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THE ROLE OF *mPer1* IN MORPHINE DEPENDENCE IN MICE

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Abstract

Investigations using *Drosophila melanogaster* have shown that the circadian clock gene *period* can influence behavioral responses to cocaine, and the mouse homologues, *mPer1* and *mPer2*, modulate cocaine sensitization and reward. In the present study, we applied DNAzyme targeting *mPer1* to interfere the expression of *mPer1* in CNS in mice and studied the role of *mPer1* on morphine dependence. We found that the DNAzyme could attenuate the expression of *mPer1* in CNS in mice. Mice treated with DNAzyme and morphine synchronously did not show preference to the morphine-trained side, whereas the control group did. In contrast, mice treated with DNAzyme after morphine showed preference to the morphine-trained side as well as the control group did. These results indicate that drug dependence seems to be influenced at least partially by *mPer1*, but *mPer1* cannot affect morphine dependence that has been formed.

Keywords

drug dependence; DNAzyme; learning and memory; circadian; i.c.v.

Circadian clocks are molecular time-keeping mechanisms that reside in a diverse range of cell types in a variety of organisms. The primary role of these cell-autonomous clocks is to maintain their own approximately 24 h molecular rhythm and to drive the rhythmic expression of genes involved in physiology, metabolism and behavior. Components of the endogenous master clock were first identified in the fruit fly *Drosophila melanogaster*. The *Period* (*Per*) encodes one of the essential elements involved in the transcription/translation-based auto-regular loop of the endogenous master clock (Reppert and Weaver, 2001). Three homologues of *Drosophila Per* genes were subsequently identified in mice (*mPer1*, *mPer2*, and *mPer3*) (Albrecht, 2002), leading to great progress in elucidation of the molecular mechanism underlying circadian rhythm in the CNS.

It has been shown that repeated administration of methamphetamine caused behavioral sensitization as well as sensitized expression of *mPer1* (Nikaido et al., 2001). Some studies implicate a role for *Per* genes in drug-induced behavioral sensitization processes. This suggestion is supported by investigations using *Drosophila* flies. Flies mutant in the *Per* gene did not sensitize after repeated exposure to volatilized free-base cocaine (Andretic et al., 1999; Hirsh, 2001). In mice, the *mPer1* and *mPer2* genes influence cocaine-induced sensitization and reward in an opposite manner. The lack or dysfunction of the *mPer1* gene

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abolishes cocaine sensitization and reward whereas the dysfunction of the *mPer2* gene induces a hypersensitized response to cocaine (Abarca et al., 2002).

DNAzyme is a suitable tool for studying gene function. The typical DNAzyme, known as the "10–23" model, is capable of cleaving single-stranded RNA at specific sites. The "10–23" model of DNAzymes has a catalytic domain of 15 highly conserved deoxyribonucleotides, flanked by two substrate-recognition domains, which can cleave effectively between any unpaired purine and pyrimidine of mRNA transcripts (Santoro and Joyce, 1997).

To further understand the role and the mechanism of *mPer1* gene in morphine dependence, we first studied morphine-induced reward to be involved in morphine dependence by blocking the expression of *mPer1* gene with the "10–23" DNAzyme.

EXPERIMENTAL PROCEDURES

Animals

In all experiments, 4- to 6-week-old male BALB/C mice were used. Mice were housed in groups of five and provided with food and water *ad libitum*. Artificial light was provided daily from 8:00 a.m. to 8:00 p.m. with room temperature and humidity kept constant (temperature: 22–24 °C; humidity: 55–65%). All procedures were performed in compliance with the local, international and institutional guidelines. All efforts were made to minimize the number of animals used and their suffering.

Conditioned place preference (CPP)

Place conditioning was conducted as described previously according to Suzuki et al. (1993). The apparatus consisted of a shuttle box (30×15×15 cm: length×width×height) made of an acryl-resin board. The box was divided into two compartments of equal size by means of a sliding partition. One compartment was white with a textured floor, and the other was black with a smooth one. When the CPP was measured, the partition separating the two compartments was raised to 7 cm above the floor. Preference for a particular place was assessed. The time spent in black compartment during a 900-s session was measured automatically. To avoid the introduction of systematic errors, the CPP experiment was carried out in a light and sound-controlled environment.

