The role of deubiquitinase USP2 in modulating behavior and circadian light response in mice

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This thesis is dedicated to all the mice that were used in this project.

Science is all the richer for your contribution.

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The journey of a PhD graduate is akin to a child growing up. You start off clueless, knowing very little and needing a lot of supervision, and by the end, you are somewhat competent, pretty independent and are ready to move out of the lab, to newer avenues! However, the person you are at the end of this winding road is hugely influenced all the people who you interact with and all your experiences along the way and it would be amiss to not acknowledge all the positive influences that shaped this journey.

In this analogy, the supervisor is obviously the parent, and I can't stress how well my supervisor, Dr. Nicolas Cermakian, handles this role [1–6]! He has always encouraged ideas, experiments and my crazy thought processes, while at the same time keeping me grounded by insisting on sound basics, strong rationales and by intense discussions on the subject. Thanks Nico, for taking a chance on me 5 years ago, for allowing me to carry out amazing research with the freedom I have always desired, for pushing me to be a better, detail-oriented scientist, for all the patience, corrections and umpteen versions of each document and for joining me on this roller-coaster ride of a PhD!

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Abstract

Circadian rhythms, with a periodicity of approximately 24 hours, regulate various aspects of physiology, such as sleep and body temperature. The suprachiasmatic nucleus (SCN) is the body's master pacemaker that maintains the phase relationship between the different cells and organs. These rhythms are controlled by transcription-translation negative feedback loops of clock genes and proteins. Post-translational modifications (PTMs) such as ubiquitination play an essential role in maintaining the rhythmicity of clock proteins. Ubiquitin specific peptidase 2 (USP2) is a deubiquitinase that interacts with multiple clock proteins and affects photic entrainment. Further, USP2 shows widespread expression across the brain. This thesis investigates the roles of USP2 in the brain and how it modulates the light response of the clock.

We assayed the role of USP2 in the brain by subjecting mice lacking USP2 (*Usp2* KO) and WT mice to a battery of behavioral tests related to the areas of the brain showing highest Usp2 expression. We found that *Usp2* KO mice showed reduced motor coordination and reduced anxiety-like behaviors. Further, we found a reduction in recognition memory, but not spatial memory, in *Usp2* KO mice. We also assayed pre-pulse inhibition, but the effects in the *Usp2* KO mice were mild. Therefore, USP2 substantially regulates animal behavior.

Having determined the effect of USP2 on non-circadian behaviors, we looked at the effects USP2 on circadian behaviors. Based on previous data from our lab, we constructed a phase response curve focussing on the early night. In *Usp2* KO mice, early-night entrainment as well as entrainment at the day-night transition were affected. To further study the role of USP2 in in the early night, we used a modified skeleton

photoperiod protocol (mSPP) and found that *Usp2* KO mice entrained better to both phase-delays and advances in the mSPP. Thus, USP2 controls fidelity of entrainment to early night light exposure.

To localize USP2 action, we injected a Cre-expressing viral construct into the SCNs of *Usp2* floxed mice to knockout Usp2 specifically in the SCN. Strikingly, their entrainment to the mSPP recapitulated the entrainment patterns of *Usp2* KO mice. To understand the effect of *Usp2* on entrainment within the clock, we observed *Per1* expression in the SCN in response to the mSPP light pulse. In *Usp2* KOs, *Per1* induction was abrogated, showing a molecular correlate for the differences in entrainment. Since photic input to the clock is also informed by the retina, we checked intrinsically photosensitive retinal ganglionic cell (ipRGC) function by testing pupillary light response. We found no differences between genotypes, which further supports a role of USP2 specifically in the SCN. Since ipRGCs innervate the ventral SCN, which contains VIP-ergic neurons, we knocked out Usp2 in these cells. These KO mice showed no differences in entrainment to mSPP compared to WTs. Further, *Usp2* expression seemed to be even across the SCN.

Hence, to understand how USP2 affects light response in the whole SCN, we exposed *Usp2* KO and WT mice to an early-night light pulse and subjected the SCN lysates to shotgun proteomics. Over 3000 proteins were detected and quantified, of which 173 proteins were differentially expressed between the genotypes exclusively in the light-pulsed condition. Among these, pathways for ERK, EIF2 and mTOR cascades were enriched. Using IHC, we showed that phosphorylation of ERK and EIF2α were

differentially affected between the genotypes, pointing towards the pathways affected by USP2.

In conclusion, we showed that USP2 has a significant role in maintaining circadian and non-circadian behaviors. Our work is the first to characterize the specific role and molecular underpinnings of a deubiquitinase in the process of entrainment in mammals, and this opens avenues to study PTMs upstream of the clock as an additional layer of control on entrainment.

Resumé

Les rythmes circadiens, d'une période d'environ 24 heures, régulent plusieurs aspects de la physiologie. Le noyau suprachiasmatique (NSC) est l'horloge principale maintenant la phase entre les différents organes et cellules. Ces rythmes sont contrôlés par des boucles de rétroaction de transcription-traduction de gènes et protéines. Les modifications post-traductionnelles (PTM), telles que l'ubiquitination jouent un rôle essentiel dans le maintien de la rythmicité de l'horloge. L'ubiquitine peptidase spécifique 2 (USP2) est une déubiquitinase (DUB) interagissant avec plusieurs protéines de l'horloge et pouvant affecter l'entraînement, c'est-à-dire la capacité d'un organisme à s'adapter à des changements des cycles de lumière. Cette thèse étudie les rôles d'USP2 dans le cerveau et sa capacité à moduler la réponse circadienne à la lumière.

Le rôle d'USP2 dans le cerveau fut évalué en soumettant des souris déficientes en USP2 (*Usp2* KO) et des souris contrôles (WT) à des tests comportementaux liés aux régions exprimant fortement *Usp2*. Les souris *Usp2* KO montrent des comportements moteurs, anxieux et de reconnaissance mnémonique réduits, mais une mémoire spatiale et des comportements liés au striatum comparables aux WT. Ainsi, USP2 régule substantielle le comportement.

Après avoir déterminé l'effet d'USP2 sur les comportements non circadiens, l'effet d'USP2 sur les comportements circadiens a été étudié. Notre laboratoire ayant démontré qu'USP2 a un effet sur l'entraînement en début de nuit, une courbe de réponse de phase axée sur cette période a donc été construite. En absence d'USP2, l'entraînement en début de nuit ainsi qu'à la transition jour-nuit sont affectés. Afin d'étudier davantage USP2 en début de nuit, un protocole de photopériode squelettique modifié (mSPP) a été

développé et a démontré un meilleur entraînement aux retards et aux avances de phase pour les souris *Usp2* KO. USP2 contrôle donc la robustesse de l'entraînement suite à la lumière en début de nuit.

Pour localiser son action, USP2 fut éliminé uniquement dans le NSC via un système Cre-Lox, résultant en un entraînement similaire aux *Usp2* KO. Pour comprendre l'effet des *Usp2* KO sur l'entraînement, l'expression de *Per1* dans le NSC a été étudiée avant et après une pulsion lumineuse. En l'absence d'*Usp2*, l'induction de *Per1* à été abolie, ce qu pourrait expliquer les différences d'entraînement entre génotypes. Le contrôle photique du NSC étant médié par la rétine, nous avons vérifié la fonction des cellules ganglionnaires rétiniennes intrinsèquement photosensibles (ipRGC) en utilisant le réflexe pupillaire, et aucune différence n'a été trouvée. Puisque les ipRGC innervent principalement la région ventro-latérale du NSC, contenant majoritairement des neurones VIP, *Usp2* a été éliminé spécifiquement dans ces cellules, menant à un entraînement similaire aux WT.

Une analyse d'expression a prouvé qu'USP2 est exprimé partout dans le NSC. Pour comprendre comment la réponse à la lumière dans l'ensemble du NSC est liée à USP2, des souris *Usp2* KO et WT ont été soumisse à une stimulation lumineuse en début de nuit alors que d'autres sont restées dans le noir. Une étude protéomique du NSC a détecté 107 protéines différemment exprimées entre les génotypes après la stimulation lumineuse. Parmi celles-ci, les cascades comprenant ERK, eIF2 et mTOR étaient enrichies et l'effet du génotype sur la phosphorylation d'ERK et eIF2α fut confirmé par IHC.

En conclusion, nous avons montré qu'USP2 joue un rôle important dans le maintien des comportements circadiens et non circadiens. USP2 affecte l'entraînement photique en modulant les voies moléculaires impliquées dans la réponse à la lumière du NSC. Ce travail est le premier à caractériser le rôle spécifique et les fondements moléculaires d'une DUB dans le processus d'entraînement chez les mammifères. Notre étude ouvre des avenues pour étudier les PTM dans l'horloge en tant que contrôle de son entraînement.

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12:12 LD	12 hours: 12 hours light-dark cycle
4EBP	Elongation factor 4E binding protein
AAV	Adeno-associated virus
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor
ANOVA	Analysis of variance
ASPP	Phase advance with modified skeleton photoperiod
AVP	Arginine vasopressin peptide
BMAL1	Bone and muscle ARNT-like 1
Ca2+	Calcium ion
CALB1	Calbindin 1
CAMK2	Ca2+/Calmodulin-dependent protein kinase 2
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CLOCK	Circadian locomotor output cycles kaput
CREB	cAMP-response element binding protein
CRY	Cryptochrome
CT	Circadian time
DAPI	4',6-diamidino-2-phenylindole
dB	Decibels
DBP	D-site binding protein
DD	Constant darkness
DEC	Differentially expressed in chondrocytes
DIG	Digoxigenin
DR	Discrimination ratio
DSPP	Phase delay with modified skeleton photoperiod
DUB	Deubiquitinase
E4BP4	E4-promoter binding protein 4
EIF2	Elongation initiation factor 2
EIF2α	Elongation initiation factor 2, α subunit
EIF4E	Elongation initiation factor 4E
EPM	Elevated plus maze

ERK	Extracellular signal-regulated kinase
GABA	Gamma amino butyric acid
(e)GFP	(Enhanced) Green fluorescent protein
GRIA	Glutamate receptor, ionotropic, AMPA
GRIK	Glutamate receptor, ionotropic, kainate
GRIN	Glutamate receptor, ionotropic
GRP	Gastrin releasing peptide
IGL	Intergeniculate nucleus
IHC	Immunohistochemistry
IPA	Ingenuity pathway analysis
ipRGC	Intrinsically photosensitive retinal ganglionic cells
IS	Interdaily stability
ISH	In-situ hybridization
kDa	Kilo Dalton
KO	Knockout
LC-MS	Liquid Chromatography with tandem mass spectrometry
LP	Light pulse
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
MSK1	Mitogen- and stress-activated protein kinase 1
mSPP	Modified skeleton photoperiod
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MWM	Morris water maze
N.S.	Not significant
NLP	No light pulse
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NMS	Neuromedin S
NOR	Novel object recognition test
NSF	Novelty supressed feeding test
OFT	Open field test
OPN	Olivary pretectal nucleus
OPN1LW	Opsin 1 long-wave

OPN1MW	Opsin 1 medium-wave
OPN1SW	Opsin 1 short-wave
OPN2	Opsin 2, also called rhodopsin
OPN4	Opsin 4, also called melanopsin
PAC1R	PACAP type 1 receptor
PACAP	Pituitary adenylate-cyclase-activating polypeptide
PAE	Phase angle of entrainment
pEIF2α	Phosphorylated elongation initiation factor 2, α subunit
PER	Period
pERK	Phosphorylated extracellular signal-regulated kinase
PI3K	Phosphoinositide 3-kinase
PLR	Pupillary light reflex
PP2A	Protein phosphatase 2A
PPI	Prepulse inhibition assay
PRC	Phase response curve
pS6	phosphorylated S6 kinase
PTM	Post-translational modification
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
REV-ERB	Reverse erythroblastosis virus proteins
RHT	Retinohypothalamic tract
ROR	RAR-related orphan receptors
RPMPM	Rotations per minute per minute
RPS6KA1	Ribosomal protein S6 kinase A1
S6	S6 kinase
SCN	Supra chiasmatic nucleus
SD	Standard deviation
SEM	Standard error of mean
SPP	Skeleton photoperiod
TTFL	Transcription-translation feedback loop
USP	Unique selling point (exclusively for the pun on page 194)
USP2	Ubiquitin specific peptidase 2
VIP	Vasoactive intestinal peptide
WT	Wildtype

Contribution to original knowledge

Publications

- Authored a review article on the roles of ubiquitin ligases and deubiquitinases on the circadian clock, published in Journal of Neurochemistry, in 2021.

