The transcriptional repressor Rev-erba regulates circadian expression of the astrocyte Fabp7 mRNA

William M. Vanderheyden a, b, Bin Fang c, Carlos C. Flores a, Jennifer Jager d, Jason R. Gerstner a, b, c, *

a Elson S. Floyd College of Medicine, Washington State University, Spokane, WA 99202, USA
b Sleep and Performance Research Center, Washington State University, Spokane, WA 99202, USA
c Genomics Institute of the Novartis Research Foundation, 10675 John Jay Hopkins Dr, San Diego, CA 92121, USA
d Université Côte d’Azur, INSERM, Centre Mediterranéen de Médecine Moléculaire (CIM), Cellular and Molecular Physiopathology of Obesity, Nice, France

* Corresponding author. Elson S. Floyd College of Medicine, Washington State University, Spokane, WA 99202, USA.
E-mail address: j.gerstner@wsu.edu (J.R. Gerstner).

Keywords: Lipid Metabolism Glia BLBP B-FABP Clock

ARTICLE INFO

ABSTRACT

The astrocyte brain-type fatty-acid binding protein (Fabp7) circadian gene expression is synchronized in the same temporal phase throughout mammalian brain. Cellular and molecular mechanisms that contribute to this coordinated expression are not completely understood, but likely involve the nuclear receptor Rev-erbas (NR1D1), a transcriptional repressor. We performed ChIP-seq on ventral tegmental area (VTA) and identified gene targets of Rev-erbas, including Fabp7. We confirmed that Rev-erbas binds to the Fabp7 promoter in multiple brain areas, including hippocampus, hypothalamus, and VTA, and showed that Fabp7 gene expression is upregulated in Rev-erbas knock-out mice. Compared to Fabp7 mRNA levels, Fabp3 and Fabp5 mRNA were unaffected by Rev-erbas depletion in hippocampus, suggesting that these effects are specific to Fabp7. To determine whether these effects of Rev-erbas depletion occur broadly throughout the brain, we also evaluated Fabp mRNA expression levels in multiple brain areas, including cerebellum, cortex, hypothalamus, striatum, and VTA in Rev-erbas knock-out mice. While small but significant changes in Fabp5 mRNA expression exist in some of these areas, the magnitude of these effects are minimal to that of Fabp7 mRNA expression, which was over 6-fold across all brain regions. These studies suggest that Rev-erbas is a transcriptional repressor of Fabp7 gene expression throughout mammalian brain.

1. Introduction

Fatty-acid binding proteins (Fabp) comprise a family of small (~15 kDa) hydrophobic ligand binding carriers with high affinity for long-chain fatty-acids for intracellular transport, and are associated with metabolic, inflammatory, and energy homeostasis pathways (Furuhashi and Hotamisligil, 2008; Storch and Corsico, 2008). These include three that are expressed in the adult mammalian central nervous system (CNS), and are Fabp3 (H-Fabp), Fabp5 (E-Fabp), and Fabp7 (B-Fabp). Fabp3 is primarily expressed in neurons, Fabp5 is expressed in various cell types, including both neurons and glia, and Fabp7 is most abundant in astrocytes and neural progenitors. While performing microarray analysis of transcripts in mouse brain to characterize novel diurnally regulated genes, Fabp7 was identified as a unique transcript elevated in multiple hypothalamic brain regions during the sleep phase (Gerstner et al., 2006). Unlike other circadian regulated gene products, Fabp7 has a synchronized pattern of global diurnal expression in adult murine brain (Gerstner et al., 2006, 2008; Gerstner et al., 2012), is regulated by the core clock gene BMAL1 (Gerstner and Paschos, 2020) and has a general role in governing aspects of sleep behavior in multiple species, including flies, mice, and humans (Gerstner et al., 2017). Fabp7 has been shown to regulate dendritic morphology and excitatory cortical neuron synaptic function (Ebrahimiet al., 2016), as well as locomotor responses to NMDA-receptor activity (Watanabees et al., 2007), and other behavioral conditions including fear memory and anxiety (Owadaet al., 2006). Therefore, Fabp7 may play an important role in regulating time-of-day dependent changes in astrocyte-derived and evolutionarily conserved plasticity-related processes (Lavialleet al., 2011; Nagaiet al., 2020; Gerstner, 2012).
Since the time-of-day profile of Fabp7 mRNA expression is abolished in BMAL1 KO mice (Gerstner and Paschos, 2020), we performed bioinformatic analysis to locate core canonical E-box elements (CACGTG) within the Fabp7 promoter. We did not detect any canonical E-box elements, so we considered whether other cis-acting elements influenced by circadian output in the Fabp7 promoter exist. Analysis of the promoter for Fabp7 gene revealed several sites known to be involved in the metabolic arm of the clock (Choet al., 2012; Bugge et al., 2012; Zhang et al., 2015), including multiple sites for the transcriptional co-repressor nuclear receptor Rev-erbα (NR1D1), termed Rev-erbα response elements (RORE) (Table S1).