I.c.v. injection

The i.c.v. injection procedure was adapted from the method described earlier (Mistry et al., 1997). Briefly, the i.c.v. injections were given as follows: under light ether anesthesia, bregma was exposed. An injection volume of 20 μl was delivered over a 60-s period, 2 mm lateral and caudal to bregma at a depth of 2 mm by using a syringe. Proper placement was verified in the experiments by injection and localization of Methylene Blue dye.

DNAzyme

We designed a "10–23" DNAzyme, as described previously by Santoro and Joyce (1997), targeting *mPer1* gene in mice (Fig. 1). The 15-nt catalytic domain is flanked by two eight-nt arms that recognized the *mPer1* mRNA substrate from 287 to 303 nt except 296 nt (GenBank accession number: U49930). The 5′ and 3′ termini of the molecule are protected from exonucleases by a phosphorothioate linkage and a CPG-C3 cap respectively. To inactivate the DNAzyme and to generate a control oligonucleotide (ODN), two nts were changed in the catalytic domain of the DNAzyme (Fig. 1C). Transversion of two nts in the disabled ODN is sufficient to inactivate the catalytic activity (Santoro and Joyce, 1997; Wu et al., 1999; Sriram and Banerjea, 2000; Unwalla and Banerjea, 2001). The DNAzyme and the disabled DNAzyme ODN (control ODN) were synthesized by Invitrogen (Invitrogen, USA).

In vitro transcript of sequence of *mPer1* mRNA for cleavage

A double stranded ODN containing the sequence of the *mPer1* cDNA 140–845 nts (GenBank accession number: U49930) plus appropriate cloning sites was synthesized and introduced into the *Hind*III and *Bam*H I sites of plasmid pBluescript II SK(+). This recombinant was prepared for cleavage experiments.

Cleavage experiments

DNAzyme cleavage experiments were performed as described previously (Santiago et al., 1999). The oligoribonucleotide substrate of the DNAzyme was labeled at the 5′ end with [^{33}P] ATP (2500 Ci/mmol; Amersham Pharmacia Biotech) by using T4 polynucleotide kinase (New England Biolabs). DNAzyme (50 pmol) was added to 4 pmol of the *in vitro* transcript substrate. The reaction was then stopped at several time points. In these cleavage experiments, the cut and uncut substrates were separated by electrophoresis on a 5% urea denaturing polyacrylamide gel and detected by autoradiography at 4 °C. Signals were then scanned by Storm 840 instrument and analyzed by Image-Quant 5.0 software (Molecular Dynamics).

Treated with DNAzyme and morphine synchronously

DNAzyme and control ODN were enclosed with Lipofectamine (Invitrogen) according to the description respectively. After mice's acclimatization, the basic CPP of mice was measured. The animals were divided into two groups (n=30 per group), including DNAzyme group (DMS) and control ODN group (CMS). The animals were given saline (the same volume as morphine, s.c.) at zeitgeber time (ZT) 2 (10:00 a.m.) before placed into black section of the shuttle boxes for 30 min. On the next day, the animals were given morphine (10 mg/kg, s.c.) at ZT2 (10:00 a.m.) and then placed into white section of the shuttle boxes for 30 min. These procedures were repeated four times in 8 days. At ZT12 (8:00 p.m.) mice of different group were injected intracerebroventricularly with DNAzyme and control ODN enclosed with Lipofectamine respectively once a day from the 1st to the 7th day of the experiment according to different group. At ZT8 (4:00 p.m.), the CPP of the mice was measured on the 8th day of the experiment. Thus four mice of the each group were killed at ZT12 (8:00 p.m.) and ZT16 (12:00 p.m.) on the 6th day and at ZT20 (4:00 a.m.), ZT0 (8:00 a.m.), ZT4 (12:00 a.m.) and ZT8 (4:00 p.m.) on the 9th day respectively. The brains of the killed mice were prepared for Western blot.

Treated with DNAzyme after morphine

Mice were also divided into two groups (n=30 per group), including DNAzyme group and (DMA) control ODN group (CMA). The basic CPP of mice was measured. The animals were given saline (the same volume as morphine, s.c.) at ZT2 (10:00 a.m.) before placed into black section of the shuttle boxes for 30 min. On the next day, the animals were given morphine (10 mg/kg, s.c.) at ZT2 (10:00 a.m.) and then placed into white section of the shuttle boxes for 30 min. These procedures were repeated four times in 8 days, and then the mice were injected intracerebroventricularly with DNAzyme and control ODN once a day respectively according to different group in the next 7 days at ZT12 (8:00 p.m.). The remained procedures were the same as the abovementioned.

