12 h dark conditions (lights were on from 07h00 to 19h00) and fed *ad libitum*. Behavioural experiments started one or two weeks after the arrival of the animals in the experimental facilities. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe), and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directives.

2.2. Real-time RT-PCR screening

2.2.1. Mouse brain tissue dissection

Morphine-naïve mutant and control mice were sacrificed by decapitation at ZT5 (ZT stands for Zeitgeber Time, where ZT0 and ZT12 corresponds to the beginning of the light and the beginning of the dark cycle phase, respectively). Brains were quickly removed and submerged for 5 min in (dry)ice-cooled iso-pentane (Sigma-Aldrich Co., St. Louis, MO, USA). Afterwards, brains were wrapped with parafilm and aluminum foil and stored at −80 °C. For dissection, mouse brains were placed at −20 °C overnight and mounted in a Leica CM3000 Cryostat (Leica, Bensheim, Germany). Brains were sliced in coronal sections of 120 μm. Different regions were extracted by punching with a set of self-constructed needles of several diameters ranging from 0.75 to 1.5 mm (FMI, Seeheim, Germany) and collected into vials. The identification of regions was based on landmarks from the stereotaxical descriptions of The Mouse Brain in Stereotaxic Coordinates (Paxinos & Franklin, Academic Press, Inc.). To assess the punching precision, Nissl-Stained punched slices were checked by microscopy. The following brain sites were collected and stored at −80 °C: prefrontal cortex (PFC), nucleus accumbens (NAC), caudate-putamen (CPU), amygdala and the hippocampus. These brain sites were chosen as it was previously shown that the basal expression of several transcripts in these areas can influence the development of morphine dependence (Ammon-Treiber and Hollt, 2005; Falcon and McClung, 2009).

2.2.2. Total RNA isolation and quality control

Punched tissue was processed for each animal separately. The RNA isolation and quality control have been performed as described previously (Vengeliene et al., 2006). First the tissue was lysed in TRIzol®Reagent (Invitrogen, Karlsruhe, Germany) and homogenized by passing the suspension 30 times through a 22 gauge needle. Total RNA was extracted by adding chloroform. To achieve better separation of organic and aqueous phases, Phase Lock Gel™ Heavy tubes (Eppendorf, Hamburg, Germany) were used. Upper phases were carefully removed by pipetting and total RNA was purified using RNeasy®Micro Kit (Qiagen, Hilden, Germany). Total RNA was quantified with Quant-iT™ RiboGreen® RNA Reagent and Kit (Invitrogen) by measuring in a Wallac Victor^2 1420 Multilabel Counter (Perkin Elmer, Jügesheim, Germany). Total RNA quality was evaluated by OD measurements (260 nm/280 nm) in a NanoDrop (peqLab, Erlangen, Germany) and its integrity was determined by measuring ribosomal 28S/18S ratios using RNA 6000 Nano Assay RNA chips run in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Ratios of 1.9–2.2 (OD 260/280) and >1.6 (28S/18S rRNA) as well as an absence of a peak of DNA contamination in electropherograms were chosen as inclusion criteria.

2.2.3. qRT-PCR

Relative quantification by real-time RT-PCR (qRT-PCR) was carried out for gene expression profiling of a panel of mouse transcripts known to be involved in the development of morphine dependence (Ammon-Treiber and Hollt, 2005; Falcon and McClung, 2009; Robledo et al., 2008). The panel of mouse transcripts consists of "dopamine receptor D1a" (Drd1a), "dopamine receptor 2" (Drd2), "dopamine receptor 3" (Drd3), "solute carrier family 6 member 3" (Slc6a3), "tyrosine hydroxylase" (Th), "opioid receptor, delta 1" (Oprd1), "opioid receptor, kappa 1" (Oprk1), "opioid receptor, mu 1" (Oprm1), "cannabinoid receptor 1" (Cnr1), "prodynorphin" (Pdyn), "preproenkephalin 1" (Penk1), "pro-opiomelanocortin-alpha" (Pomc), "neuropeptide Y" (Npy), "corticotropin releasing hormone receptor 1" (Crhr1), "period homolog 1 (Drosophila)" (Per1), "circadian locomoter output cycles kaput" (Clock), and "neuronal PAS domain protein 2" (Npas2). Primers for each target were designed by considering exon–exon junctions based on NCBI information. Amplicons were 90–110 bp length and melting temperatures ranged from >75–90 °C. qRT-PCR was carried out in a total reaction volume of 20 μl using *Power* SYBR®Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) and run in an ABI 7900 HT RT-PCR System (Applied Biosystems). Rat β-actin (Actb) was used as internal standard, since it showed highly stable expression

between samples. Cycling conditions were 95 °C for 10 s, 60 °C for 40 s.

