Rhythmic clock gene expression in the spinal cord may underlie sympathetic nerve control of adrenal function

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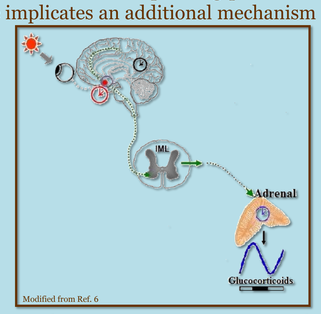
Background

•Circadian glucocorticoid (GC) secretion by the adrenal cortex is dependent on an endogenous molecular clock that is entrained by the hypothalamic suprachiasmatic nucleus (SCN).[1] Environmental cues such as light, feeding, and activity entrain biological clocks in peripheral tissues and help maintain a variety of normal metabolic, behavioral, and physiological processes.

•Glucocorticoid hormone release is regulated by the endocrine hypothalamic-pituitary-adrenal (HPA) axis as well as sympathetic nerve activity.[2] The finding that rhythmic corticosterone (CORT) secretion in rodents can occur without a corresponding pattern of pituitary adrenocorticotropic hormone (ACTH) release implicates an additional mechanism for regulating circadian GC rhythms.[3]

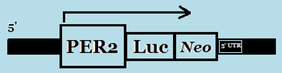


•Tracing studies reveal connections between the SCN and the adrenal medulla, which may communicate with the adrenal cortex to control GC secretion. These projections occur via the intermediolateral (IML) cell column, located in the thoracic spinal cord, which contains preganglionic sympathetic neurons that transmit information to the adrenal via the splanchnic nerve.[4] Ablation of splanchnic nerve projections to the adrenal results in blunted amplitudes of GC rhythms.[5]

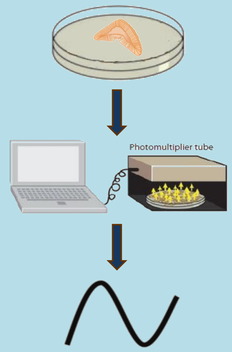
•Still, the neuroendocrine mechanism through which sympathetic nerve activity entrains the adrenal clock to environmental signals is unknown. We hypothesized that IML neurons possess an endogenous molecular clock in which oscillating clock genes transmit rhythmic information to the adrenal. We used immunohistochemistry to search for these neurons and monitored real-time expression of Period2 (Per2), a core clock gene, in transgenic mice expressing the PER2::Luciferase fusion protein. Addition of luciferin to tissue explants from these animals produces bioluminescence and allows us to continuously monitor PER2 expression.

Methods

Immunohistochemistry. Mouse spinal cord tissue was fixed in Zamboni's fixative for 24 h followed by 20% sucrose. Coronal thoracic and lumbar (30 µm sections) were washed with phosphate buffered saline-Triton X-100 (PBS-X) and blocked with 10% normal donkey serum (NDS) in PBS-X. Primary antibodies were diluted in 1% NDS and PBS at 1:50 (Guinea pig anti-BMAL1), 1:500 (Rabbit anti-PER2; Goat anti-vesicular acetylcholine transporter. Secondary antibodies were diluted 1:400 (donkey anti-goat Cy3; donkey anti-guinea pig Cy3) and 1:200 (donkey anti-rabbit Cy5) with 10% normal rat serum (NRS) and 5% NDS in PBS.



Real-time monitoring of bioluminescence by a photomultiplier tube (PMT) detector. Fresh harvested tissue was extracted from PER2::Luc transgenic mice housed in 12:12 LD cycle between CT 8 and CT 9 and kept on ice in Hanks Buffered saline solution (HBSS). Adrenal tissue was hemisected; spinal cords were cut coronally and bisected down the midline to expose grey matter. Tissues were placed in 35-mm dishes atop Millicell membranes in 1.2 mL of media containing penicillin-streptomycin and luciferin at 37◦C. Bioluminescence in the explants was monitored for 3-5 days. Period and phase data were analyzed using the Lumicycle Analysis software.