Western blot

Mice were deeply anesthetized with ether. Whole brain homogenates were obtained as described (Hastings et al., 1999; Lee et al., 1998). Briefly, tissues were homogenized at 4 °C in buffer 1 (0.4 M NaCl, 20 mM HEPES [pH 7.5], 1 mM EDTA, 5 mM NaF, 1 mM dithiothreitol, 0.3% Triton X-100, 5% glycerol, 0.25 mM phenylmethylsulfonyl fluoride, 10 mg of aprotinin per ml, 5 mg of leupeptin per ml, 1 mg of pepstatin A per ml). Homogenates were cleared by centrifugation (twice, 12 min each, 12,000×g). Proteins were separated by

electrophoresis through sodium dodecyl sulfate–6% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 and then incubated with affinity-purified antisera to mPER1 (Alpha Diagnostics International). Immunoreactive bands were visualized using antigoat immunoglobulin G secondary antisera and enhanced chemiluminescence detection. Signals were then scanned by Storm 840 instrument and analyzed by Image-Quant 5.0 software (Molecular Dynamics).

Data analysis

Data were analyzed by one-way ANOVA for group differences and time difference respectively, and by two-way ANOVA for time and group differences (SPSS 11.5).

RESULTS

In vitro cleavage of *mPer1* mRNA

We tested the efficacy of the DNAzyme targeting *mPer1* to cleave the substrate of *mPer1* mRNA prepared an *in vitro* transcript. The DNAzyme had the effect on cleaving *mPer1* mRNA *in vitro*. Two expected cleavage products, 255-nt and 451-nt, were produced (Fig. 2A). With 4 pmol of the RNA transcript as the target, the DNAzyme at 0 min, 30 min, 60 min, 90 min and 120 min digested 0, 18.1, 38.9, 46.5 and 51.9%, respectively, of the substrate (Fig. 2A). Cleavage products were progressively increased until the last time point at 120 min. In contrast, the disabled DNAzyme ODN (control ODN) did not show any enzymatic activity (Fig. 2B).

In vivo interfering expression of mPER1 protein in CNS

In this study, the results showed that the DNAzyme could attenuate the expression of *mPer1* in whole brain in mice. Fig. 3 displayed examples of mPER1 protein with Western blotting in CNS. In mice treated with control ODN, the rhyme of the density of mPER1 immunoreactivity was display on Fig. 3A, whereas the density of mPER1 immunoreactivity of mice treated with DNAzyme was arrhythmic and lower than that of mice treated with control ODN (Fig. 3B). One-way ANOVA displayed significant difference of mPER1 immunoreactivity in the whole brain in DMS, CMS, DMA and CMA, and revealed significant difference in DMS to CMS, DMS to CMA, DMA to CMS and DMA to CMA, and non-significant difference in DMS to DMA and CMS to CMA (Fig. 4).

Significant difference of mPER1 immunoreactivity in the whole brain in groups and time points was revealed (two-way ANOVA). One-way ANOVA revealed a significant daily rhythm of mPER1 immunoreactivity in the whole brain in mice treated with control ODN and morphine synchronously (CMS) and treated with control ODN after morphine (CMA). In contrast, there were non-significant daily rhythms of mPER1 in the whole brain in mice treated with DNAzyme and morphine synchronously (DMS) and treated with DNAzyme after morphine (DMA; Fig. 4).

Morphine-induced reward under treatment with DNAzyme

CPP has been widely used to measure the rewarding properties of drug dependence (Tzschentke, 1998). Therefore, we studied the response of mice treated with DNAzyme in the CPP paradigm to detect differences in morphine-induced reward. As expected, one-way ANOVA displayed DMS, unlike CMS, did not show preference to morphine-trained side (Fig. 5).

Treatment with DNAzyme when morphine addiction had been formed

When animals were given morphine for three to four times, the morphine dependence was formed, and the CPP would last for 3 weeks (Schecter, 1998). We studied the response of mice treated with the DNAzyme after given morphine in the CPP paradigm. DMA showed preference to the morphine-trained side as well as CMA group did (Fig. 5). One-way ANOVA showed non-significant difference.