2.3. Behavioural procedure

2.3.1. Measurement of morphine-induced analgesia and tolerance

The development of tolerance to the analgesic effects of morphine was studied by means of two different behavioural procedures: the tail-immersion and the hotplate tests. The protocols used for these tests were based on previously described methods (Simonin et al., 1998). All the pharmacological treatments and behavioural tests were completed between ZT3 and ZT5.

For the tail-immersion test, the water temperature was kept constant at 48 ± 0.5 °C by using a thermo regulated water bath. The mice were maintained in a plastic cylinder and their tails were immersed in the heated water. The latency to the occurrence of a rapid flick of the tail was considered as the endpoint of the test. Mice that still did not respond after a cut-off time of 60 s were removed from the water and put back in their home cage. Mice were tested on three different days. On the first day (D0), in order to establish a drug-free pain threshold, the mice were tested 30 min after receiving an intraperitoneal saline injection. On the following day (D1), they were tested 30 min after receiving a morphine injection (5 mg/kg, i.p.; Sigma-Aldrich Germany), providing then a measure of the acute analgesic effects of morphine. From the next morning on, mice received daily morphine injections (5 mg/kg, i.p.) in their home cages for 5 consecutive additional days. Finally, on the 7th day from the first morphine injection (D7), mice were re-tested in the tail-immersion test 30 min after a last morphine injection (5 mg/kg, i.p.).

For the hotplate test, mice were placed on the heated surface (53 ± 0.1 °C) of a hotplate (22 × 60 cm) (ATLab, Vendargues, France) and latencies to the first response to one of the following signs were assessed: licking forepaws, flipping hind paws and jumping. Mice that still did not respond after a cut-off time of 120 s were removed from the water and put back in their home cage. The experimental design and treatment conditions were similar to those used for the tail-immersion study. Similarly, a drug-free pain threshold was first assessed (D0) by testing the animals 30 min after an acute intraperitoneal injection of saline. On the following day (D1), the acute analgesic effect of morphine (20 mg/kg; i.p.; 30 min prior) was established. Across the 5 following days, mice received identical morphine injections in their home cages, and on D7, they were re-tested in the hotplate under the same conditions as on D1.

2.3.2. Naloxone-induced withdrawal responses in morphine-dependent mice

Opioid dependence was induced by repeated injections of morphine based on the procedure described by Maldonado et al. (1997). Thus, mice received morphine injections twice a day, at ZT3 (3 h after lights on) and ZT15 (3 h after lights off), with progressively increasing doses (20, 40, 60, 80 and 100 mg/kg, i.p.) for 5 consecutive days. On the 6th day, mice were injected with morphine (100 mg/kg, i.p.; ZT2) and, after one hour and a half, were individually placed in the observation boxes (22 × 22 × 40 cm; Tru Scan Photobeam activity monitors, Coulbourn Instruments, Allentwon, USA). Thirty minutes later, morphine withdrawal was precipitated by a subcutaneous naloxone injection (1 mg/kg; Sigma-Aldrich) and an observer blind to the experimental conditions evaluated the withdrawal signs during a period of 30 min. Two kinds of signs were evaluated: (i) behavioural signs, scored as the number of jumps, rearings, orofacial movements, forepaw tremors and shakes and (ii) physiological signs such as diarrhoea and loss of body weight (calculated as the percentage of body weight 30 min after vs. before the naloxone challenge) were estimated.

2.3.3. Naloxone-precipitated morphine withdrawal-induced conditioned-place aversion

Behavioural signs of withdrawal from acute and chronic opioid dependence seem to share common mechanisms (Azar et al., 2003). Therefore acute opioid dependence can be a useful tool for assessing the neurobiological mechanisms involved in the initial development of opioid dependence. Hence, we studied the conditioned-place aversion (CPA) induced by a naloxone-precipitated acute morphine withdrawal in the *Per2*^{Brdm1} mutant and WT mice. The place preference boxes we used (Panlab, Barcelona, Spain) consisted of 3 distinct compartments: an entry/corridor compartment and 2 different conditioning compartments (different visual and tactile cues). Based upon the experimental protocol from Azar et al. (2003), we have developed a simplified procedure to measure naloxone-precipitated CPA with only two training sessions. Complete testing lasted 5 days and consisted of a preconditioning phase, a conditioning and a test phase. For the CPA experiment a non-biased apparatus and a biased conditioning protocol was used. On the first day, mice were put in the test room for 30 min of habituation. On the second day, a pre-test to assess the compartment preference was conducted in the conditioning boxes with the 3 compartments available to the animal for 20 min. On the third day, first conditioning day, the animals were first injected with saline (s.c.) at ZT2 in their keeping room, and then injected with naloxone (0.3 mg/kg, i.p.) and put in the non-preferred compartment for 30 min at ZT6. On the fourth day, second conditioning day, the animals were first injected with morphine (30 mg/kg; s.c.) at ZT2 in their keeping room, and then injected with naloxone (0.3 mg/kg, i.p.) and put in the preferred compartment for 30 min at ZT6. On the last day, avoidance to the preferred compartment was then assessed putting the animals in the boxes with the 3 compartments available for 20 min. Avoidance to the preferred compartment, or percentage of CPA, was then calculated as the percentage of the reduction of time spent in the preferred compartment on the last day compared to the 1st day.