  “Srikanta SB, Cermakian N. To Ub or not to Ub: Regulation of circadian clocks by ubiquitination and deubiquitination. Journal of Neurochemistry. Wiley; 2021. pp. 11–30. doi:10.1111/jnc.15132”
- Second author of a review article on the different deubiquitinases involved in the circadian clock, published in the American Journal of Physiology – Cell Physiology

  “Harris-Gauthier N, Srikanta SB, Cermakian N. Deubiquitinases: key regulators of the circadian clock. Am J Physiol Cell Physiol. 2022;323: C1539–C1547. doi:10.1152/ajpcell.00289.2022”
- Co-first author on a collaborative project investigating the long-term effects of dim light at night and social jet lag on circadian and non-circadian behaviors.

  “Delorme TC, Srikanta SB, Fisk AS, Cloutier ME, Sato M, Pothecary CA, et al. Chronic Exposure to Dim Light at Night or Irregular Lighting Conditions Impact Circadian Behavior, Motor Coordination, and Neuronal Morphology. Front Neurosci. 2022;16. doi:10.3389/fnins.2022.855154”
- First author of a scientific article investigating the role of deubiquitinase USP2 in modulating circadian and non-circadian behaviors.

  “Srikanta SB, Stojkovic K, Cermakian N. Behavioral phenotyping of mice lacking the deubiquitinase USP2. Oster H, editor. PLoS One. 2021;16: e0241403. doi: 10.1371/journal.pone.0241403”

- Two manuscripts investigating the mechanisms of action of deubiquitinase USP2 in modulating photic entrainment, to be submitted in 2023.

Chapter 2:

- Showed that USP2 is involved in maintenance of motor coordination in mice.
- Showed that USP2 is involved in the regulation of anxiety-like behavior in mice.
- Showed that USP2 is involved in maintaining normal recognition memory, in mice.
- Published these results in *PLoS ONE*.

Chapter 3:

- Established that USP2 acts in the early night, to modulate entrainment.
- Established the SCN as the site for USP2's action in modulating entrainment.
- Showed that USP2 is required for photic induction of light-responsive clock gene, hence, for the first time, pointing towards the specific pathways affected by USP2 action, to modulate entrainment.
- Developed a new photic phase shifting protocol to delineate effects of direction of phase shift from timing of phase shift.

Chapter 4:

- Created a profile of *Usp2* expression in various regions within the SCN.
- Discovered and validated the specific pathways affected by USP2, to modulate entrainment.
- The first report to establish a role for deubiquitinases in light-response pathways.

Contribution of authors

Chapter 2

S.B.S. carried out the wheel running experiments, NSF, NOR, PPI, rotarod and most iterations of the EPM experiment and was involved in data curation, formal analysis, validation, visualization, writing, revising and editing of the manuscript

K.S. carried out the MWM and some iterations of the EPM experiment and was involved in data curation, investigation and editing of the manuscript.

N.C. was involved in the conceptualization of the study, funding acquisition, project administration, supervision, writing, revising and editing of the manuscript.

Chapter 3

S.B.S. was involved in conceptualization of the study, conducted all the wheel-running experiments, intracranial surgeries, ISH, tissue collection, microscopy and performed the data curation, formal analysis, validation, visualization, writing, revising and editing of the manuscript.

A.M. and R.R. conducted and analyzed the PLR experiment.

L.Z. and K-F.S. provided assistance and reagents for the ISH experiment.

C.C. and O.D.B. carried out the RNA extraction of retina tissues and validated and carried out the qRT-PCRs of retina tissues as well.

N.C. was involved in the conceptualization of the study, funding acquisition, project administration, supervision, writing, revising and editing of the manuscript.

Chapter 4

S.B.S. conducted all the wheel-running experiments, IHCs and performed the data curation, formal analysis, validation, visualization, writing, revising and editing of the manuscript.

V.R. and R.Z. carried out protein lysate preparation and LC-MS/MS experiments (along with analysis)

M.E.C. collected and processed brain tissue and carried out the RNAscope fluorescent in-situ hybridization.

M.M. assisted with brain tissue collection and processing and carrying out IHC.

N.C. was involved in the conceptualization of the study, funding acquisition, project administration, supervision, writing, revising and editing of the manuscript.

Chapter 1: Introduction, rationale and objectives of the thesis

Introduction

“The whole universe is based on rhythms.

Everything happens in circles, in spirals.”

-John Hartford

One of the constants in my PhD journey has been music. There is a sense of calmness to my experiments when I am listening to certain songs while working; I have observed that the constant rhythmicity of the beats of these songs help me focus better on my work. However, I don’t think my cognizance and appreciation of constant rhythmicity is anything out of the ordinary! Constant rhythmicity is something ubiquitous in this world and it affects most spheres of our daily lives, from the division of cells to the change in seasons. Among all the rhythms, the most noticeable and forceful rhythm in the world, is the daily, 24-hour-long day-night cycle. Ever since the Earth came to be, as a cloud of gases orbiting the sun, it has been rotating around its axis. By the time the first multicellular forms of life came to be, less than 1 billion years ago, the planet had a rotational period between 22 and 24 hours. This has meant that modern life forms have mostly evolved within the constraints of an approximately 22- to 24-hour day. Hence, it is not surprising that most organisms have an internal rhythm-maintaining system with a period between 23 and 24 hours, which keeps them synchronized to the periodicity of the external environment.

1.1. Circadian rhythms: an introduction

The endogenous rhythms in living organisms with a period close to 24 hours are called circadian rhythms (from the Latin words *circa* = approximately, *diem* = day) [7]. These remarkably stable endogenous oscillations are involved in the control of most aspects of an organism's physiology [8]. Additionally, these rhythms can adapt or entrain to external stimuli such as light, nutrient availability or temperature, thus shifting the phase of the rhythm, to match the external environment.

In most organisms, the circadian system consists of a transcription-translation negative feedback loop of clock genes and proteins, with a period of 24 hours. However, the specific clock proteins and mechanisms vary between different classes of organisms. For instance, the plant clock, primarily studied in *Arabidopsis thaliana*, consists of clock proteins specifically expressed in the morning, afternoon and evening. Each successive set of proteins repress the proteins of the previous phase and activate the proteins in the next phase, to create a TTFL [9,10]. The fungal clock, one of the first characterized circadian systems, which is studied using the model organism *Neurospora crassa*, on the other hand, is a very simple loop with only four known clock genes and proteins [11]. The insect and mammalian clocks are two other extensively studied clock systems. Both systems consist of two interconnected feedback loops, which together form the TTFL. As the above examples show, there is a diversity of clocks in the living world, each more fascinating than the next! However, in this thesis, we will be concentrating on mammalian clock.

1.2. The mammalian circadian system

1.2.1. Organization of the circadian cycle

The primary model organisms for study of the mammalian circadian system have been the house mouse (*Mus musculus*), the brown rat (*Rattus norvegicus*) and the Syrian hamster (*Mesocricetus auratus*).

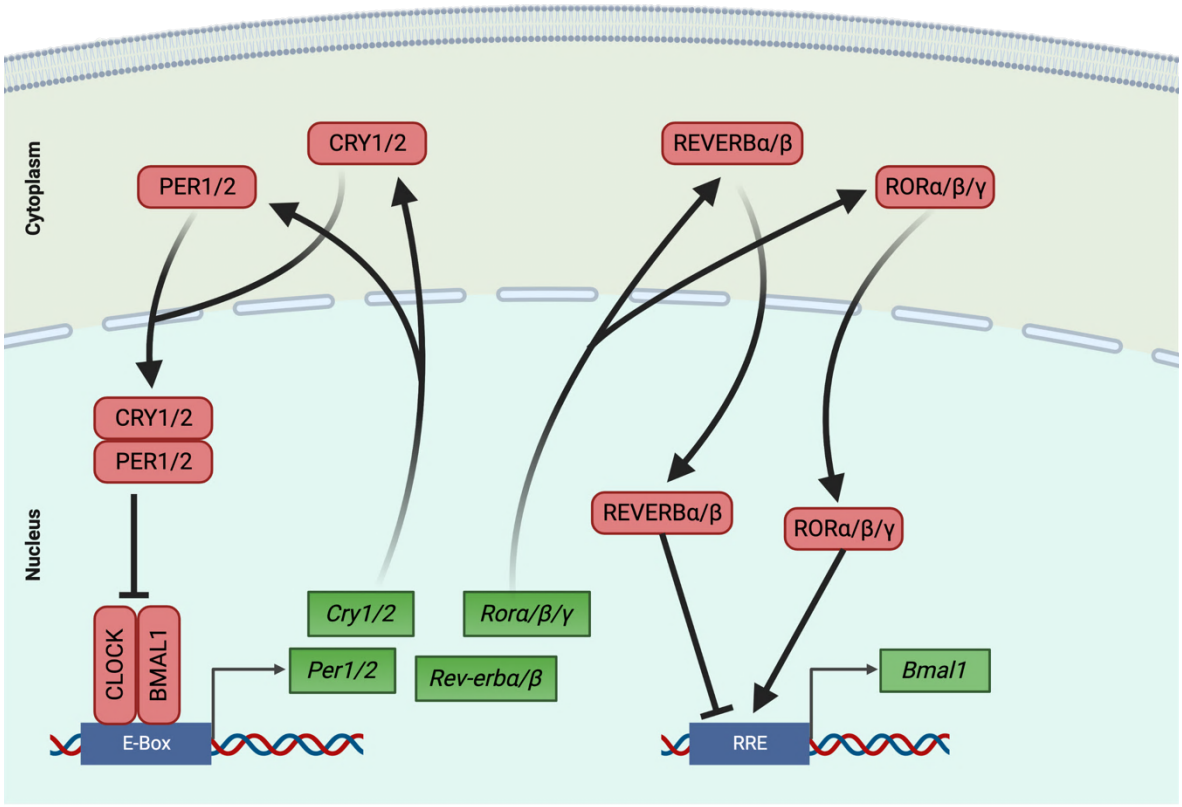

Figure 1: The primary feedback loops of the mammalian circadian clock*. (Created with BioRender.com)*

Figure 1 is a simplified schematic of the primary feedback loops driving the mammalian circadian cycle. Briefly, “In the mammalian clock, the transcription factors