DISCUSSION

The mechanism of circadian oscillation based on the molecular feedback loops consists of several circadian genes and their protein products. Several clock genes including the *Per* genes have been cloned in the last few years. *Drosophila* and mice were the models in these performed experiments (Wager-Smith and Kay, 2000; Forger and Peskin, 2003), which gave us viewpoints that circadian clock components affect many complex neuron activities like drug reward (Andretic et al., 1999). *Clock, cycle, period*, and *timeless, doubletime* were the object to be manipulate in experiment performed on *Drosophila*. *Clock, cycle, period*, and *doubletime* mutant phenotype showed no sensitization to cocaine. Mice with *mPer1* gene mutation did not sensitize to cocaine and CPP test also indicated the block of reward to cocaine (Abarca et al., 2002). Those results showed that regulation of circadian clock gene expression might influence the process of drug dependence significantly.

DNAzymes are short DNA molecules that have the potential to cleave any target RNA in a sequence-specific manner, and two major catalytic motifs (10–23 and 8–17) have been described (Santoro and Joyce, 1997). DNAzymes are suitable tools for studying gene function, since they can down-regulate endogenous gene expression. This approach was used to interfere specifically with the intracellular function of the target genes (Famhe and Khachigian, 2004; Ordoukhanian and Jouce, 2002; Khachigian et al., 2002). Our study reveals that DNAzyme is an effective tool of researching gene function, which can cleave the targeting mRNA and attenuates its protein product *in vivo* and *in vitro*.

There is strong expression and significant circadian rhythm of mPER1 in SCN in mice, and furthermore *Per1* mRNA and proteins are expressed in other brain areas (Hastings et al., 1999; Yan et al., 1999; Takumi et al., 1998; Field et al., 2000). The mPER1 in SCN starts to rise in the late morning, reaches its maximum around the usual time of the afternoon, and then declines, whereas the *mPer1* mRNA cycle precedes the mPER1 protein cycle by 4–6 h, consistent with a role for the mPER protein in the autoregulation of its transcription. The rhythm of mPER1 in the whole brain in mice was demonstrated in the present results, and the pattern of circadian expression of mPER1 is similar to that in SCN. Masashi et al. (1999) used antisense ODN to inhibit *mPer1* expression *in vivo* and *in vitro*, which showed that *mPer1* mRNA in the SCN could was reduced by ODNs injected intracerebroventricularly (Masashi et al., 1999), but mPER1 protein was not detected in that report. In our research, the expression of mPER1 could be attenuated by i.c.v. injection of DNAzyme, and the pattern of circadian expression was interfered also.

The degree of response to cocaine-induced behavioral sensitization and reward is under the influence of the diurnal state of the animal (Abarca et al., 2002; Tei et al., 1997; Sun et al., 1997). A similar time-dependent profile was reported in methylphenidate-sensitized animals (Gaytan et al., 1999, 2000). The lack or dysfunction of the *mPer1* gene abolishes cocaine sensitization and reward whereas the dysfunction of the *mPer2* gene induces a hypersensitized response to cocaine (Abarca et al., 2002). Consistent with those results, animals attenuated *mPer1* expression with the DNAzyme when treated with morphine synchronously did not show a preference to the morphine-paired side in this study. These results support that drug addiction seems to be influenced at least partially by the expression of *mPer1*.

It is known that different components of the midbrain dopamine system and the glutamatergic system play a critical role in drug-induced sensitization and reward (Spanagel and Wess, 1999; Vanderschuren and Kalivas, 2000; Cornish and Kalivas, 2001), and mesolimbic dopaminergic neurons are involved in strengthening formation of associations between salient contextual stimuli and internal rewarding or aversive events (Spanagel and Wess, 1999). The common long-term adaptations produced by opioids and other drugs of abuse in this system could enhance these processes and thereby play a major role in initiation and maintenance of compulsive drug use. Drugs of abuse cause long-lasting changes in the brain that underlie the behavioral abnormalities associated with drug addiction. Similarly, experience can induce memory formation by causing stable changes in the brain. Learning and memory and drug addiction are modulated by the same neurotrophic factors, share certain intracellular signaling cascades, and depend on activation of the transcription factor CREB. They are associated with similar adaptations in neuronal morphology, and both are accompanied by alterations in synaptic plasticity at particular glutamatergic synapses in the brain (Eric, 2002). In this study, animals interfered *mPer1* expression with DNAzyme when treated with morphine synchronously did not show preference to the morphine-paired side in the CPP paradigm in comparison with the controlled. In contrast, animals with interfered-with *mPer1* expression with DNAzyme after treatment with morphine showed the same preference to the morphine-trained side in the CPP paradigm in comparison with the controlled. These experiments indicate that *mPer1* and its product do not influence morphine dependence when morphine dependence has been memorized. In fact, *mPer1* is not related to learning and memory processes. The *mPer1* and *mPer2* mutants did not differ from wildtype animals in a fear conditioning paradigm, demonstrating that *Per* mutants do not differ in their learning abilities from wildtype animals (Abarca et al., 2002). This may be interpreted that the morphine dependence was not interfered with when mice were treated with DNAzyme after morphine.