2.4. Data analysis and statistics

All data analyses were performed using the software Statistica 6.1 (StatSoft, Inc., Tulsa, OK, USA).

The PCR relative quantification was done according to the ΔΔCT method. Differences in transcript abundance in the different brain regions were analysed using a two-way MANOVA. One-way MANOVA analyses were used for each separate brain region. Differences were considered statistically significant when $P<0.003$ after Bonferroni corrections.

Since the data set on the latencies to the first response obtained in the tolerance tests failed to follow a normal distribution, we transformed these data sets using a square root transformation. These transformed data were then analysed with two-way ANOVAs for repeated measures followed, when appropriate, by Newman–Keuls post-hoc tests. The degree of tolerance was defined as the difference between the theoretical 100% of tolerance to the analgesic effect of morphine and the percentage of maximum effect observed on D7 over D1, and the following formula was therefore applied: Degree of tolerance (%) = 100% − (100 * (LatencyD7 − LatencyD0) / (LatencyD1 − LatencyD0)). Student's *t*-tests for independent samples were used to analyse the calculated degrees of tolerance.

For analysing the withdrawal responses, a one-way MANOVA analysis was performed, and group means for each behavioural or physiological withdrawal sign was then assessed using Student Newman–Keuls post-hoc tests. Student's *t*-tests for independent samples were used to compare global indexes of behavioural and physiological withdrawal symptomatology. These indexes were calculated assigning weight values to each sign accordingly with those previously published (Maldonado et al., 1997). For those signs which were not included in that published study, a weight value of 1 was used.

Table 1 Differences in ΔΔCT from the WT. Gene expression profiling of a panel of transcripts in several brain regions of *Per2*^{Brdm1} mutant and WT littermates under basal conditions. The panel of mouse transcripts consists of "dopamine receptor D1a" (Drd1a), "dopamine receptor 2" (Drd2), "dopamine receptor 3" (Drd3), "solute carrier family 6 member 3" (Slc6a3), "tyrosine hydroxylase" (Th), "opioid receptor, delta 1" (Oprd1), "opioid receptor, kappa 1" (Oprk1), "opioid receptor, mu 1" (Oprm1), "cannabinoid receptor 1" (Cnr1), "prodynorphin" (Pdyn), "preproenkephalin 1" (Penk1), "pro-opiomelanocortin-alpha" (Pomc), "neuropeptide Y" (Npy), "corticotropin releasing hormone receptor 1" (Crhr1), "period homolog 1 (Drosophila)" (Per1), "circadian locomotor output cycles kaput" (Clock), and "neuronal PAS domain protein 2" (Npas2). Values represent the average ± SEM of ΔΔCT between genotypes. Fold change (FC) is calculated as $FC = 2^{\Delta\Delta CT}$.

