

# The Circadian Clock Gene, *mPer2*, Controls Circadian Rhythm of Pain Sensitivity

Tiffany H. Lee, Kori L. Brewer<sup>1</sup>, Todd A. Nolan<sup>2</sup>, Brian McMillen<sup>3</sup>, Jian M. Ding<sup>2</sup>

Departments of <sup>1</sup>Emergency Medicine, <sup>2</sup>Physiology, <sup>3</sup>Pharmacology & Toxicology, Brody School of Medicine, East Carolina University, Greenville, NC 27834.

## Abstract

Pain is a pervasive medical problem especially among the elderly and cancer patients. Clinically, pain intensity has been shown to vary following a circadian pattern. Patients with arthritis or undergoing dental procedures experienced the lowest pain threshold early in the morning and the highest threshold 12 hours later. Similar fluctuation has been described in the endogenous opioid, enkephalin, suggesting the circadian clock's role in enkephalin production and alteration of pain perception over a 24-hour period. Despite the observation of this circadian pattern, the evidence that links pain threshold and the circadian clock gene is lacking. We hypothesized that the *mPer2* clock gene, co-localized with enkephalin in the Dorsal Horn of the spinal cord, regulates the pain threshold. Immunologic DAB staining as well as Western Blot techniques were used to detect co-localization in Dorsal Horn. Results indicate a clear circadian variation of pain threshold and proenkephalin expression in Wild Type(WT) as compared to *mPer2* knock-Out (KO) mice. There is no co-localization of enkephalin and *mPer2* gene in the Dorsal Horn of the spinal cord during the immunologic DAB staining suggesting further study of co-localization in the Rostral Ventral Medulla is warranted.

## Introduction

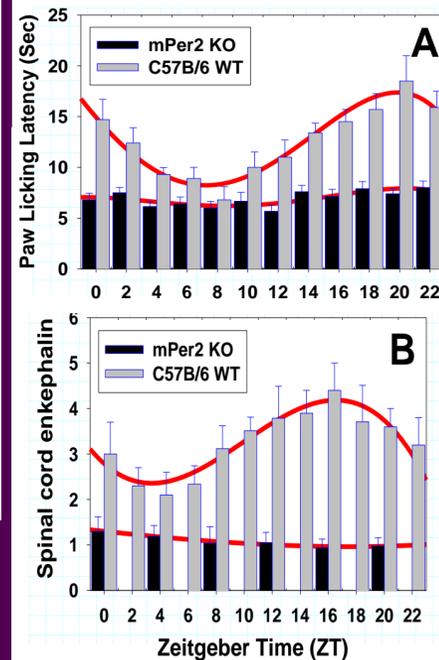
Circadian rhythms are important physiological functions that manifest a near 24-hour period. In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus serves as the master circadian clock. While at the cellular level, circadian rhythms are regulated by the oscillatory expression of a set of circadian clock genes. One of these genes, *mPer2*, is involved in diverse functions, including cocaine addiction and alcohol consumption. Mice lacking the *mPer2* gene demonstrate increased alcohol and cocaine preference.

Pain has been described clinically and shown experimentally to have circadian variations in onset and intensity. Healthy volunteers exhibit a circadian pattern of pain thresholds, with the lowest threshold for pain occurring in the early morning (end of rest period) and the highest threshold for pain occurring 12 hours later. Therefore, an intervention that effectively combats pain at one point in time, may not be effective if administered at another time.

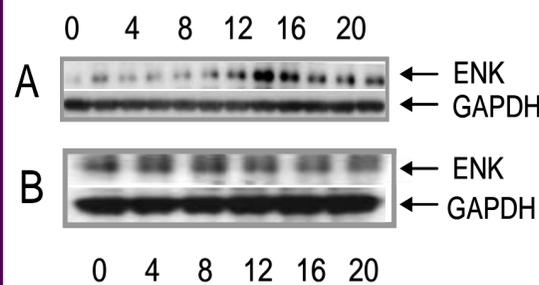
Typically, the opioid system provides an endogenous pain control mechanism at spinal and supraspinal levels. The level of endogenous opioids fluctuates over a 24-hour period with the timing of increased enkephalin corresponding to that of increased pain threshold in mice as tested using tail-flick or hot plate tests, while the lowest level of enkephalin corresponds to a lower pain threshold.

Despite this circadian pattern in pain thresholds and opioid peptide levels, no data exists to demonstrate a possible link between pain and circadian clock genes. Because drug addiction and alcohol consumption are increased in humans and animals that suffer from chronic pain states, and *mPer2* mutation predisposes mice to alcohol and cocaine preference, we previously hypothesized that the *mPer2* KO animals would be more susceptible to pain than WT animals. Mice that lack the *mPer2* gene, were found to have decreased pain thresholds and peptide levels of pro-enkephalin (ENK) in the spinal cord over a 24-hour period compared to WT animals. These data suggest that the *mPer2* gene can control the expression of ENK in the spinal cord. The current study sought to determine if *mPer2* is working as a transcription factor for ENK by assessing the expression of *mPer2* in the dorsal horn and determining if it is co-localized with ENK in this region.