In summary, the expression of the circadian clock gene, *mPer1*, can be attenuated by i.c.v. injection of DNAzyme targeting *mPer1* in CNS in mice. Our data also show that animals with interfered-with expression with DNAzyme when treated with morphine synchronously did not show morphine dependence, but in contrast, animals with interfered-with *mPer1* expression with DNAzyme after treatment with morphine display morphine dependence. These results indicate that drug dependence seems to be influenced at least partially by *mPer1*, but *mPer1* cannot affect morphine dependence that has been formed.

Abbreviations

CPP, conditioned place preference; ODN, oligonucleotide; *Per*, *Period*; ZT, zeitgeber time..

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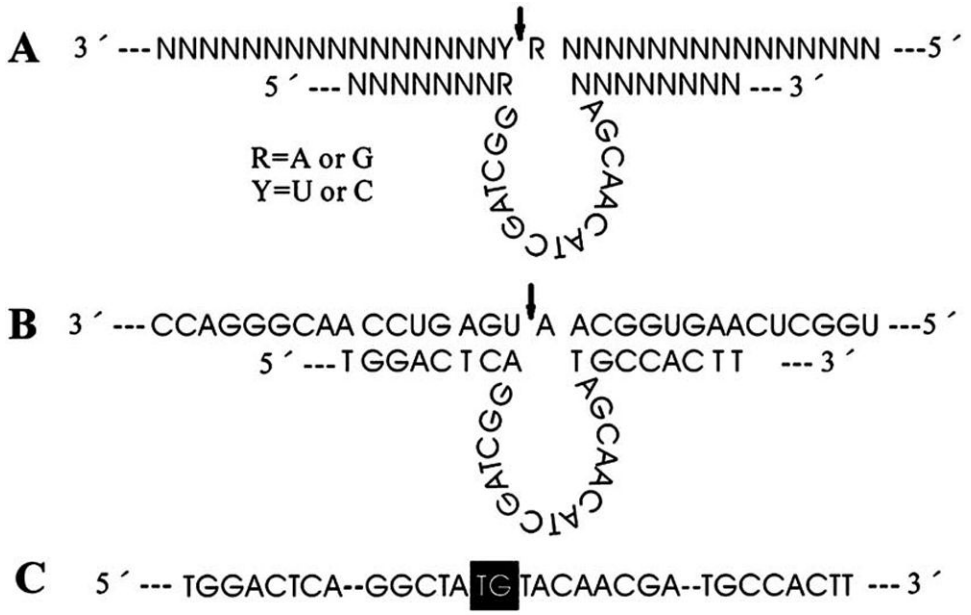

Fig. 1. DNAzyme and its mRNA substrate of *mPer1*

(A) A 10–23 DNAzyme structure with substrate cleavage occurring at the position indicated by the arrow. (B) Sequence and structure of *mPer1* DNAzyme annealed to the *mPer1* substrate. The arrow indicates the cleavage site. (C) Sequence of the control ODN. To inactivate enzymatic activity of the DNAzyme, two nts (black) in the intervening 15-nt catalytic domain were altered.