Circadian Locomotor Output Cycles Kaput (CLOCK) [12] and Brain and Muscle ARNT-Like 1 (BMAL1) together bind E-box elements upstream of the Period (*Per1, 2, 3*) and Cryptochrome (*Cry1, 2*) genes, activating their transcription. After translation, the PER [13,14] and CRY [14–17] proteins translocate into the nucleus, interact with each other and suppress the activity of the CLOCK/BMAL1 complex, reducing the transcription of *Per* and *Cry* and hence completing one 24-hr cycle of the circadian clock. This main feedback loop is flanked by subsidiary feedback loops such as those of reverse erythroblastosis virus proteins (REV-ERBs) [18,19] and RAR-related orphan receptors (RORs) [19,20]. The genes encoding these proteins are transcribed by the CLOCK/BMAL1 complex. While the REV-ERBs inhibit *Bmal1* expression, the RORs activate it. All these proteins also have targets outside the clock machinery. Because of the circadian patterns of expression and activity of clock transcription factors, their downstream targets can also be expressed in a circadian fashion. Such genes are called clock-controlled genes [21]. These clock-controlled genes have a circadian pattern of expression and have short half-lives, but they are not a part of the circadian clock."[22]

1.2.2. Localization of the circadian system

"Most cell types in the body express clock genes and therefore have the capacity to generate autonomous circadian rhythms. A master clock, present in a brain region called the suprachiasmatic nucleus (SCN), synchronizes the other body clocks via various neuronal, humoral, and systemic cues." [8,22,23] In order to maintain the phase relationships between the different clocks in the body, the SCN projects to many regions all over the brain and controls various processes like hormonal rhythms, temperature

rhythms, appetite, blood pressure and the sleep-wake cycle [24,25]. In return, the SCN is innervated by different regions of the brain as well [26]. The SCN itself is a small and dense cluster of cells (20,000 cells in rodents and 100,000 cells in humans) in the anterior hypothalamus [7,23]. The necessity of the SCN for mammalian timekeeping was shown by surgical ablation. Multiple studies have shown a loss of locomotor rhythms, feeding and drinking rhythms in the absence of the SCN [27,28]. Further, studies that grafted neonatal SCN tissues into SCN-ablated animals showed the sufficiency of SCN to generate circadian rhythms in mammals [29,30]. Together, the SCN is necessary and sufficient to maintain circadian rhythmicity in mammals.

The neurons within the SCN express a variety of neuropeptides such as vasoactive intestinal peptide (VIP), arginine vasopressin peptide (AVP), gastrin releasing peptide (GRP), and Cholecystokinin (CCK) [31,32]. However, most neurons in the SCN are GABA-ergic. Apart from GABA, based on the other predominantly expressed neuropeptides, the SCN is divided into the dorso-medial “shell” region, which primarily expresses AVP and the ventro-lateral “core” which is populated by GRP and VIP [31,33,34]. Within the core, most of the neurons express VIP and are localized more to the ventral side, while the dorsal part is populated predominantly GRP-expressing neurons [33,35,36]. The AVP-expressing SCN shell shows strong cell to cell coupling and high amplitude clock rhythms [34]. The cells in this region are responsible for the maintenance of the regular periodicity of circadian rhythms in mammals [37]. The core, on the other hand, shows relatively damped rhythms and is innervated by glutamatergic projections from the retina [31]. In order to facilitate entrainment, VIP signalling reduces synchrony between cells, allowing easier phase shifting of each cell. Hence, VIP-ergic

projections from the core innervate both, the core and the shell, leading to entrainment of the whole SCN and eventually, the organism.

1.2.3. Photic transduction in mammals

1.2.3.1. Physiology of the eye

The process of photic entrainment starts in the eye. The eye contains the retina, a photoreceptive structure. Studies have shown that different cell types in the retina also have circadian rhythms. However, studying circadian rhythms of the whole retina shows damped rhythms. Currently, this is assumed to be a result of different retinal layers having different phases of clock genes [38,39] or the result of some cells in the retina just not having rhythms [40]. The retina is made up of over 60 different types of cells, which are divided among three major zones [41].

At the back of the eye, the rods and cones, which detect light intensity and color information, respectively, make up the photoreceptor layer. The rods are marked by the expression of rhodopsin (RHO or OPN2) and detect the brightness of the image, as well as detect very low levels of light. Cones have 3 distinct types, based on the opsin they express. The S-opsin (OPN1SW) detects shorter wavelengths of light like violet and blue, the M-opsin (OPN1MW) detects intermediate wavelengths like green, while the L-opsin (OPN1LW) detects longer wavelengths like red [17,42–44]. Different animals see different color sets, based on the expressed cone-opsins. For example, since rodents don't express OPN1LW, their sensitivity to red light is extremely low [45].

The horizontal, bipolar and amacrine neurons in the intermediate layer transfer the photic information to the outermost layer, which is made up of retinal ganglionic cells (RGCs) [46].

The RGCs transfer photic information from the retina to the visual centers of the brain. Among the RGCs, ~1% of the cells are intrinsically photosensitive RGCs (ipRGCs) and are involved in non-image-forming responses [47]. These ipRGC respond directly to presence and intensities of light. The ipRGCs, being photoreceptors, express melanopsin (OPN4) [41]. There are multiple subtypes of ipRGCs, based on the morphology of the cells, as well as the specific proteins they express [48].

1.2.3.2. Transduction of photic information from the eye to the brain

The axons of all the ipRGCs project via the optic nerve, to the brain [41]. The point where the two optic nerves meet is called the optic chiasm. The SCN is located directly above this meeting point and hence derives its name (from Latin, *supra* = above). The ipRGC innervations to the SCN are glutamatergic in nature and predominantly synapse with VIP-expressing neurons in the core of the SCN [49]. Treating *ex-vivo* organotypic SCN slices with NMDA, a glutamate receptor agonist, mimics the responses to light seen *in-vivo* [50], thus showing the sufficiency of glutamate signalling for photic information transfer to the SCN. Additionally, these neurons also release the neurotransmitter pituitary adenylate cyclase activating polypeptide (PACAP), which is structurally very similar to VIP [51]. PACAP knockout mice show normal circadian rhythms but blunted phase shifting responses [52]. Thus, photic information is transferred to the core of the SCN by the combination of glutamate and PACAP signalling.

From the optic chiasm, the ipRGCs project to the habenula (center involved in mood control), lateral geniculate nucleus, the intergeniculate nucleus, then the olivary pretectal nucleus (OPN) and finally to the visual cortex via the superior colliculus [41,53].

The OPN mediates the response of the eye to bright lights by controlling the constriction of the pupil in response to light intensity. This physiological response is, unimaginatively, called the pupillary light constriction response (PLR) [54]. While the light information for PLR is transmitted from the eye to the OPN via the ipRGCs as well, the subtypes of ipRGCs that project to the SCN and those that are involved in PLR are different from each other [55].

1.2.4. Photic entrainment in mammals

Why is photic transduction to the SCN important? The solar day-night cycle is 24-hours long, while the endogenous clock has a period slightly different from 24-hours. Thus, to be well synchronized to the external environment on a daily basis, the mammalian circadian system needs to be adaptable to or entrained by environmental light information on a daily basis. The circadian system can also be entrained by environmental factors such as temperature [56], food availability [57], exercise [58] or by administering exogenous compounds such as melatonin [59–61] or caffeine [62–64]. However, light is the strongest entraining stimulus or “Zeitgeber” (from German, *Zeit*: time, *Geber*: giver) for the SCN clock, superseding the other entrainment stimuli[65,66].

1.2.4.1. Types of photic entrainment

Let us take a closer look at photic entrainment! At the level of the organism, photic entrainment can be broadly divided into two classes: parametric and non-parametric. Parametric entrainment refers to the ability of an animal to entrain to a cycle that has a long light stimulus, followed by a long dark phase. A 12 h :12 h light:dark (12:12 LD) cycle is one such example. Light, as a strong zeitgeber, can inhibit or mask activity in nocturnal animals [7,65], so that they aren't active at a phase detrimental to their survival. The inhibition of locomotor activity by light is called "masking". By having a 12:12 LD cycle all the activity of a nocturnal animal can be restricted to the imposed dark phase due to masking in the imposed daytime, resulting in the overall "speed" of the clock being changed to match the LD cycle. This results in the activity patterns (and hence, the circadian cycle) oscillating with a period of 24 hours, rather than with the endogenous period of the animal. Thus, by subjecting an animal to a parametric cycle with a period of 24 hours, the period of the clock is being modulated on a daily basis to be 24 hours as well. The start and end of the 12:12 LD cycle can be changed, to simulate jetlag-like conditions, to shift the phase of the clock (phase-shifting response) [67]. While masking, biologically, allows for parametric entrainment and hence, synchronization to the external light-dark cycle, it is less helpful for scientific studies. In phase shifting studies, it might sometimes be hard, to understand whether the clock has phase-shifted and entrained or if the clock hasn't entrained to the new LD cycle, but rather, the activity is being masked by light [67].

Non-parametric entrainment on the other hand, uses smaller pulses of light to discretely shift the clock's phase. Unlike a parametric lighting condition, the absence of

light during the subjective daytime prevents masking, which allows us to fully visualize the activity rhythms and hence clock entrainment. Considering the abundance of light during the daytime, the circadian clock has evolved to be insensitive to light in the daytime. Light at night, however, has the ability to shift the clock. Taking the example of a burrowing nocturnal rodent, these phase shifts can easily be understood. If the rodent emerges too early in the evening, the circadian system needs to be delayed the following day. Thus, light in the early part of the night delays the clock, so that the subsequent cycles start later. However, if the rodent stays out too late, the circadian cycle needs to be advanced so the rodent can go back to its burrow earlier. Hence, light towards the end of the night advances the clock so that the subsequent circadian cycles start earlier. By providing light at different times of the night, the degree of phase shifting of the rhythm can be assessed and graphed as a Phase response curve (PRC). A PRC in rodents, under normal lighting intensities shows no phase shift in the daytime (phase shift = 0). In the early part of the night, light increasingly delays the clock, till ~CT 14 (CT = circadian time; the time after the start of the circadian day), after which the delays reduce till CT 18 (phase delays are denoted as negative numbers). At ~CT 18, the phase shift is 0 again, following which the clock begins to advance increasingly till CT 22 (phase advance are denoted as positive numbers). The phase shift then gradually declines, till the clock begins to show no phase shift during the day again [67,68].

Following the nocturnal, burrowing rodent, it is also evident that the highest chances of this creature being exposed to light is at dawn and at dusk, due to which animals show maximum phase shifts at these times. Further, unlike parametric entrainment, rather than affecting the speed of the clock, non-parametric entrainment

facilitates entrainment by shifting the cycle according to the timing of light. Thus, an animal in the laboratory can be entrained to a 24-hour cycle using 2 flanking light pulses to define the subjective daytime: one pulse at the beginning of the subjective day (the morning or M-pulse) and one at the end (The evening or E-pulse) [67,69]. These light pulses can be as short as two seconds, in some rodents. Such a light regimen is called a Skeleton photoperiod (SPP) [67–70]. Experiments have shown that any increase in the duration of light pulses doesn't affect entrainment to the SPP. Extrapolating this, it can be argued that light pulses of 6h duration could very well be used, in which case the non-parametrically based SPP entrainment paradigm becomes equivalent to the parametric 12:12 LD protocol. Thus, many chronobiologists conclude that the two types of entrainment are, in fact, just two theories of the mechanism of photic entrainment in mammals [7].