	PFC		NAc		CPu		Amy		Hipp	
	WT	Per2^{Brdm1}	WT	Per2^{Brdm1}	WT	Per2^{Brdm1}	WT	Per2^{Brdm1}	WT	Per2^{Brdm1}
Drd1a	0.00 ± 0.16	0.20 ± 0.56	0.00 ± 0.08	−0.16 ± 0.37	0.00 ± 0.12	−0.16 ± 0.05	0.00 ± 0.28	−0.11 ± 0.15	0.00 ± 0.15	0.25 ± 0.12
Drd2	0.00 ± 0.06	0.64 ± 1.02	0.00 ± 0.10	−0.53 ± 0.48	0.00 ± 0.11	−0.08 ± 0.06	0.00 ± 0.38	0.18 ± 0.29	0.00 ± 0.11	−0.04 ± 0.09
Drd3	0.00 ± 0.52	1.36 ± 1.59	0.00 ± 0.12	−0.69 ± 0.33	0.00 ± 0.14	−0.27 ± 0.15	0.00 ± 0.43	−0.21 ± 0.15	0.00 ± 0.22	−0.27 ± 0.20
Slc6a3	0.00 ± 0.52	−0.93 ± 0.45	0.00 ± 0.10	0.44 ± 0.14	0.00 ± 0.09	−0.26 ± 0.22	0.00 ± 0.21	0.52 ± 0.11	0.00 ± 0.72	−0.85 ± 0.14
Th	0.00 ± 0.52	−0.82 ± 0.37	0.00 ± 0.15	0.48 ± 0.19	0.00 ± 0.14	0.17 ± 0.04	0.00 ± 0.25	0.77 ± 0.21	0.00 ± 0.44	0.18 ± 0.14
Oprd1	0.00 ± 0.05	−0.31 ± 0.05	0.00 ± 0.11	−0.06 ± 0.24	0.00 ± 0.06	−0.09 ± 0.06	0.00 ± 0.04	−0.27 ± 0.13	0.00 ± 0.07	−0.23 ± 0.14
Oprk1	0.00 ± 0.14	−0.08 ± 0.26	0.00 ± 0.05	−0.09 ± 0.16	0.00 ± 0.09	0.03 ± 0.11	0.00 ± 0.06	−0.37 ± 0.04	0.00 ± 0.15	−0.38 ± 0.32
Oprm1	0.00 ± 0.09	−0.05 ± 0.56	0.00 ± 0.12	0.02 ± 0.07	0.00 ± 0.11	−0.25 ± 0.23	0.00 ± 0.18	−0.03 ± 0.09	0.00 ± 0.04	−0.08 ± 0.13
Cnr1	0.00 ± 0.11	−0.1720 ± 0.05	0.00 ± 0.11	−0.01 ± 0.17	0.00 ± 0.14	−0.17 ± 0.06	0.00 ± 0.12	0.009 ± 0.11	0.00 ± 0.11	−0.41 ± 0.18
Pdyn	0.00 ± 0.10	0.06 ± 0.15	0.00 ± 0.09	−0.47 ± 0.53	0.00 ± 0.11	−0.004 ± 0.13	0.00 ± 0.42	0.32 ± 0.19	0.00 ± 0.15	−0.10 ± 0.10
Penk1	0.00 ± 0.16	0.07 ± 0.35	0.00 ± 0.07	−0.31 ± 0.29	0.00 ± 0.67	−0.33 ± 0.30	0.00 ± 0.19	0.25 ± 0.10	0.00 ± 0.21	−0.21 ± 0.15
Pomc	0.00 ± 0.21	0.24 ± 0.10	0.00 ± 0.21	0.34 ± 0.22	0.00 ± 0.16	0.12 ± 0.08	0.00 ± 0.11	0.63 ± 0.30	0.00 ± 0.11	0.16 ± 0.13
Npy	0.08 ± 0.16	−0.06 ± 0.10	0.00 ± 0.09	−0.22 ± 0.37	0.00 ± 0.11	0.02 ± 0.07	0.00 ± 0.10	0.08 ± 0.09	0.00 ± 0.16	−0.02 ± 0.05
Crhr1	0.00 ± 0.16	0.21 ± 0.13	0.00 ± 0.06	0.49 ± 0.23	0.00 ± 0.11	0.09 ± 0.12	0.00 ± 0.19	0.53 ± 0.21	0.00 ± 0.09	−0.13 ± 0.14
Per1	0.00 ± 0.05	0.10 ± 0.07	0.00 ± 0.09	−0.07 ± 0.19	0.00 ± 0.04	0.35 ± 0.04	0.00 ± 0.54	0.08 ± 0.08	0.00 ± 0.06	0.23 ± 0.13
Clock	0.00 ± 0.03	−0.24 ± 0.04	0.00 ± 0.07	−0.18 ± 0.14	0.00 ± 0.06	−0.22 ± 0.03	0.00 ± 0.10	−0.13 ± 0.03	0.00 ± 0.03	−0.08 ± 0.05
Npas2	0.00 ± 0.04	−0.15 ± 0.03	0.00 ± 0.55	−0.51 ± 0.49	0.00 ± 0.04	0.12 ± 0.008	0.00 ± 0.07	−0.16 ± 0.07	0.00 ± 0.05	−0.16 ± 0.20