Results

Characterization of clock genes in the mouse spinal cord

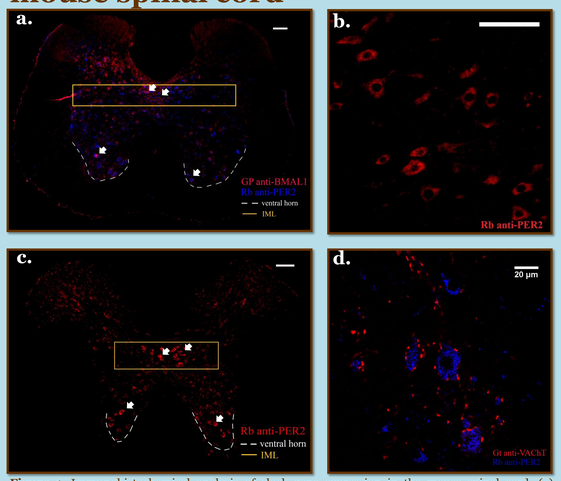


Figure 1. Immunohistochemical analysis of clock gene expression in the mouse spinal cord. (a) BMAL1 and PER2 co-localize in neurons within ventral horn and IML regions and appear to be differentially recruited in the cytoplasm or nucleus. (b) PER2-positive cell bodies in the ventral horn. (c) Expression of PER2 in various grey matter areas of the thoracic spinal cord. (d) Co-localization of PER2 and VAChT in lumbar spinal cord neurons. VAChT, vesicular acetylcholine transporter; BMAL1, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; PER2, Period 2. All scale bars denote 100 µm unless otherwise noted.

Thoracic spinal cord explants exhibit PER2Luc rhythms

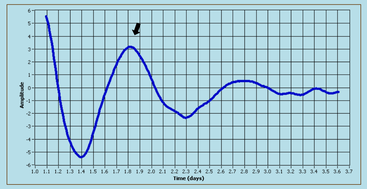


Figure 2. Example of PER2Luc bioluminescence rhythms obtained from spinal cords in mPER2::Luc mice. Explants were monitored for four days and rhythms were normalized and adjusted by a smoothing average using Lumicycle Analysis software. Due to background-light exposure during the initial tissue incubation, the first 24 hrs typically contained arrhythmic noise and thus bioluminescence data was calculated after the first day. The first peak phase analyzed occurred during the second day *in-vitro*, denoted here by the peak occurring at 1.8 days.

Real-time monitoring of mPER2Luc activity in spinal cord and adrenal explants

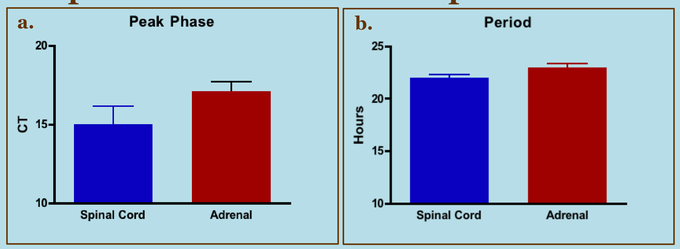


Figure 3. PER2 expression patterns in spinal cord explants are similar to that seen in the adrenal gland. (a) Peak phase of PER2Luc activity in circadian time (CT) in the spinal cord (n=4) occurs before the adrenal (n=5; P=0.1266, unpaired t-test). (b) Periods of PER2Luc activity in the spinal cord and adrenal gland do not differ significantly. Length of period was calculated from sine wave fits from an average of 3 peak-to-peak times (P=0.0992).

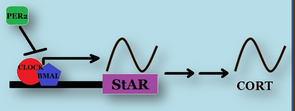
Conclusions

•We identified IML neurons that express PER2 and BMAL1, two core clock genes that are expressed in a circadian fashion. Double-labeling for VAChT, a marker of cholinergic neurons, also revealed clock gene-positive IML neurons in thoracic intermediate grey and in motor neurons in thoracic and lumbar ventral horns.

•To examine whether IML neurons express clock genes in a circadian fashion, we used the reporter *mPer2*^{Luciferase} knockin mice to monitor real-time gene expression and found that the spinal cord exhibits rhythms in PER2 expression. Although non-significant, PER2Luc activity in the spinal cord appears to peak before the adrenal, supporting the notion that the spinal cord clock is upstream of the adrenal.

•Future aims include pinpointing the specific group of spinal cord neurons that exhibits PER2Luc rhythms by dissecting out ventral horn vs. IML neurons and monitoring their bioluminescence separately. In addition, we are currently using a retrograde tracer injected into the adrenal to identify IML neurons and subsequently label them for clock genes to see whether this group of cells drives GC rhythms in the adrenal.

•Our results suggest a mechanism by which sympathetic nerve activity could modulate adrenal glucocorticoid and catecholamine release. Sympathetic nerve activity has been proposed to modulate adrenal sensitivity to pituitary hormones, which is dysregulated in diseases such as depression and post-traumatic stress disorder.[7,8,5]



Acknowledgements

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