In either case, the flexibility offered by the lack of light in SPP (resulting in reduced masking) is unequivocally accepted in literature. Further probing this SPP paradigm, it can be argued that one pulse should be sufficient for entrainment. For an animal with circadian period less than 24 hours, where the endogenous cycle advances each day, one daily delaying E-pulse is sufficient to maintain a 24-hour rhythm. Similarly, a daily advancing pulse entrains the clock of animals with a period greater than 24 hours [7]. Thus, this single daily light pulse can mimic a full 24-hour circadian cycle and, it can also be used to phase shift the clock, as we will see in chapter 3 of this thesis.

1.2.4.2. Molecular pathways mediating photic entrainment in the SCN

In the above sections, we have discussed photic entrainment in the animal, as well as photic transduction to the SCN, from the retina. To fully understand entrainment, understanding of the molecular mechanisms that feed into the circadian clock within the SCN cells is key. Functionally, since the SCN has a large number of neurons, the activity of the SCN can be recorded by electrophysiology. Electrophysiological recordings of the SCN have shown that the firing rate of the whole SCN shows a circadian rhythm, with the firing rate peaking around the middle of the subjective day [7]. Thus, light at night causes neuronal firing during the circadian trough of neuronal electrical activity. Further, the individual cells are also rhythmic, with a larger proportion of cells in the shell showing rhythmicity compared to the core, supporting the observation that the neurons in the shell have more robust rhythmicity [71].

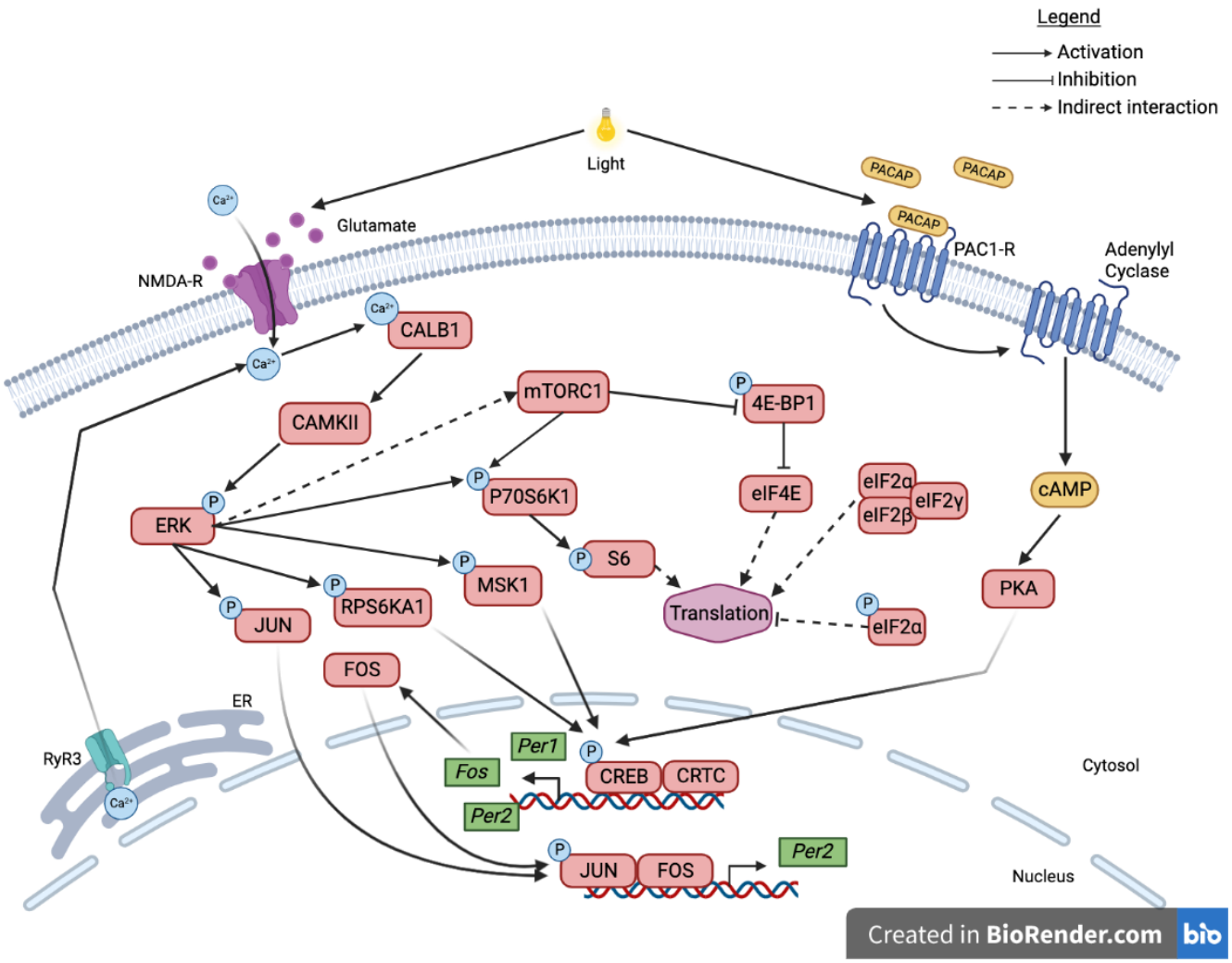

Figure 2: Molecular pathways involved in photic entrainment of the SCN.

(Created with BioRender.com)

When light information reaches the SCN in the night, via the RHT, it causes the activation of cells in the core region, and they fire in response to light [48]. The molecular mechanisms involved in photic entrainment of the SCN in the early night are shown in Figure 2. Specifically, the glutamate and PACAP released by the RHT projections [52] bind to the glutamate [72,73] and PAC-1 receptors [74,75] of the neurons in the SCN.

The activation of glutamatergic receptors leads to an influx of Ca^{2+} and Na^{+} ions into the cell [73] The resulting neuronal depolarization leads to the activation of voltage gated channels which further increase Ca^{2+} influx into the cell. The increased Ca^{2+} leads to the activation of Ca^{2+} channels on the endoplasmic reticulum [76,77], further increasing cytosolic Ca^{2+} levels [76]. The calcium binding protein, calbindin 1 (CALB1) then binds to this cytosolic Ca^{2+} [78,79] and activates Ca^{2+}/calmodulin dependent kinase 2 (CAMKII) [80]. CAMKII then leads to the phosphorylation of extracellular signal-regulated kinase (ERK) [81] (will be important for chapter 4). This phosphorylation and activation of ERK, leads to the phosphorylation of multiple proteins including the ribosomal protein S6 Kinase A1 (RPS6KA1), mitogen stress activated kinase 1 (MSK1) and Jun proto-oncogene (JUN) [82–84]. The phosphorylated forms of RPS6KA1 and MSK1 in turn phosphorylate the cAMP responsive element binding protein (CREB).

The binding of PACAP to PAC-1R leads to the activation of adenylyl cyclase [75], causing an increase in cyclic adenosine monophosphate (cAMP) levels [85]. cAMP binds and activates protein kinase A (PKA), which then phosphorylates CREB [63,86].

CREB, a transcription factor constitutively bound to CRE promoters, when phosphorylated, associates with CREB regulated transcription coactivator 1 (CRTC1) to initiate transcription of the light responsive genes[63,82,84,85,87,88] such as *Fos*, *Per1* and *Per2* [85,87,89–92]. The FOS protein associates with pJUN to then transcribe *Per2* [63,85]. *Per1* and *Per2* are both clock proteins induced by light. *Per1* is induced quickly after light exposure (which we will return to, in chapter 3), but *Per2* is induced about an hour after light exposure [89].

The kinase cascade activated by pERK also activates the mammalian target of rapamycin complex 1 (mTORC1) [93]. mTORC1 phosphorylates the p70 kinase p70 S6K1, which in turn phosphorylates the kinase S6 (this will be important in chapter 4) [93,94]. The phosphorylated kinase S6 is able to initiate further kinase cascades to activate proteins involved in protein translation [93]. mTORC1 also phosphorylates and inhibits initiation factor 4E binding protein 1 (4EBP1). 4EBP1 can bind elongation initiation factor 4E (eIF4E), thus sequestering it and inactivating it [95]. Thus, by inactivating 4EBP1, mTORC1 allows eIF4E to function freely.

eIF4E is involved in the first steps of the protein translation pathway. The initiation complex forms when eIF2 (composed of 3 subunits: α, β and γ), in conjunction with other eIFs, recruits the Met-t-RNA to the 40S subunit of the ribosome. eIF4E is involved in mRNA recognition and positioning, within the translation initiation complex [96]. One of the central processes for the assembly of the initiation complex is the interaction of eIF2 with the GDP to GTP exchange factor eIF2B. This process is mediated by the eIF2α subunit as its phosphorylation blocks the recruitment of the met-t-RNA to the initiation complex. Thus, pEIF2α reduces translation efficiency (this will also be important in chapter 4) [97]. Eventually, this translation pathway leads to the formation of light responsive proteins such as cFOS, light-responsive neuropeptides such as VIP [95] and light-responsive clock proteins PER1 and PER2. These clock proteins further feed back into the circadian cycle of the cell, to affect the phase of the oscillation, thus leading to photic entrainment, at the molecular level.

1.3. Post-translational control of the clock

The periodicity and entrainment of the clock is maintained by the transcription-translation negative feedback loop of clock genes and clock proteins. The rhythmicity of these proteins, however, is modulated by an additional layer of control known as post-translational modifications (PTMs). Physiologically, PTMs involve the addition or removal of smaller protein or non-protein moieties on specific residues of a protein, to regulate its activity, localization and/or half-life. Thus, in the context of the clock, PTMs decide, when each protein is activated, where it is localized and for how long until it gets degraded, to cumulatively regulate the period of the endogenous circadian cycle to be the same, on a daily basis. The post-translational PTMs studied in the mammalian clock include phosphorylation, acetylation, poly ADP-ribosylation, O-GlcNAcylation, SUMOylation and ubiquitination and their balance is essential to maintain protein homeostasis in the circadian clock[98,99]. Among these, ubiquitination, which can regulate activity, localization and half-life of clock proteins, and its counter-process deubiquitination, are especially important PTMs [22,100,101].

1.3.1. Ubiquitination and deubiquitination in the mammalian clock

Ubiquitin is a small, ubiquitously present 8.6 kDa regulatory protein found in most eukaryotes [102]. The process of tagging a protein with ubiquitin is called ubiquitination and removing this tag is called deubiquitination. A protein can be tagged with one single ubiquitin tag (mono ubiquitination) or by a chain of ubiquitin tags (poly ubiquitination). While ubiquitination of the protein could happen at multiple residues or domains, poly-ubiquitination happens predominantly at K43 (most abundant), K63 (non-degradative

roles) [103], K11 or M1 (leads to a linear ubiquitin chain) [102,104,105]. A set of 3 enzymes called the E1, E2 and E3 enzymes go through a complex, cascading process to affix ubiquitin tags onto proteins. Of the three, the E3 ubiquitin ligase is directly involved with protein ubiquitination. Tagging a protein with ubiquitin could regulate its activity, localization or half-life.