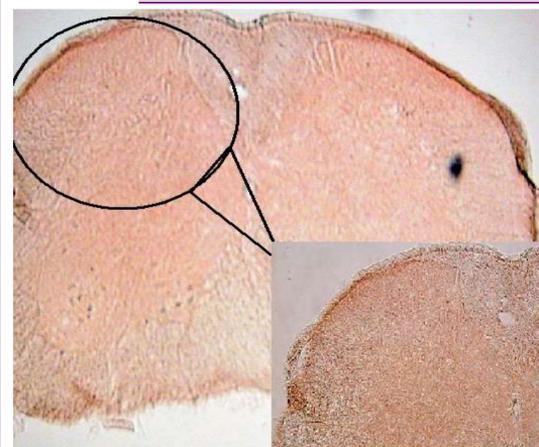
## Results



**Figure 1:** Thermal pain thresholds (A) and spinal cord proenkephalin expression (B) in wild type and *mPer2* mutant mice. WT animals show a circadian variation in pain thresholds as measured by hot plate test (n=10). Pain thresholds in *mPer2* mutant animals are flat over time and similar to the lowest threshold measured in WT animals (n=10). Similarly, spinal proenkephalin levels vary over 24 hours in WT animals (n=6), with a reduction in proenkephalin preceding the lowest pain threshold by 2 hours. Spinal proenkephalin in *mPer2* mutant animals does not fluctuate over time and is significantly lower than WT levels (n=4). All animals are kept in 12 hour light and 12 hour dark schedule.



**Figure 2.** Levels of proenkephalin protein in spinal cord. Western blot analysis shows the circadian variation of proenkephalin levels in the spinal cord of the wild type mice, higher during the dark phase and lower during the light phase, normalized to GAPDH (A), (n=6 per ZT). In contrast, there is a lack of circadian variation of proenkephalin levels in the spinal cord of the *mPer2* mutant mice (B), (n=4 per ZT). Total protein levels in the spinal cord are constant over the 24 hour circadian cycle (C), (n=6 per ZT).



Absence of DAB Immunostaining in *mPer2* sections.



DAB Immunostaining is present in Enkephalin sections

## Method

Male C57BL/6 WT mice and *mPer2* gene knock-out (KO) mice (Jackson Lab *Per2*<sup>Brdm1</sup>, 8-12 week old) were housed in light proof chambers with 12 on:12 off Light-Dark schedule at constant room temperature. The moment of light on and light off is designated as ZT 0 and ZT 12 respectively. Beginning at ZT 0, mice from each group were tested for sensitivity to thermal stimuli using a hot plate (at 56°C) test every 2 hours over a 24-hour period. Pain reflex latencies to the nearest 0.1 second were measured using a Hot Plate Analgesia Meter. Each animal was tested 3 times, and the average response latency calculated. Animals were euthanized at every other hour throughout the 24-hour circadian cycle. A 5mm length of spinal cord from the lumbar region was removed and detection of pro-enkephalin was performed using Western Blot analysis. Ratios of signal intensity of ENK/GAPDH are reported for each time point. A least square best fit regression analysis determined variations in pain thresholds over time.

For co-localization of ENK and *mPer2*, DAB Immunologic staining was performed on 25µm cross sections of lumbar cord from WT animals at ZT16. For *mPer2* staining, sections were incubated in 1<sup>o</sup> antibody for 48 hours, followed by 1 hour of DAB-conjugated 2<sup>o</sup> antibody. For detection of ENK, sections were incubated in 1<sup>o</sup> antibody for 2 hours followed by 1 hour in 2<sup>o</sup>. Cords were examined under light microscopy for expression of ENK and *mPer2*.

## Discussion

The loss of circadian variation in thermal pain threshold and pro-enkephalin expression in *mPer2* KO mice suggests a regulatory role of a circadian clock gene, *mPer2*, on the daily rhythm of pain thresholds and enkephalin production in the spinal cord. Thus far, our research provides a novel approach to the study of circadian clock genes and pain in vivo and further provide a rationale for optimal timing for intervention to maximize pain management in chronic pain sufferers.

The lack of immunologic DAB staining for *mPer2* in the Dorsal Horn of the spinal cord suggests that expression of ENK is not directly regulated by *mPer2* at the spinal level as hypothesized. It is possible that an alternative mechanism by which *mPer2* may affect ENK expression in the supraspinal level. *mPer2* is known to be expressed in brain regions that provide descending modulation of pain transmission through the dorsal horn such as the Rostral Ventral Medulla.

## Conclusions

A clear circadian variation of pain threshold and pro-enkephalin expression in WT as compared to *mPer2* knock-Out mice further support a plausible reason for the varying effect of pain treatments across time. Future research must characterize this interaction and determine optimal timing for the intervention in chronic pain sufferers.

The lack of co-localization of enkephalin and the *mPer2* gene in the Dorsal Horn region of the spinal cord indicates that expression is being regulated by an alternative pathway for their research in the Rostral Ventral Medulla is indicated.