To determine whether these RORE sites were functional, we performed chromatin immunoprecipitation experiments followed by DNA-sequencing (ChIP-seq) on tissue from the ventral tegmental area (VTA), a brain region known to regulate motivational/reward behaviors (Moraes and Margolis, 2017; Russo and Nestler, 2013), wakefulness, and sleep (Takata et al., 2018; Yue et al., 2019; Eban-Rothschild et al., 2016). Here we identified positive Rev-erbα interactions within the first kilobase upstream of the transcription start site of the Fabp7 promoter, but not in the Fabp3 or Fabp5 promoters (Fig. 1A–C). The top 20 Rev-erbα binding site loci, peak score, distance to the translational start site and gene names are listed in Table 1. Gene Ontology (GO) analysis revealed significant enrichment of several biological processes, molecular functions, and cellular components (Table 2) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis shows the top 20 pathways in Rev-erbα ChIP-seq genes (Fig. 2). The complete list of Rev-erbα ChIP-seq genes is provided in [SUPPLEMENTAL dataset 1].

To confirm that Rev-erbα binds the Fabp7 promoter in multiple brain regions, we compared Rev-erbα binding in the Fabp7 promoter against the negative control insulin, and the positive control Npas2 in WT and Rev-erbα KO mice. We observed Rev-erbα binding to the Fabp7 and Npas2 promoters in WT, but not in the Rev-erbα KO mice, in both hippocampus (Fig. 3A) and hypothalamus (Fig. 3B). Binding of Rev-erbα was not observed for insulin, regardless of genotype (Fig. 3A and B). Since BMAL1 is known to transactivate Rev-erbα (Mohawk et al., 2012; Albrecht, 2012), a transcriptional repressor, BMAL1 could influence Fabp7 gene expression (Gerstner and Paschos, 2020) indirectly through Rev-erbα.

To test the hypothesis that Rev-erbα represses Fabp7 gene expression, we examined the diurnal profile of Fabp7 mRNA in Rev-erbα KO mice. If Fabp7 expression is repressed by Rev-erbα, this predicts that Fabp7 mRNA should be elevated in the Rev-erbα KO. We confirmed that Fabp7 mRNA is elevated in hippocampus of Rev-erbα KO mice, while Fabp3 and Fabp5 mRNA levels are not affected (Fig. 4A–C). To determine whether time-of-day mRNA levels are affected by Rev-erbα, we analyzed the normalized mRNA expression for Fabp3, Fabp5, and Fabp7 from six time-points over 24h of Rev-erbα KO and WT mice. While Fabp3 (Fig. 4D) and Fabp5 (Fig. 4E) mRNA do not oscillate in WT mice and remain unaffected in Rev-erbα KO, the Fabp7 mRNA circadian oscillation is disrupted in the Rev-erbα KO compared to WT hippocampus (Fig. 4F). Since Fabp7 expression is diurnally regulated throughout murine brain (Gerstner et al., 2006; Gerstner, 2008; Gersmer et al., 2012), we wanted to determine if Fabp7 mRNA levels were regulated by Rev-erbα broadly in multiple brain regions. Analysis of multiple brain regions including striatum, VTA, cerebellum, hippocampus, hypothalamus, and cortex of Rev-erbα KO compared to WT mice revealed analogous increases in Fabp7 mRNA levels (~6–15 fold), but not Fabp3 or Fabp5 mRNA levels (Fig. 5).
Together, these data suggest that the circadian clock control of Fabp7 mRNA expression requires Rev-erβ broadly across many brain regions.

3. Discussion

The astrocyte Fabp7 gene expression is known to cycle in a synchronized fashion throughout the mammalian CNS (Gerstner et al., 2006; Gerstner, 2008; Gerstner et al., 2012; Schnellet al., 2014). Previous studies have shown Fabp7 circadian gene expression is under control of the core clock transcription factor BMAL1 (Gerstner and Paschos, 2020), however the Fabp7 circadian expression via Rev-erβ, a transcriptional repressor, and known BMAL1 target (Guillaumond et al., 2005). Here we provide evidence that Fabp7