While there are hundreds of E3 ligases in the mammalian body, there are only about 110 DUBs, divided into 5 distinct families, based on the motifs they recognise. Of these the ubiquitin specific peptidases (USPs) form the largest family [102]. These DUBs are antagonistic to the E3 ligases and remove or shorten ubiquitin tags on proteins. Thus, ubiquitination and deubiquitination modulate the timing of activity, localization or half-life of proteins. Hence, unsurprisingly, that they are intricately linked with circadian rhythms.

In figure 3, we see all the ubiquitin ligases and deubiquitinases (DUBs) identified so far, in the mammalian circadian clock.

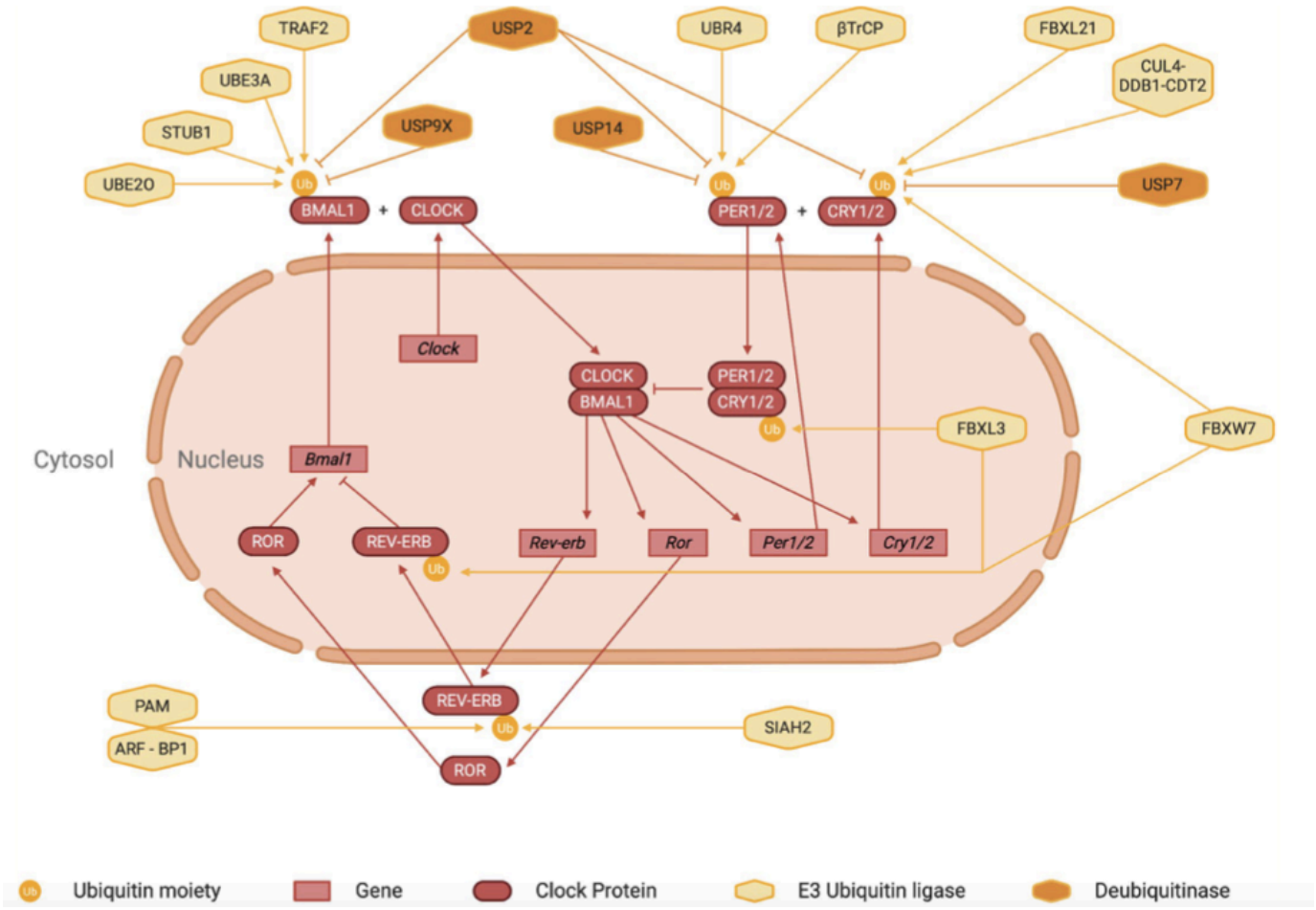

Figure 3: Schematic representation of the main components of the mammalian circadian clock, along with the E3 ubiquitin ligases and deubiquitinases known to regulate them. *Adapted from Srikanta S. B. et. al., J. NeuroChem (2021) (Created with BioRender.com)*

1.3.2. Ubiquitin specific peptidase 2

Ubiquitin specific peptidase 2 or USP2 is one of ~110 DUBs found in the mammalian body. In humans, the *Usp2* gene is on chromosome 11, while it is present on chromosome 9 in mice. Human USP2 has 4 isoforms, of which only two have been well characterized, while mouse USP2 has 5 protein coding isoforms, of which three are

characterized. Between the two systems, USP2-69 and USP2-45 are the common and best studied isoforms. The catalytic C-terminus end of the protein is common in both isoforms, while the N-terminus varies, leading to mostly similar functionalities between isoforms [106–108].

Physiologically, “USP2 is a well-studied DUB with multiple established functions. The first known substrate of USP2 was the fatty acid synthase protein (FAS). In 2004, Graner and colleagues showed that USP2 binds to and stabilizes FAS, a protein known to be upregulated in many cancers[109] including prostate cancer [110]. USP2 was shown to be involved in regulating the degradation of oncogene p53 by targeting the ubiquitin ligase MDM2 [111]. MDMX, another target of MDM2, is also deubiquitinated by USP2. Further, USP2 controls the cell cycle by deubiquitinating Aurora-A, a centrosomal kinase required for mitosis [112], as well as cyclins such as cyclin A1 and cyclin D [113–115], which are regulators of meiosis. Hence, unsurprisingly, the dysregulation of USP2 levels was associated with the development of various kinds of cancers, such as colorectal cancer [114], prostate cancer [113] and oral squamous cell carcinoma [116]. In the circadian system, USP2 regulates the ubiquitination of several clock proteins and contributes to the response of the circadian clock to light [117–120]. Apart from its functions within the clock, USP2 also mediates clock output. For instance, USP2 regulates the membrane scaffolding protein NHERF, in a circadian manner [121]. NHERF, in turn, regulates cellular homeostasis of calcium absorption in a clock-dependent manner [121], hence making USP2 a clock output mediator.”[122]

One of the differences between USP2-69 and USP2-45 is that USP2-45 shows localization in the brain, while USP2-69 does not [123]. The ubiquity of USP2 in the brain

has been shown by multiple studies [21,124–126]. However, the roles of USP2 in the brain are not well studied, even though currently available literature makes strong correlations between USP2 and normal brain functioning. Studies have shown a correlation between stress and USP2 regulation [126], as well, USP2 levels and schizophrenia and bipolar disorder severity [127]. Hence, USP2 seems to have far reaching effects on brain functioning and health.

1.3.2.1. USP2 and circadian rhythms

Among all the deubiquitinases involved in the clock, USP2 is the only DUB that is rhythmic in all the tissues it has been studied. In a study by Zhang and colleagues where they created a circadian gene expression atlas in mammals, they sampled 12 different tissues and regions in the mouse body around the clock, including three brain regions (the cortex, the cerebellum and the brain stem), and subjected them to RNAseq and DNA arrays. *Usp2* was one of only 10 genes that showed rhythmicity in all the tissues assayed [21]. Corroborating these findings, *Usp2* has shown circadian patterns of expression in other studies as well [128–130]. Additionally, a study has shown that *Usp2* transcription in the liver is under BMAL1 control [131]. Thus, it seemed that USP2 is strongly under the influence of the clock.

Studies by Scoma and colleagues, Tong and colleagues and our lab (Yang and colleagues), have showed that USP2 affects BMAL1 [120], CRY1 [119] and PER1 [117,118], respectively, thus pointing towards a role for USP2 in controlling the clock. The studies from our lab further found that USP2 directly interacts with PER1 and promotes

its nuclear localization. However, there was no evidence of direct interactions with either BMAL1 or CRY1 [118].

1.3.2.2. USP2 in photic entrainment

In the 2012 paper from our lab, with the premise that USP2 is circadian, we investigated the role of USP2 in the circadian clock, using a full-body germline *Usp2* KO mouse model (which I will refer to, simply, as *Usp2* KO, here on). While USP2 seemed to affect multiple proteins in the clock, there were only mild effects on the regular function of the clock, with a modest 15-minute increase in period, compared to WT mice, while most other parameters didn't change between genotypes. However, entrainment phenotypes significantly varied between genotypes [117].

When subjected to a 6-hour phase advance under a 12:12 LD cycle, *Usp2* KO mice showed slower entrainment to the shift, as compared to WT mice. However, when subjected to a 6-hour delay under a 12:12 LD cycle, *Usp2* KO mice showed significantly quicker entrainment. Further, when subjected to non-parametric phase shifts, *Usp2* KO mice showed a significantly larger delay to light pulses at CT 14, in keeping with the phenotype observed in the parametric phase shift condition. Similarly, *Usp2* KO mice showed a significantly reduced advance to a light pulse at CT 22, compared to WT mice [117].

Additionally, Scoma and colleagues tested the sensitivity of *Usp2* KO mice to different light intensities [120]. At low light intensities, they found that *Usp2* KO mice were more sensitive to phase shifts and even showed larger phase delays at CT 12, compared to WT mice. This phenotype was not replicated in the middle of or late at night [120].

Put together, the results from the different studies showed that USP2 affected entrainment and that it had different effects on the clock in the dawn and dusk phases of the circadian cycle. While this establishes a role for USP2 in the photoreception pathway and photic entrainment of the clock, these studies did not delve any further into understanding the basis for or mechanism of USP2's role in entrainment. Since *Usp2* was knocked out in the whole body, can the behavioral phenotypes observed in *Usp2* KO mice be explained by effects on the non-circadian behavioral systems? If the phenotypes are indeed emergent from the circadian system, does USP2 act on the retina, the SCN and/or elsewhere, to mediate its role in photic entrainment? What molecular pathways are affected by USP2 to give rise to the observed effects on entrainment? As a DUB, what are the specific proteins deubiquitinated by USP2 and how does this affect them? These are the main questions we aimed to answer during my PhD, as you will see from this thesis.