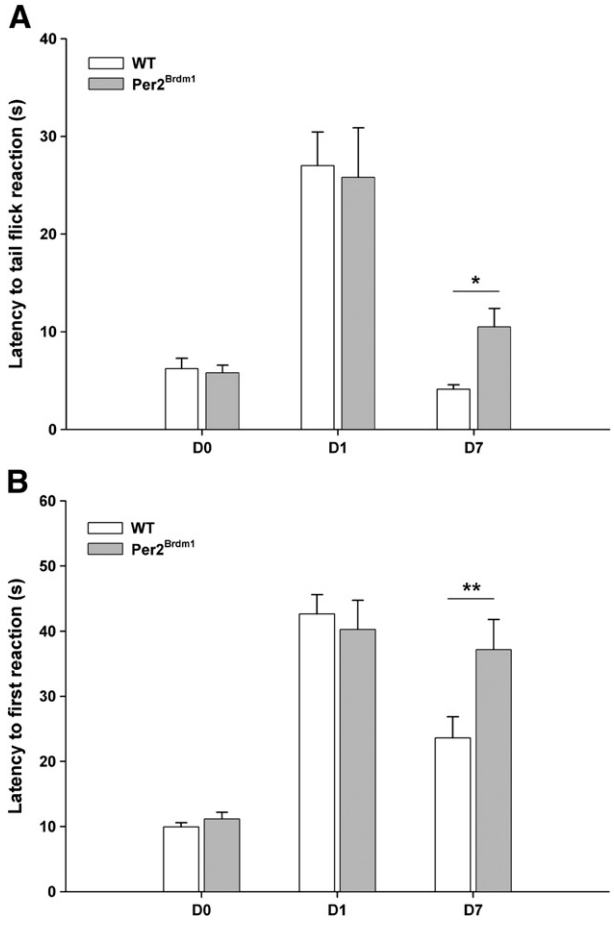

Figure 1 Tolerance to the analgesic effects of morphine in the tail-immersion (A) and hotplate (B) tests in *Per2*^{Brdm1} mutant and WT mice. (A) Latencies to tail-flick reaction after saline, acute and chronic morphine injections in the respective genotypes (n=8–10 mice per genotype). (B) Latencies to first response on the hotplate plate after saline, acute and chronic morphine injections in the respective genotypes (n=23–25 per genotype). *p<0.05 and ** p<0.01 post-hoc tests from the two-way ANOVA for repeated measures analysis realised on the transformed data. Mean values ± SEM of latencies (s) to a rapid flick of the tail (A) or to the first response (B).

For the conditioned-place aversion test, the time spent in the preferred compartment before and following the acute withdrawal experience was analysed for the two genotypes with a two-way ANOVA for repeated measures. The percentages of CPA of the respective genotypes were then analysed with a Student t-test for independent samples.

3. Results

3.1. qPCR analyses in the *Per2*^{Brdm1} mice

When one compares the difference in ΔΔCT to the WT littermates of the different gene transcripts chosen for all the brain regions (Table 1), a global difference in genotype (Wilks lambda = 0.16, $F_{Genotype}(17, 19) = 5.8$, $p<0.001$), as well as a global region effect (Wilks lambda = 0.00001, $F_{Region}(68, 77) = 39.31$, $p<0.0001$) could be seen. However, the differences on the transcripts of those genes among the different brain regions do not seem to be influenced by the genotype (Wilks lambda = 0.085, $F_{Genotype*Region}(68, 77) = 0.99$, $p = 0.52$).

Furthermore, separate MANOVA analyses done for each specific brain region did not reach statistical significance (PFC: Wilks lambda = 0.03, $F_{Genotype}(7, 1) = 4.84$, $p = 0.34$; NAc: Wilks lambda = 0.28, $F_{Genotype}(6, 1) = 0.43$, $p = 0.82$; CPU: Wilks lambda = 0.03, $F_{Genotype}(7, 1) = 4.14$, $p = 0.36$; Amy: Wilks lambda = 0.002, $F_{Genotype}(7, 1) = 72.29$, $p = 0.09$; Hipp: Wilks lambda = 0.08, $F_{Genotype}(8, 1) = 1.40$, $p = 0.58$), indicating that, in each region, the expression of these genes did not significantly differ between the two genotypes.

3.2. Acute analgesic effects of morphine and development of tolerance

Since the data in latencies presented in Fig. 1 for both the tail-immersion (Fig. 1A) and the hotplate (Fig. 1B) test did not follow a normal distribution (Shapiro–Wilk test: $W = 0.79$ and $p<0.0001$, and $W = 0.87$ and $p<0.0001$, respectively), we transformed the data using a square root transformation. The two-way ANOVA for repeated measures performed on these normalised sets of data revealed clear differential effects of the chronic morphine treatment depending on the genotype ($F_{Days*Treatment}(2, 34) = 3.39$, $p<0.05$ and $F_{Days*Treatment}(2, 92) = 4.66$, $p<0.01$ for the tail-immersion and hotplate tests, respectively). When compared to their respective littermates, the *Per2*^{Brdm1} mutant mice did not show any significant difference neither in pain thresholds (Post-hoc test: $p = 0.89$ and $p = 0.77$, for the tail-immersion and hotplate tests, respectively) nor in acute sensitivity to morphine (Post-hoc test: $p = 0.63$ and $p = 0.44$, for the tail-immersion and hotplate tests, respectively). However, after receiving repeated morphine injections, the analgesic effect of the same dose of morphine (5 mg/kg in the tail-immersion test or 20 mg/kg in the hotplate test) was much more preserved in *Per2*^{Brdm1} mutant than in WT mice. Indeed for both tests, the *Per2*^{Brdm1} mutant showed higher latencies than the WT mice on Day 7 (Post-hoc test: $p<0.05$ and $p<0.01$, for the tail-immersion and hotplate test, respectively).