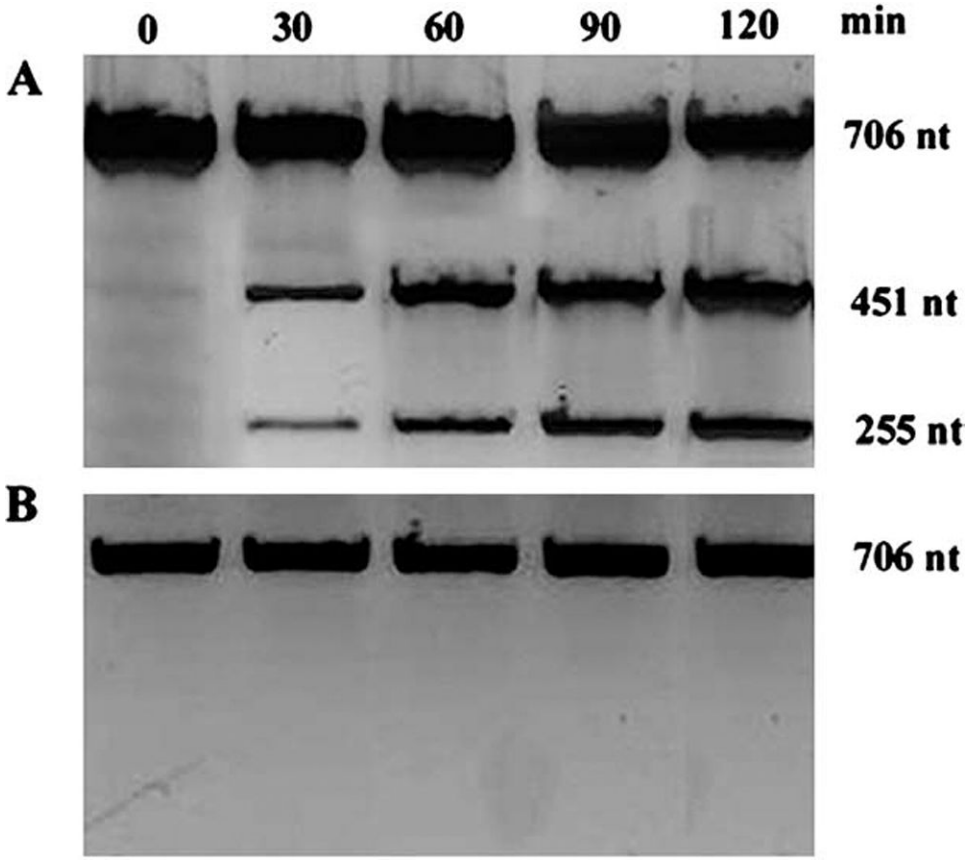

Fig. 2. In vitro cleavage of *mPer1* RNA

(A) For DNAzyme cleavage of an *in vitro* transcript, 4 pmol of a 706-nt *mPer1* mRNA transcript were digested with 50 pmol of DNAzyme. The expected 255-nt and 451-nt cleavage products were generated. (B) The control ODN did not show any enzymatic activity. The substrate and cleavage products were separated and analyzed on a urea denaturing polyacrylamide gel.