1.4. Rationale and objectives of the thesis

Considering that USP2 is expressed in multiple regions in the brain, and it is correlated to brain health, we hypothesized that it affects neuronal functioning, hence regulating various pathways in the brain that control various behaviors. Additionally, the involvement of USP2 in regulating circadian rhythms and its own circadian expression, suggested that it is closely involved with the clock. Adding to the hypothesis about the role of USP2 in the brain, previous findings regarding USP2 affecting photic entrainment in mice further pointed towards a role for USP2 in the central pacemaker. Hence, in this thesis, I tackle three main questions, in the next three chapters:

1. *What role does USP2 play in regulating behaviors regulated by different parts of the brain?*
2. *What is the location and mechanism of USP2 action, in modulating the circadian clock's photic entrainment response?*
3. *What are the molecular pathways affected by USP2 in response to light, to give rise to the observed entrainment response?*

Chapter 2: Behavioral phenotyping of mice lacking deubiquitinase USP2

Behavioral phenotyping of mice lacking the deubiquitinase USP2

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2.1. Abstract

Ubiquitin specific peptidase 2 (USP2) is a deubiquitinating enzyme expressed almost ubiquitously in the body, including in multiple brain regions. We previously showed that mice lacking USP2 present altered locomotor activity rhythms and response of the clock to light. However, the possible implication of USP2 in regulating other behaviors has yet to be tested. To address this, we ran a battery of behavioral tests on *Usp2* KO mice. Firstly, we confirmed our prior findings of increased daily activity and reduced activity fragmentation in *Usp2* KO mice. Further, mice lacking USP2 showed impaired motor coordination and equilibrium, a decrease in anxiety-like behavior, a deficit in working memory and in sensorimotor gating. On the other hand, no effects of *Usp2* gene deletion were found on spatial memory. Hence, our data uncover the implication of USP2 in different behaviors and expands the range of the known functions of this deubiquitinase.

2.2. Introduction

Ubiquitination is the process of covalent attachment of a 76 kDa long protein called Ubiquitin to other proteins. While ubiquitination has multiple roles in the functioning of the cell, including protein localization and trafficking [1], its most salient function is the targeting of tagged proteins for degradation [2]. Ubiquitination is carried out by a group of enzymes known as E3 ubiquitin ligases [2, 3]. The process of ubiquitination is counteracted by the process of deubiquitination, which is mediated by enzymes known as deubiquitinases or DUBs. These opposing processes can, in essence, determine the timing of various processes such as degradation, activity and localization of proteins.

While various DUBs are found in different tissues and cell types, Ubiquitin Specific Peptidase 2 or USP2 is one of the DUBs that has been found to be expressed almost ubiquitously in the organism [4–7]. More interestingly, it is the only DUB that has been found to have a circadian pattern of expression in virtually all the tissues where it is expressed [7,8].

Circadian rhythms are self-sustaining oscillations with a period of approximately 24 hours, which are sustained at the molecular level by negative feedback loops involving circadian clock genes [9]. The counterbalance between ubiquitination and deubiquitination is essential to accurately control the timing of degradation of several clock proteins and hence, to the maintenance of periodicity in the clock [10]. Previous studies have shown that the deubiquitinase USP2 modulates the stability and/or localization of clock gene products BMAL1, PER1 and CRY1 [11–14]. Surprisingly though, despite its interaction with many of the core clock components, knocking out *Usp2* in mice did not lead to proportionally severe circadian deficits [11, 12]. Our group showed

that USP2 modulates the response of the clock to light [11]. We also showed that *Usp2* KO mice have a slightly longer period than WT littermates [11]. While no other significant phenotypes were noticed under a normal light:dark cycle, a trend towards increased total activity over 24 hours was observed in the *Usp2* KO mice, compared to WT mice [11].

Ubiquitination, and ubiquitinating/deubiquitinating enzymes, have been associated with various behaviors as well as neurological and psychiatric disorders, such as bipolar disorder [15, 16], neurodevelopmental disorders [15, 17, 18], and Parkinson's disease [19]. Searching the mouse brain expression data in the Allen Mouse Brain Atlas [20], we noticed that *Usp2* is expressed in many brain regions. Hence, we aimed to test the hypothesis that USP2 plays a role in modulating various behaviors beyond circadian rhythms. To test this, we subjected *Usp2* KO and WT mice to a battery of tests, to assay various behaviors such as daily wheel running behavior, motor coordination, anxiety-like behavior, sensorimotor gating and memory. We confirmed that the *Usp2* KO mice showed increased and more consolidated running wheel activity, which was not due to increased motor coordination. Further, based on high *Usp2* expression in the hippocampus and cortex, we tested the mice for anxiety-like behavior and memory, and noticed a reduction in anxiety-like phenotypes and deficits in the working memory of *Usp2* KO mice, but not in their spatial memory. Based on *Usp2* expression in the olfactory bulb, striatum, thalamus and prefrontal cortex, we assessed sensorimotor gating, for which limited effects of *Usp2* gene deletion were found.

2.3. Materials and Methods

2.3.1. Animals

Usp2 KO mice [21] were obtained from Dr. Simon S. Wing and bred in house. *Usp2* KO and WT littermates (on a C57BL/6J background) were generated by breeding heterozygotes. In some cases, PER2::LUC knockin mice (on a C57BL/6J background) were used as controls. This PER2 gene modification was shown not to affect PER2 function, mouse health, general activity or wheel-running activity [22]. Four such mice were used in the wheel running, rotarod, novel object recognition and pre-pulse inhibition tests. In all cases, similar effects of the KO were seen irrespective of whether or not these mice were included in the analyses.

Mice were weaned at 3 weeks of age, into cages containing no more than 5 mice per cage. They were maintained on a 12h:12h light:dark cycle (with light at ~300 lux). At 2 to 3 months of age, male mice were designated to running wheel or (non-circadian) behavioral cohorts. All procedures involving animals were carried out in accordance with guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committees of McGill University and Douglas Mental Health University Institute (protocol no. 2001-4586). For euthanasia at the end of the experiments, the animals were first anesthetized by isoflurane inhalation, followed by CO_2 euthanasia; all efforts were made to minimize suffering.

2.3.2. Wheel-running behavior

For running wheel experiments, 2 to 3-month-old WT and KO mice (n = 6) were transferred to running wheel-equipped cages (Actimetrics, Wilmette, IL, USA), where they

were singly housed, with ad-libitum access to food and water. After a baseline week for acclimatization, wheel running activity was recorded for three weeks, in the same LD cycle. The last 10 days of recorded data were analysed on the Clocklab software Version 6 (Actimetrics, Wilmette, IL, USA). Daily wheel rotations, number of bouts of activity per day, length of each activity bout and number of wheel rotations in each bout were quantified. One mouse (KO) was excluded from the analysis because its wheel running activity was not recorded on the software.

2.3.3. Non-circadian behavioral tests

WT mice (6-12/group) and *Usp2* KO mice (10-12/group) went through Rotarod, elevated plus maze (EPM), Novelty suppressed feeding (NSF), Novel object recognition (NOR), Morris water maze (MWM) and Pre-pulse inhibition (PPI) of acoustic startle. To account for time-of-day effects on behavior, all behavioral tests were carried out between one and five hours after lights on. Tests were performed under dim light conditions (~15 lux) unless noted otherwise. The groups (WT and KO) were counterbalanced between tests and between trials. Mice were habituated to the testing room for 30 minutes, prior to the start of each session of behavioral testing. EPM, NSF and NOR tests were video recorded to minimize interaction with the mice and to reduce the resulting stress levels. All the mice in the video recordings of the behaviors were tracked using the TopScan 2.0 software (Clever Sys, Restin, VA, USA). Since some tests measured anxiety-like behaviors or could be affected by stress, all mice were given 2 “recovery days” between each test, during which they were not handled or disturbed.

2.3.3.1. Rotarod

The Rotarod test assays motor coordination, equilibrium and balance [23]. This test was carried out over two days. On the first day, each mouse was habituated to the rotarod (Bioseb, Valbonne, France). For the habituation phase, the ridged cylinder was made to rotate at a constant speed of 5 rotations-per-minute (rpm) for two 3-minute-long trials and 10 rpm for two 3-minute-long trials. During each of these trials, the mice were put back on the rod if they fell, until the timer ran out. Each mouse got a rest period of at least 15 minutes in its home cage between each subsequent trial.

For the testing day, each mouse was tested for three trials. It was placed on the rotarod, which accelerated at a constant rate, from 4 to 40 rpm, over a 5-minute span (acceleration = 7.2 rpmpm). When the mouse fell off the cylinder, onto the press plate placed 20 cm below the rod, the time taken to fall off the rod was recorded. After the fall, they were returned to their home cage. The greater their latency to fall, the greater is their motor coordination, equilibrium and/or balance. Two mice were excluded from this analysis (1 WT and 1 KO) as they refused to run on the rotarod.

2.3.3.2. Open field test

The open field test measures general locomotor activity in mice [24]. For this test, we used a VersaMax Legacy Open Field setup (AccuScan Instruments, Inc., Columbus, OH, USA). Mice were left to explore the VersaMax acrylic activity chamber with infrared sensors for 50 minutes while the experimenter was absent from the room. Data were collected using the Versamax Software (version 4.0, 2004; AccuScan Instruments, Inc.,

Columbus, OH, USA). From the collected data, total distance travelled in 50 minutes was plotted.

2.3.3.3. Elevated plus maze

Anxiety-like behavior was measured using the elevated plus maze (EPM) [25]. The maze is elevated 75 cm from the floor and consists of 4 arms shaped like a cross and painted black. Two opposing arms are enclosed by 10 cm high walls on three sides, while the other two are open. For the assay, each mouse was placed in the center of the maze, facing an open arm. The mouse was then allowed to freely explore the maze for 5 minutes, after which it was returned to its home cage. The total time spent in the open and closed arms, as well as in the center were measured. The longer a mouse spends in the open arms, the less anxiety-like behavior it is considered to show. Proportion of time spent in open arms was calculated as:

$$\%\ time\ in\ open\ arms = \frac{(time\ spent\ in\ open\ arms\ (s)) + 0.5 * (time\ spent\ in\ the\ middle\ (s))}{300\ s}$$

2.3.3.4. Novelty-suppressed feeding

The novelty suppressed feeding (NSF) test is an assay of anxiety-like behavior in a conflict-based environment [26, 27]. Mice were fasted for 24 hours before the start of the test. Then, each mouse was placed in a grey 48 cm X 48 cm X 48 cm box, in a well-lit area. At the center of the box, a fixed food pellet (standard chow) was available for the

mouse to feed on. Thus, the mouse needs to choose between the anxiogenic setting of feeding in an open, well-lit area as opposed to the safety of walls, but without any food availability. The mouse was allowed to explore this setting for 10 minutes and the latency to feed was measured, being careful to not include food manipulation or sniffing behaviors in this measure. The longer a mouse takes to feed, the higher its anxiety-like phenotype.

After this, the mouse was transferred to a quiet area, into a cage containing pre-weighed quantities of food and the amount of food they ate over a further 10-minute span was recorded. This measure assesses the hunger levels of the mice and whether differences due to the food deprivation itself influences the observations within the arena.

Additionally, to factor in the possibility of a general difference in appetite between KOs and WTs, at the end of the NSF protocol, the mice were weighed daily for 9 days and the amount of food that they consumed was noted over these days.

2.3.3.5. Novel object recognition

The novel object recognition (NOR) test measures learning and memory in mice [28]. NOR involves three phases spread over two days. On the first day, each mouse was acclimatized to an empty grey 48cm X 48cm X 48cm box by allowing it to freely explore the box for 10 minutes. On the second day, the first phase of the day is the habituation phase where the mouse was allowed to freely explore the grey box for 10 minutes. Each box contained two identical objects (either two 75 mm tissue culture flasks filled with corn cob bedding, or two boxes of coverslips) placed at opposite corners of the box, 12 cm away from each of the two nearest walls. The mouse was then returned to its home cage. The second phase of the day is the novel object recognition phase, which tests recall

from working memory. In this phase, which starts 4 hours after the habituation phase, one of the objects in each box was exchanged for a differently shaped object (tissue culture flask as a replacement for coverslip box and vice-versa). The mouse was then allowed to freely explore the arena for 10 more minutes. The time spent exploring the two objects was separately recoded, in both phases. A mouse was considered to be involved in object exploration if its head was directed towards the object, within a radius of approximately 2 to 3 cm from it. This analysis was carried out using the TopScan 2.0 software. Mice tend to prefer exploring novel objects, over familiar objects. Hence, the exploration of the novel object is a measure of the extent to which the animal remembers the previous encounter with the familiar object. The ability to discriminate between the novel and familiar object was measured by the Discrimination Ratio (DR), calculated as:

$$DR = \frac{Time\ spent\ exploring\ novel\ object}{Time\ spent\ exploring\ familiar\ object}$$

A DR of 1 shows a lack of discrimination between the two objects. DR > 1 shows that the mouse is interacting with the novel object more than the familiar object.