What is more, the degree of tolerance calculated for both the tail-immersion and the hotplate tolerance tests in *Per2*^{Brdm1} mutant (53.91 ± 32.43% and −17.85 ± 17.31%, respectively) were significantly lower ($T(17) = 2.21$, $p<0.05$ and $T(46) = 2.80$, $p<0.01$, respectively) than the one calculated in WT mice (157.32 ± 37.50% and 37.94 ± 8.78%, respectively). Therefore, the results of this experiment clearly revealed that although *Per2*^{Brdm1} mutant had no different pain threshold than WT mice and did not differ in their sensitivity to the acute analgesic effects of morphine, they clearly developed a lower degree of tolerance to morphine.

3.3. Naloxone-induced withdrawal responses in morphine-dependent mice

Following the first acute morphine injection, *Per2*^{Brdm1} mutants did not differ in locomotion (9460 ± 1310.7 cm/30 min) nor in rearing (27.5 ± 7.0 events/30 min) from their WT littermates ($T(24) = 1.08$, $p = 0.29$ and $T(24) = 1.28$, $p = 0.21$, respectively). These results further confirm that the mutation

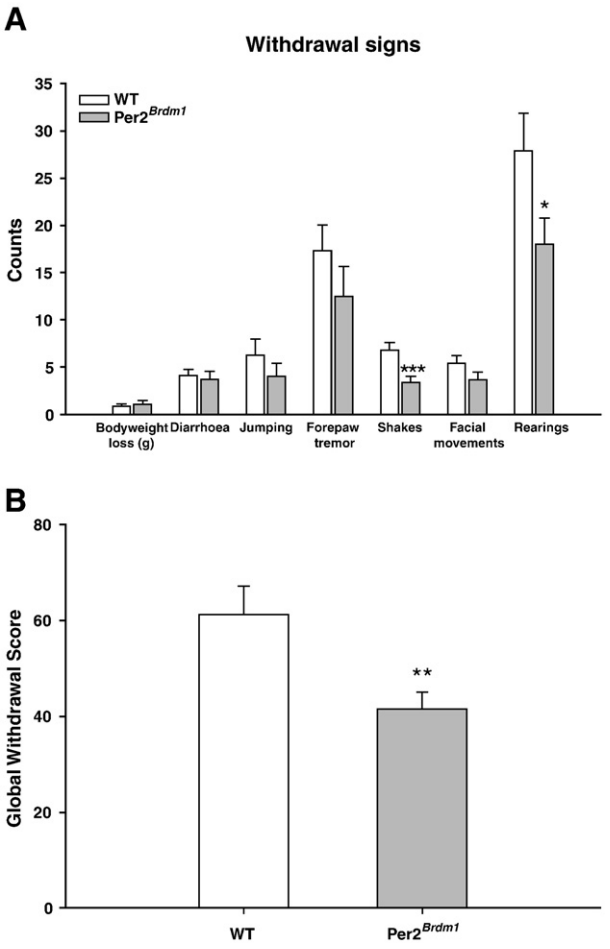

Figure 2 Morphine withdrawal in *Per2*^{Brdm1} mutant and WT littermates. (A) Naloxone-precipitated morphine withdrawal signs. After chronic treatment with morphine, withdrawal signs were assessed 30 min after naloxone precipitation. *$p<0.05$, and ***$p<0.001$ indicate the withdrawal signs that are revealed to be significantly different from the WT littermates following the post-hoc analyses. (B) Global indexes of morphine withdrawal. Global indexes of the intensity of all withdrawal signs were calculated for each subject as described in the Experimental procedures section. These indexes were then averaged by genotype. Data are expressed as mean value ± SEM ($n=21–24$ per genotype) and compared by means of Student's *t*-test for independent samples (**$p<0.01$).

Table 2 Conditioned-place aversion in *Per2*^{Brdm1} mutant and WT littermates. Comparison of the time spent in the preferred compartment before and after the acute morphine- and naloxone-induced conditioning treatment. Data are expressed as mean value ± SEM ($n=10–12$ mice per genotype).