![Fig. 2. Analysis of the top 20 KEGG pathways enriched in Rev-erβ ChIP-seq genes plotted with number of hits per pathway.](image)
contains canonical ROREs and that Rev-erb binds to the RORE regions in the Fabp7 gene locus in the VTA (Fig. 1A). The current study validates a previous report that also showed Rev-erb binding to Fabp7 in the hippocampus (Schnell et al., 2014) (Fig. 3A), and extends these findings to show this also occurs in the hypothalamus (Fig. 3B). Taken together, these results suggest that the coordinated and synchronized expression of Fabp7 mRNA expression in glial cells, however, to rule out a direct role of compensatory mechanisms that are in response to the large increases in compared to WT mice. These reductions in and no differences in hippocampus (Schnellet al., 2014) (Fig. 3A), and extends these signaling may also contribute to alterations in periodicity of gene expression (DeBruyne et al., 2015). Future studies determining the cell-type specificity of these observations are also needed to better understand lipid-mediated signaling cascades (Gooley and Chua, 2014; Gooley, 2016) downstream of circadian- and metabolically (Buggeet al., 2012; Kumar Jha et al., 2015; Bass and Takahashi, 2010; Eckel-Mahan and Sassone-Corsi, 2013) driven changes in Rev-erb expression both within and between neurons and glia.

Understanding the molecular and cellular components that regulate Fabp7 expression will have important implications for public health. For example, pathological states associated with Fabp7 overexpression exist for a variety of diseases, including multiple types of cancer (Zhout al., 2015; Liet al., 2012; Mita et al., 2010; Corderoet al., 2019; Kagawaet al., 2019; Maet al., 2018), and neurodegenerative disease, including Alzheimer’s disease (Teunissenet al., 2011; Johnsenet al., 2018). Given the role of the circadian clock in cancer (Masri and Sassone-Corsi, 2018; Sulliet al., 2018, 2019) and neurodegeneration (Musiek and Holtzman, 2016; Hood and Amir, 2017; Lanannaet al., 2020), future studies determining the role in how circadian Fabp7 and Fabp7 lipid-signaling may feedback onto metabolic (Choet al., 2012; Bass and Takahashi, 2010; Panda, 2016) and inflammatory pathways (Carteret al., 2016; Schierermann et al., 2013; Castanon-Cervanteset al., 2010) may provide novel links between clock-regulated mechanisms, fatty-acid pathways, and disease.

4. Materials and methods

Animals. The Rev-erb knock out (KO) mice were obtained from B. Vennström and were backcrossed for >7 generations with C57/B6 mice. Mice (N = 3–7 per group) were housed under standard 12h-light/12h-dark (LD) cycles and were sacrificed at specific times (zeitgeber time (ZT) 2, 6, 10, 14, 18, 22 with ZT0 corresponding to 7 a.m.). Animal care and use procedures followed the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania in accordance with the guidelines of the US National Institutes of Health.

Chromatin immunoprecipitation (ChIP). ChIP experiments were performed as previously described (Fenget al., 2011) with minor changes. Mouse brain tissue was harvested at ZT10, minced and cross-linked in 1% formaldehyde for 20min, followed by quenching with 1/20 volume of 2.5M glycine solution for 5 min, and then two washes with PBS. Cell lysates with fragmented chromatin were prepared by probe sonication in ChIP dilution buffer (50 mM HEPES, 155 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a complete protease inhibitor tablet (pH 7.5)). Proteins were immunoprecipitated in ChIP dilution buffer, using 1 µg of Rev-erb antibody (Cell signaling). Cross-linking was reversed overnight at 65 ºC in elution buffer (50 mM Tris-HCL, 10 mM EDTA, 1% SDS, pH8), and DNA isolated using phenol/chloroform/isoamyl alcohol. Precipitated DNA was analyzed by quantitative PCR or high-throughput sequencing.

ChIP-qPCR. Precipitated DNA was analyzed by quantitative PCR, using the following primers: Fabp7, forward: 5’-GGG GAT CAG GAT TGT GAT GT-3’; Fabp7, reverse: 5’-AGA TGG CTCCAA TCC TCC TCT-3’; Arbp, forward: 5’-CTG GGA CGA TGA ATG AGG AT-3’; Arbp, reverse: 5’-AGC AGG TGG CAC CTA AAG AG-3’; Npas2, forward: 5’-TGG CAG AAG CTT GGG AAA AG-3’; Npas2, reverse: 5’T-CTT CCT GTG GGA GGA GAC AG-3’.

ChIP-seq and districome analysis. For ChIP-seq, material from three mice was pooled prior to library generation. ChIP DNA was prepared for sequencing according to the amplification protocol provided by Illumina, using adaptor oligo and primers from Illumina, enzymes from New England Biolabs and PCR Purification Kit and MinElute Kit from Qiagen. Deep sequencing was performed by the Functional Genomics Core (J. Schug and K. Kaestner) of the Penn Diabetes Endocrinology Research Center using the Illumina HiSeq2000, and sequences were obtained using the Solexa Analysis Pipeline. Sequenced reads were aligned to the mouse genome.
reference genome (mm9) and peak calling was performed with HOMER (Heinzen et al., 2010). ChIP-seq data are deposited in NCBI GEO GSE67973 (Zhang et al., 2015), for GSM1659684 and GSM1659685 datasets.