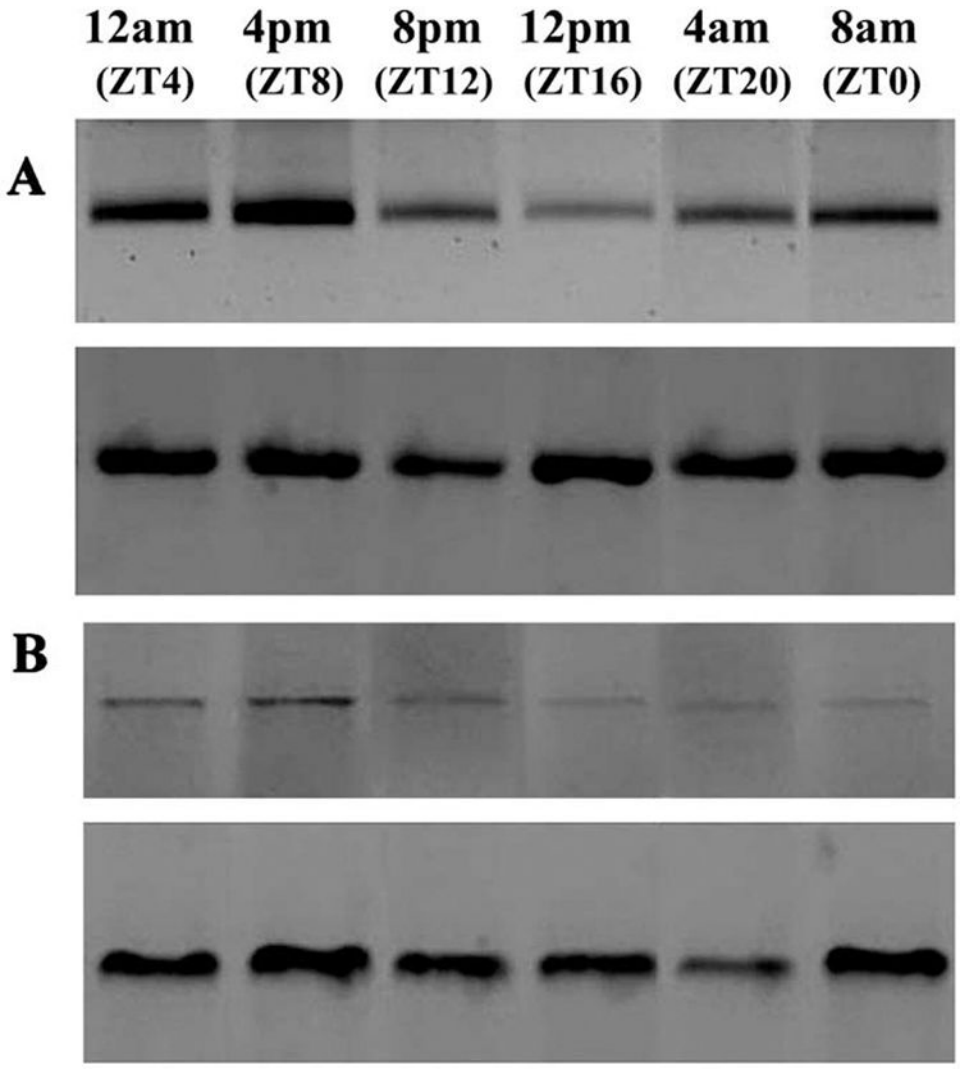

Fig. 3. Examples of mPER1 immunoreactivity in whole brain in mice

(A) Treated with control ODN (top: mPER1; bottom: actin). mPER1 immunoreactivity of mice treated with control ODN started to rise in the late morning, reached its maximum around the usual time of ZT8, and then declined. (B) Treated with DNAzyme (top: mPER1; bottom: actin). The mPER1 immunoreactivity of mice treated with DNAzyme was arrhythmic and lower than that of mice treated with control ODN.

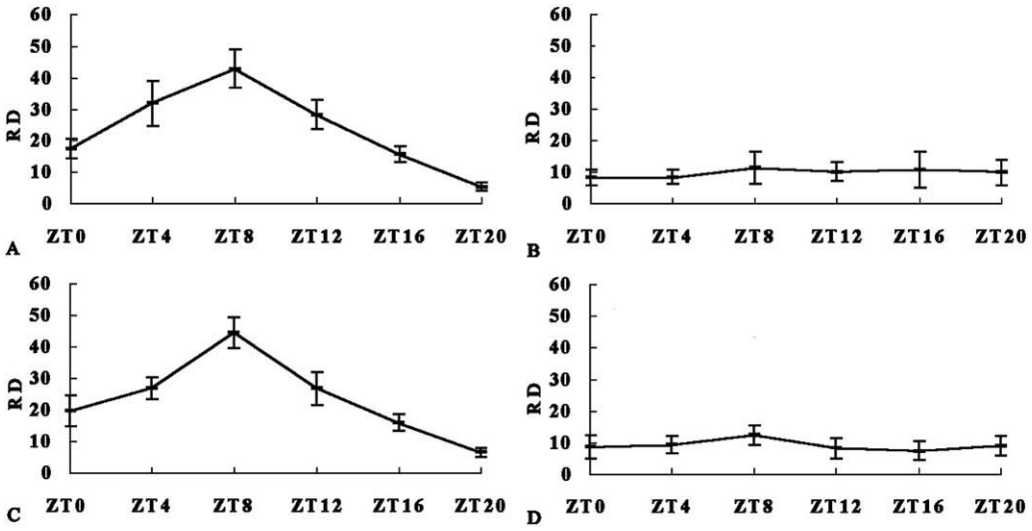

Fig. 4. mPER1 immunoreactivity in whole brain in mice of different group (mean±S.E.M.)