Time spent in the preferred compartment (s)	WT	*Per2*^{Brdm1}
Pre-treatment	604,45 ± 32.47	547.46 ± 32.26
Post-treatment	418.25 ± 58.03	461.52 ± 90.91

of this gene does not seem to modify the acute sensitivity to morphine.

After the precipitation of morphine withdrawal by an acute injection of naloxone, locomotion in *Per2*^{Brdm1} mutant mice (1928.78 ± 179.13 cm/30 min) was not significantly different ($T(24)=1.02$, $p=0.32$) from their WT littermates (2185.34 ± 177 cm/30 min). Conversely, the morphine-induced withdrawal assessed in *Per2*^{Brdm1} mutant and WT mice showed clear overall differences (MANOVA: $F_{Genotype}(7, 37)=3.64$, $p<0.01$). The number of shakes and rearing events were thus significantly lower in *Per2*^{Brdm1} mutant mice (Post-hoc test: $p<0.001$ and $p<0.05$, respectively) (Fig. 2A). In addition, although no significant difference could be observed in jumping (Post-hoc test: $p=0.25$), forepaw tremor (Post-hoc test: $p=0.17$) and orofacial movements (Post-hoc test: $p=0.09$), these behavioural signs tended to be lowered in *Per2*^{Brdm1} mutant mice (Fig. 2A). Interestingly, the physiological withdrawal signs such as the body weight loss and diarrhoea did not differ between genotypes (Post-hoc tests: $p=0.54$ and $p=0.65$, respectively).

Nevertheless, the global indexes for behavioural and physiological withdrawal signs, calculated as described in the Experimental procedures section, confirmed that WT and *Per2*^{Brdm1} mutant mice differed in their expression of morphine withdrawal responses (Fig. 2B). The general withdrawal score was then clearly significantly lower in *Per2*^{Brdm1} mutant mice ($T(43)=2.94$, $p<0.01$).

3.4. CPA

The results of the CPA experiment are presented in Table 2. The treatment clearly induced avoidance to the preferred compartment ($F_{Treatment}(1, 20)=8.14$, $p<0.01$). However, the two-way ANOVA for repeated measures failed to reveal a differential reaction towards the treatment between the genotypes ($F_{Genotype*Treatment}(1, 20)=1.10$, $p=0.31$). As well, when one compares the percentage of reduction of the time spent in the preferred compartment, WT (30.04 ± 9.29%) and *Per2*^{Brdm1} mutant (19.05 ± 13.06%) mice did not significantly differ ($T(20)=0.70$, $p=0.49$).

4. Discussion

The present study shows the involvement of the gene *mPer2* in the development of several morphine-induced behaviours reflecting the development of dependence towards that drug following chronic exposure. At first, we show that a functional mutation of the gene *mPer2* is affecting the development of tolerance to the analgesic effect of morphine, without affecting the acute effects of morphine. In addition, we demonstrate the implication of the gene *mPer2* in morphine-induced withdrawal.

The qPCR analyses of the present study, realised as a previous screening under basal conditions, did not reveal any significant difference in the Per2^{Brdm1} mutants compared to their WT littermates, revealing that there was no clear confounding factors under baseline conditions that could account for our behavioural results. This absence of significant difference is in line with our acute behaviour results. Indeed, the reduced degree of morphine tolerance and withdrawal in *Per2*^{Brdm1} mutant mice was observed in

the absence of any alteration in the acute effects of morphine as measured by its analgesic or locomotor effects. Interestingly enough, the finding that a mutation of *Per* genes affects only the chronic but not the acute drug effects was previously observed in behavioural studies on cocaine effects (Abarca et al., 2002; Andretic et al., 1999).

In the present precipitated withdrawal experiment, several morphine withdrawal signs were either significantly reduced (e.g. rearing and shakes) or showed a tendency to be less expressed (e.g. jumping, fore paw tremor and orofacial movements) in *Per2*^{Brdm1} mutant mice when compared to their respective wild-type littermates. Interestingly, the physiological withdrawal signs such as the body weight loss and diarrhoea did not differ between the genotypes. This indicates a selective implication of the *mPer2* in centrally driven as opposed to peripherally driven withdrawal signs. However, the involvement of the *mPer2* gene in the expression of withdrawal signs was clearly revealed when global indexes of morphine withdrawal were calculated according to the relative weight values used by other authors (Maldonado et al., 1997). These results are in agreement with the fact that *rPer2* expression is enhanced in the rat brain following chronic morphine treatment and induction of naloxone-precipitated withdrawal (Ammon et al., 2003; Ammon-Treiber and Hollt, 2005).