4.1. MEME package

Analysis of the Fabp7 promoter was done using the MEME package (http://meme.nbcr.net/meme/). 2000 base pairs upstream and 2000 base pairs downstream of the murine Fabp7 transcription start site (TSS) was used for promoter analysis. Reference to site location of cis-elements were expressed 0–4000, with 2000 being at the TSS.

4.2. GO and KEGG analysis

Gene ontology analysis was performed on the ranked list of Rev-erbα ChIP-seq genes with peak score >2 [SUPPLEMENTAL dataset 1], using Panther GO-Slim against the mouse gene list (http://geneontology.org release 2021-01-01: 44,091; (Ashburner et al., 2000; The Gene Ontology resource, 2021). Top non-redundant categories are presented.

KEGG pathway analysis was performed on the same gene list using KEGG Mapper https://www.genome.jp/kegg/tool/map_pathway1.html (Kanehisa and Sato, 2020) against mouse pathways.

4.3. qPCR

Total RNA was extracted from tissue using the RNeasy Mini Kit (QIAGEN) and treated with DNase (QIAGEN). The RNA was reversed transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and analyzed by quantitative PCR. Quantitative PCR was performed with Power SYBR Green PCR Mastermix on the PRISM 7500 (Applied Biosystems). Gene expression was normalized to mRNA levels of housekeeping gene 36B4 and the level of the gene of interest in the control samples. Circadian oscillations in gene expression were calculated using JTK_cyclev3.1 scripts (Hughes et al., 2010) run on R. Amplitude confidence intervals were calculated according to Miyazaki et al. (2011).

4.4. Primers

| Gene   | Forward Primer                     | Reverse Primer          |
|--------|------------------------------------|-------------------------|
| Fabp3  | TCC-AGG-CTT-TGG-GCA-TCA            |                         |
| Fabp5  | CTT-TAT-CAG-CTG-CAC-ATC-CTG-A      | Fabp3                   |
| Fabp7  | GGC-AGG-TCA-CGG-CTC-CTT            |                         |
| Fabp5  | CGA-CAG-ATG-GCA-GAA-AAA            |                         |
| Fabp5  | GAC-CAG-GGC-ACC-GTC-TTG            |                         |
| Fabp7  | TTC-CTG-CTC-GAT-AAT-CAG-AGT-TGG   |                         |

Fig. 4. A–C: Relative hippocampal mRNA expression of various Fabps in Rev-erbα KO vs. WT mice under normal (LD) conditions. Levels of Fabp3 (A) or Fabp5. (B) are unaffected by Rev-erbα deficiency, however, Fabp7 shows a significant increase in expression based on genotype. *p < 0.001, N = 4–7 per group, Student’s t-test. ZT = zeitgeber time. Hippocampal mRNA expression normalized to genome to visualize the circadian rhythmicity of Fabp3 (D), Fabp5 (E), and Fabp7 (F). Fabp7 circadian rhythmicity was significantly disrupted in Rev-erbα KO mice (adj. p = 0.184; JTK_Cycle) compared to WT mice (adj. p < 0.001; JTK_Cycle).
Funding

This work was supported by National Institute of Heath grant R35GM133440 to J.R.G.

CRediT author statement

William M. Vanderheyden: Formal analysis, Writing – Review & Editing, Visualization. Bin Fang: Formal analysis, Investigation, Data Curation, Resources, Writing – Review & Editing, Visualization. Carlos C. Flores: Formal analysis, Investigation, Data Curation, Resources, Writing – Review & Editing, Visualization. Jennifer Jager: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing – Review & Editing, Visualization. Jason R. Gerstner: Conceptualization, Methodology, Resources, Writing – Original Draft, Writing – Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Dr. A. Pack and the UPENN Center for Sleep and Circadian Neurobiology and Dr. M. Lazar and the UPENN Institute for Diabetes, Obesity, and Metabolism for advice and support.

Appendix A. Peer Review Overview and Supplementary data

A Peer Review Overview and (sometimes) Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.crneur.2021.100009.

References

Albrecht, U., 2012. Timing to perfection: the biology of central and peripheral circadian clocks. Neuron 74, 246–260.
Ashburner, M., et al., 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25, 25–29.
Bass, J., Takahashi, J.S., 2010. Circadian integration of metabolism and energetics. Science 330, 1349–1354.
Bugge, A., et al., 2012. Rev-erbalpha and Rev-erbbeta coordinately protect the circadian clock and normal metabolic function. Genes Dev. 26, 657–667.
Carter, S.J., et al., 2016. A matter of time: study of circadian clocks and their role in inflammation. J. Leukoc. Biol. 99, 549–560.
Castanon-Cervantes, O., et al., 2010. Dysregulation of inflammatory responses by chronic circadian disruption. J. Immunol. 185, 5796–5805.