The relative density of mPER1 immunoreactivity (RD) was normalized against that of actin. Two-way ANOVA displayed that RD was different in groups and time points (intercept, $F(1,87)=557.279$, $P<0.001$; group, $F(3,87)=32.775$, $P<0.001$; time, $F(5,87)=15.394$, $P<0.001$). (A) CMS. One-way ANOVA revealed a significant daily rhythm ($F(5,18)=34.516$, $P<0.001$). The maximum of mPER1 immunoreactivity was at ZT8 (ZT8–ZT4: $P=0.031$; ZT8–ZT12: $P=0.003$; ZT8–ZT16: $P<0.001$; ZT8–ZT20: $P<0.001$; ZT8–ZT0: $P<0.001$), and the minimum was at ZT20 (ZT20–ZT4: $P<0.001$; ZT20–ZT8: $P<0.001$; ZT20–ZT12: $P<0.001$; ZT20–ZT16: $P=0.05$; ZT20–ZT0: $P=0.001$). (B) DMS. One-way ANOVA revealed a non-significant daily rhythm ($F(5,18)=0.421$, $P=0.828$). (C) CMA. One-way ANOVA revealed a significant daily rhythm ($F(5,18)=33.109$, $P<0.001$). The maximum of mPER1 immunoreactivity was at ZT8 (ZT8–ZT4: $P=0.002$; ZT8–ZT12: $P=0.002$; ZT8–ZT16: $P<0.001$; ZT8–ZT20: $P<0.001$; ZT8–ZT0: $P<0.001$), and the minimum was at ZT20 (ZT20–ZT4: $P<0.001$; ZT20–ZT8: $P<0.001$; ZT20–ZT12: $P<0.001$; ZT20–ZT16: $P=0.037$; ZT20–ZT0: $P=0.003$). (D) DMA. One-way ANOVA revealed a non-significant daily rhythm ($F(5,18)=1.157$, $P=0.368$).

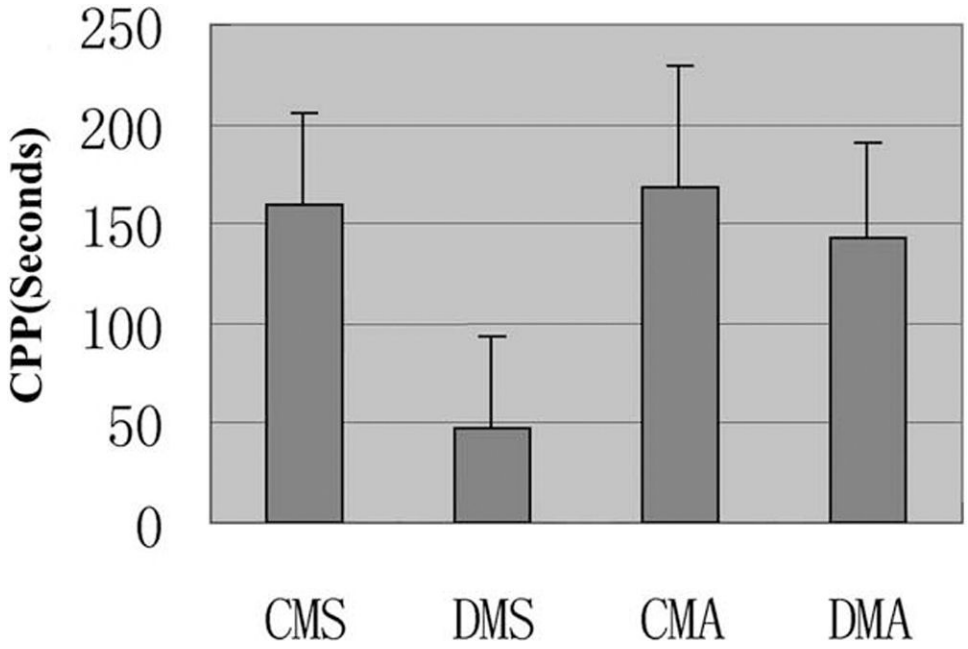

Fig. 5. CPP in mice

Data are given as mean (±S.E.M.) of difference between the basic CPP and CPP after treatment. One-way ANOVA revealed significant difference ($F(3,116)=30.436$, $P<0.001$). DMS was significant to other groups (DMS to CMS: $P<0.001$; DMS to CMA: $P<0.001$; DMS to DMA: $P<0.001$), but others were non-significant difference.