Regarding the molecular mechanisms by which the *mPer2* gene mutation could lead to our phenotypic observations i.e. reduced tolerance and withdrawal symptomatology, several candidates involved in the development of morphine dependence (Ammon et al., 2003; Ammon-Treiber and Hollt, 2005; McClung, 2006) could be envisaged and further investigated. For instance, enhanced activity of the glutamate system has been attributed to morphine withdrawal, with *in vivo* microdialysis studies showing that enhanced extra-synaptic glutamate levels parallel the expression of some withdrawal signs (Aghajanian et al., 1994; Sepulveda et al., 1998). However, we previously reported that the *Per2*^{Brdm1} mutant mice display a significant down-regulation of the glutamate transporter gene EAAT1, and subsequent augmented extra-synaptic glutamate levels in the NAC (Spanagel et al., 2005). Taken these findings into account one would have expected enhanced withdrawal responses in *Per2*^{Brdm1} mutant mice but the mutant mice showed less withdrawal symptoms. One speculative explanation would be that morphine-naïve *Per2*^{Brdm1} mutant mice do already have enhanced brain glutamate levels that are not further increased during withdrawal (ceiling effect). Thus, the net increase between withdrawal-induced glutamate levels and basal glutamate levels might be higher in WT animals as compared to the one in *Per2*^{Brdm1} mutant mice, resulting in enhanced withdrawal symptomatology in the WT mice.

Nevertheless, the dopaminergic system could also be another candidate for conveying these phenotype differences. Indeed, the dopaminergic system has been shown to be clearly affected during morphine withdrawal. Several microdialysis studies have shown that mesolimbic DA release is inhibited during withdrawal (Georges et al., 1999; Pothos et al., 1991; Spanagel et al., 1994). We recently showed that *Per2*^{Brdm1} mutant mice display enhanced dopamine levels within the NAc under basal conditions due to a reduction of the expression and the activity of the monoamine oxidase A (Hampp et al., 2008). Such hyperdopaminergic state might explain the phenotype we observed in these mice during withdrawal. Hence, we could speculate that in the *Per2*^{Brdm1} mutant mice under withdrawal DA levels are reduced to a lesser extent than in the control mice due to the impairment of this molecular mechanism.

In the same study (Hampp et al., 2008), we also suggested that *mPer2* modulates the expression and activity of the monoamine oxidase A directly or via the modulation of other clock-controlled genes. Indeed, clock genes influence the expression of other genes so-called clock-controlled genes containing canonical or non-canonical E-box sites (Dardente and Cermakian, 2007; Ko and Takahashi, 2006). So far, we ignore which clock-controlled genes are really involved in the morphine-induced behaviours. Nonetheless, Lynch et al. (2008) have recently shown the implication of several clock-controlled genes after repeated cocaine administration (i.e. dynorphin) in the mouse dorsal striatum. Furthermore, Manev and Uz (2006) have also previously proposed that several molecular targets involved in drug addiction, such as the D1 receptors or the delta-opioid receptors, could be such putative clock-controlled gene. Interestingly, the delta-opioid mutants have been reported to show a similar phenotype than the *Per2*^{Brdm1} mutant mice, displaying at the same time a higher alcohol self-administration and a lower morphine-induced tolerance (Gaveriaux-Ruff and Kieffer, 2002; Kieffer and Gaveriaux-Ruff, 2002).

In summary, these findings confirm the idea that clock genes, and the *mPer2* transcript especially, modulate the neuroplastic changes that underlie the diverse neurobehavioural changes associated to chronic drug administration. In this regard, the identification of clock-controlled genes and the brain areas that are modulated by the PER2 protein following specific drug treatment would provide better insights into the basic mechanisms leading to drug dependence.

Role of the funding source

Funding of this study was supported by DFG (SFB 636/B1 and SP383/2-1). The DFG had no further role in the study design; in the collection, analysis and interpretation of the data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

The authors Stéphanie Perreau-Lenz, Carles Sanchis-Segura, Miriam Schneider, and Rainer Spanagel designed the study and wrote the protocol. Stéphanie Perreau-Lenz and Carles Sanchis-Segura conducted the behavioural experiments and Fernando Leonardi-Essmann the molecular analyses. Stéphanie Perreau-Lenz and Carles Sanchis-Segura undertook the statistical analyses. Stéphanie Perreau-Lenz wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

All other authors declare that they have no conflicts of interest.

Acknowledgements

We would like to thank Elisabeth Roebel, Claudia Schaeffer, Martina Saar and Anna Schuler for their technical help. We sincerely acknowledge Prof. Urs Albrecht (University of Fribourg, Switzerland), who so generously provided us with the original breeding pairs of our *Per2*^{Brdm1} colony.

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