Using the TopScan 2.0 software, the total distance traveled by the mice during the trials were also measured.

2.3.3.6. Morris water maze

The Morris water maze (MWM) assesses spatial memory [29, 30]. The maze consists of a large circular swimming pool (diameter of 150 cm) filled with opaque water with a platform (225 cm^2) submerged 1 cm below the surface of the water. The mice must

reach the platform in order to be able to stop swimming and rest. Spatial cues were placed on the pool walls to allow the mice to learn the location of the platform. The learning stage was 4 days long, with each day consisting of 4 trials, each with the mouse beginning at a different location in the pool. The inter-trial interval was at least 30 min. The time to reach the platform was recorded at every trial, to compare the patterns of learning. On day 5 of the experiment, the platform was removed, and the time spent in each quadrant of the pool in the span of 1 minute was recorded, to test the robustness of spatial learning (probe trial). Then, a cue trial, where the platform is placed in a new location along with a visual cue, was administered to verify that the visual system was intact in the animals. The latency to reach the platform and time spent in the appropriate quadrant in the probe trial were analyzed and calculated using HVS Image Analysis (HVS Image, Hampton, UK).

2.3.3.7. Pre-pulse inhibition of acoustic startle

Pre-pulse inhibition (PPI) of acoustic startle reflex is a measure of sensory-motor gating [31, 32]. Testing is carried out using the SR-Lab software connected to 6 sound attenuating chambers equipped with plexiglass animal enclosure tubes (San Diego Systems, San Diego, CA, USA). These chambers are ventilated by an electrical fan that produces a constant 70 dB background. Speakers positioned directly above the enclosure present tone pulses of differing loudness and the startle of the animal is recorded by an accelerometer attached to the base of the enclosure. Following a 5-minute acclimatization period in the tube, there were 3 phases in the paradigm. In the first and third phases, 6 startle pulses of 120 dB loudness and lasting for 30 msec each, were administered. In the second phase, 38 trials were administered. The first 8 trials were pulse only (startle only)

trials. In the next 30 trials, the mouse received a 30 msec pre-pulse of 0 (pulse alone), 6-, 9-, 12- or 15-dB intensity above the background, 100 msec prior to the actual 120 dB pulse. These pre-pulses were randomly varied across the 30 trials spaced 15 msec apart, 5 of each of the pre-pulse trials presented to the animals. The average amplitude of startle in the last 15 startle-only trials is the baseline startle. % Pre-pulse inhibition (% PPI) is calculated as:

$$\% \, PPI = 100 - \frac{Startle \; response \; to \; trials \; with \; pre - pulse}{Startle \; response \; to \; pulse \; alone \; trials} * 100$$

The higher the % PPI, greater is the inhibition of acoustic startle when subjected to a low intensity sound pulse prior to the startle. In essence, a higher PPI shows higher sensorimotor gating.

Two mice were excluded from this analysis (1 WT and 1 KO) for having impossibly high baseline startle levels, pointing to a recording error in the data of these mice.

2.3.4. Statistical Analysis of Data

All the data were analyzed first for homogeneity of variances as well as normality of data. The data were plotted and analyzed on GraphPad Prism, using the appropriate statistical tests. For all the tests with data distributed normally and having equal variance across the groups (WT, KO), unpaired two-tailed t-tests were used (Wheel running data, Rotarod, EPM, NSF, NOR, probe and cue trials of MWM). In the cases where variances were not normal (Distance traveled during NOR phase), Welch's correction was applied to the two-tailed, unpaired t-test. If the normality assumption was violated (Average

startle, Average % PPI), the two-tailed Mann-Whitney test was used. For comparing between Habituation and NOR phases in the NOR task, paired, two-tailed t-tests were used. Mixed model 2-way ANOVAs were used to compare the daily consumption of food and weights of the mice in the days after NSF, learning across days (MWM) and % PPI across different pre-pulse intensities (PPI). Differences were considered to be significant if $p < 0.05$.

2.4. Results

2.4.1. Mice show altered activity patterns in the absence of USP2

WT and *Usp2* KO mice were placed in running wheel cages and locomotor activity was recorded under a 12h:12h light:dark cycle (representative actograms in Fig 1A). *Usp2* KO mice showed a trend (p = 0.06) for more total daily activity than WT mice (Fig 1B). Further, the duration of activity bouts was significantly longer in KO mice (p = 0.049, Fig 1C) and the average number of bouts per day was lower (p = 0.14, S1A Fig). The total counts of wheel rotation per bout of activity was also significantly increased in *Usp2* KOs, compared to WTs (p = 0.0481, S1B Fig.). Overall, these data indicated increased and less fragmented activity in mice lacking USP2. These data are consistent with those previously obtained in our laboratory [11].

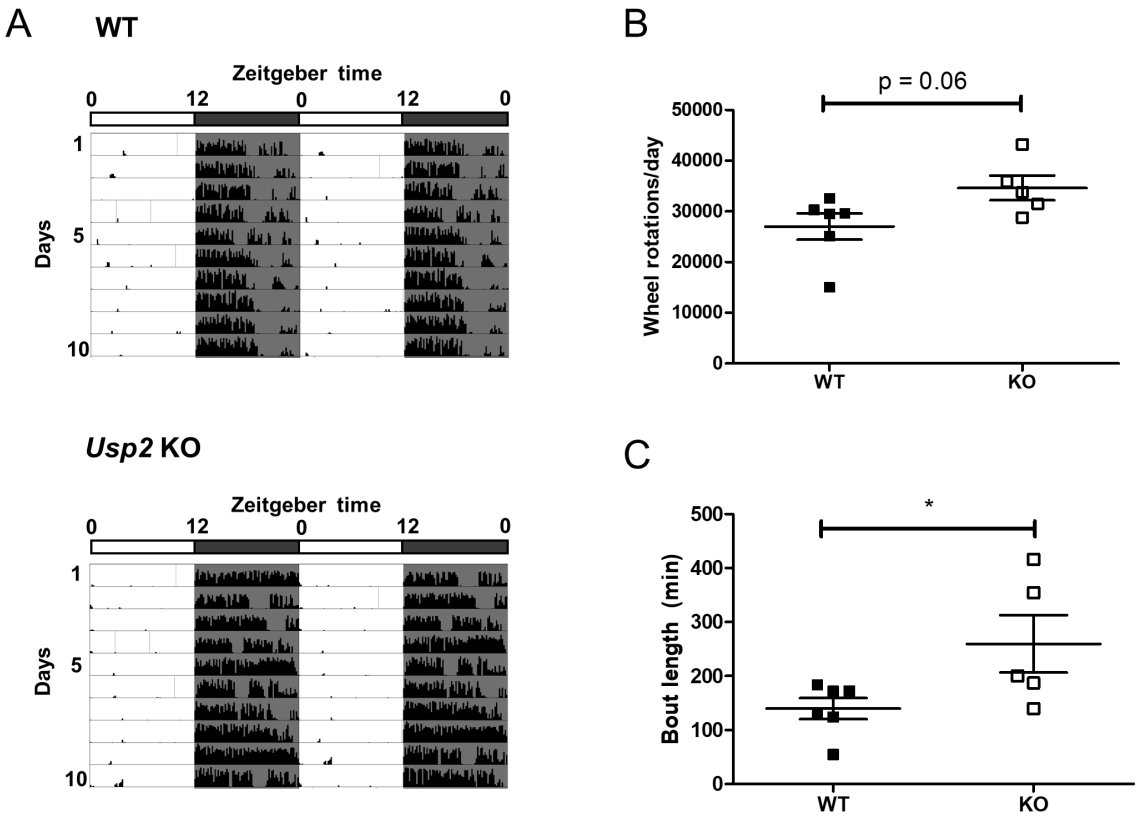

Fig 1. Increased daily activity and less fragmentation of activity in Usp2 KO mice. *(A) Representative actograms for the wheel running activity of WT and Usp2 KO mice over 10 days under a 12h:12h light:dark cycle. (B, C) Quantification, averaged over the 10 days of the total daily activity (B) and the average bout length (C). Individual data points represent independent mice (n: WT = 6, KO = 5) and data are represented as mean ± SEM. Unpaired two-tailed t-tests, *: p < 0.05.*

2.4.2. Motor coordination is reduced in mice lacking USP2

The increased activity and reduced fragmentation in running wheels led us to wonder if this phenotype could be the result of a change in motor coordination in the *Usp2* KO mice. To assay this, mice were subjected to the rotarod protocol, which assesses motor coordination and balance by evaluating the ability of the mouse to stay on top of a rotating cylinder. *Usp2* KO mice spent significantly less time on the accelerating cylinder ($t(20) = 2.49$, $p = 0.022$, Fig 2) and fell from the cylinder at lower speeds (WT: 18.11 ± 1.35 rpm, KO: 13.94 ± 1.31 rpm, $t(21) = 2.21$, $p = 0.038$) compared to WT mice, pointing towards a reduction of motor coordination in mice lacking USP2. Hence, a change in motor coordination does not explain the increased activity phenotype seen in the running wheel experiments.

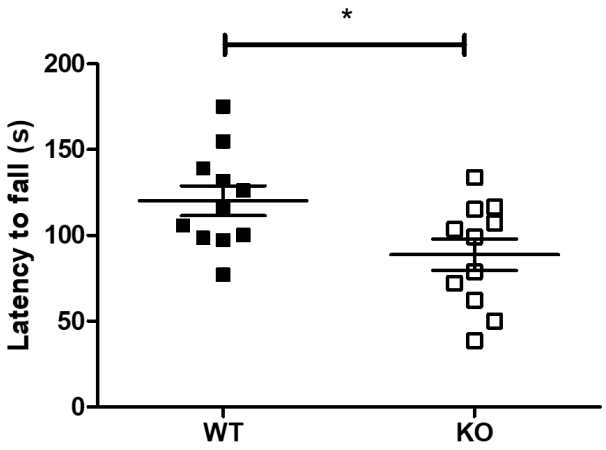

Fig 2. Reduced motor coordination in Usp2 KO mice. *Measurement of time spent on the accelerating rotarod by WT and Usp2 KO mice. Individual data points represent independent mice (n: WT = 11, KO = 11) and data are represented as mean ± SEM. Unpaired two-tailed t-tests, *: p < 0.05.*

While motivated locomotor activity did not correlate with motor coordination phenotypes, we questioned whether it might have an effect on general locomotion in the *Usp2* KO mice. This was assayed using the open field test (OFT), which assesses the general locomotion of mice when subjected to a novel environment. The total distance covered by the mice in 50 minutes showed no differences between the general locomotion of the two genotypes ($t(15) = 0.884$, $p = 0.391$, S1C Fig).

2.4.3. Anxiety-like behavior is decreased in mice lacking USP2

We surveyed *Usp2* gene expression in the mouse brain expression database in the Allen Mouse Brain Atlas (https://mouse.brain-map.org/experiment/show?id=76098316)

[20]. As shown in Table 1, *Usp2* is expressed throughout the brain. Therefore, we subjected the WT and *Usp2* KO mice to a battery of tests for affective and cognitive behaviors corresponding to these different brain regions.

Table 1. Relative expression of Usp2 transcript in the mouse brain

Brain region	***Usp2* expression level ***
Isocortex	++++++
Olfactory bulb	+++
Hippocampus	++++
Striatum	++++
Thalamus	+++
Hypothalamus	+
Midbrain	+
Pons	+
Cortical sub-plate	+++++
Pallidum	++
Medulla	+
Cerebellum	++

** Relative expression from in situ hybridization data for Usp2, in the Allen mouse brain atlas (each '+' sign represents ~1 unit of raw expression value).*

Expression of the gene in regions such as the hippocampus and cortex, which are associated with anxiety-like behavior [33], prompted us to assess such behavior, using the Elevated Plus Maze (EPM) and the Novelty-suppressed Feeding (NSF) test.

The EPM assay builds on the natural aversion of mice to heights and open spaces, counterbalanced by their drive to explore novel surroundings. When subjected to the EPM, *Usp2* KO mice exhibited a trend towards reduced anxiety-like phenotypes compared to WT mice: they spent a greater proportion of time in the open arms as compared to the WTs ($t(15) = 1.79$, $p = 0.09$, Fig 3A). The latency to enter open arms, on the other hand, did not differ significantly between genotypes (WT: 34.67 ± 29.7 s, KO: 18.23 ± 5.98 s, Mann-Whitney U test: $U = 20.5$, $p = 0.2275$). Further, the absence of differences in general locomotion between WT and KO mice in the OFT (S1C Fig) confirms that the time spent in the different arms of the EPM is based on the anxiety-like levels of the mice. Thus, the EPM data indicates a reduced anxiety-like phenotype in the *Usp2* KO mice.

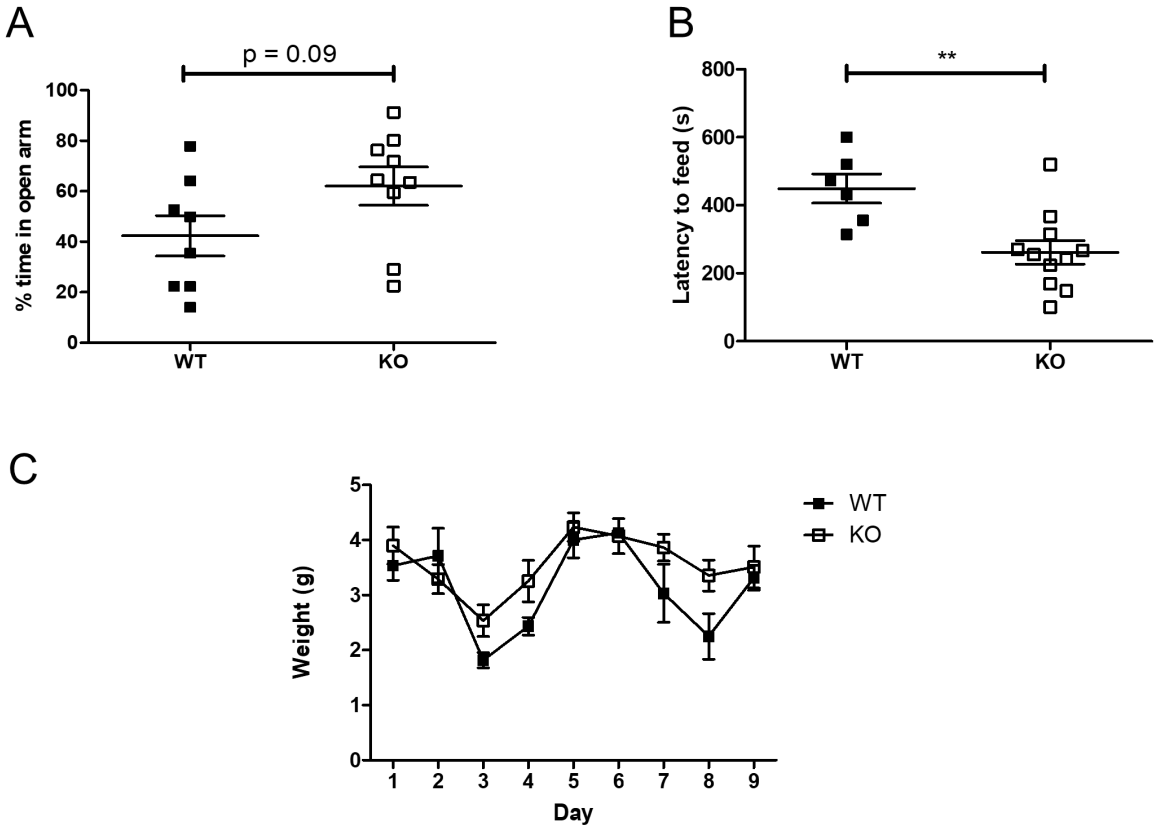

Fig 3. Reduced anxiety-like behavior in Usp2 KO mice. *(A) Proportion of time spent in the open arms of the elevated plus maze (EPM). (B) Latency to start feeding in the testing arena of the novelty-suppressed feeding (NSF) test. (C) Food consumed over 9 days following the NSF test. Individual data points represent independent mice (EPM, n: WT = 8, KO = 9; NSF, n: WT = 6, KO = 11) and data are represented as mean ± SEM. Two-way ANOVA (C) or unpaired two-tailed t-tests (A, B, D), **: p < 0.01.*

The NSF test opposes the desire for safety with the desire to feed. The *Usp2* KO mice started feeding faster than the WT mice ($t(15) = 3.323$, $p = 0.0046$, Fig 3B). When feeding was assayed in home cages right after the test, for 10 minutes, all mice ate equally ($t(15) = 0.403$, $p = 0.692$, S2A Fig). To verify if the increased latency to feed was a result of metabolic changes, or changes in hunger, mice were weighed daily, and their daily food consumption was measured. There were no differences between WT and KO mice in their general appetite, as both genotypes consumed equal amounts of food over the span of a week ($F(8, 120) = 1.63$, $p = 0.1232$, Fig 3C). Similarly, there were no differences in the weights of the mice recorded over a 9-day span ($F(4, 60) = 0.1$, $p = 0.9812$, Fig S2B). Thus, the observed phenotype of an increased latency to feed reflects mainly on a decrease in the anxiety-like behavior in mice lacking the *Usp2* gene, consistent with the trend observed in the EPM test results.

2.4.4. Recognition memory, but not spatial memory, is attenuated in mice lacking USP2

Since *Usp2* is highly expressed in the cortex and hippocampus (Table 1), we questioned whether knocking it out would affect working memory [34]. In the NOR test, WT mice had a significantly higher DR in the NOR phase of the test ($t(22) = 3.0$, $p = 0.007$, Fig 4A). On the other hand, for the *Usp2* KO mice, the DR was unchanged between the habituation and NOR phases ($t(21) = 0.364$, $p = 0.72$, Fig 4B). Comparison between the discrimination ratios of WT and KO mice showed that the KOs had a significantly lower ability to distinguish novel objects ($t(22) = 2.45$, $p = 0.023$, Fig 4C). Therefore, recognition memory is impaired in mice lacking *Usp2.*

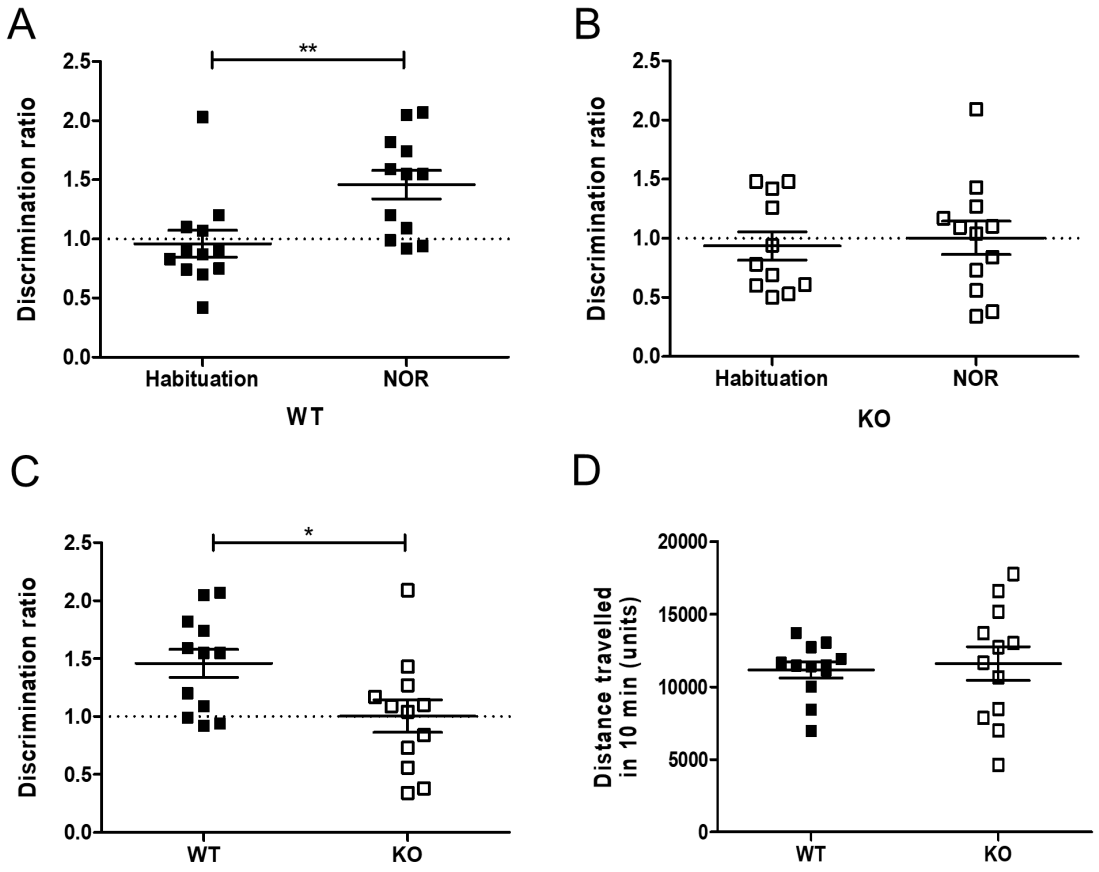

Fig 4. Impaired object recognition learning and memory in Usp2 KO mice. *(A, B) Proportion of time spent by WT mice (A) or Usp2 KO mice (B) to explore the novel object compared to the familiar object (discrimination ratio). (C) Comparison of the discrimination ratios of the WT and KO mice during the novel object recognition phase. (D) Total distance travelled by the WT and KO mice during the NOR phase of the test. Individual data points represent independent mice (n: WT = 12, KO = 12) and data are represented as mean ± SEM. Paired two-tailed t-tests (A, B), unpaired two-tailed t-tests (C) or unpaired two-tailed t-tests with Welch's correction (D), **: $p < 0.01$, *: $p < 0.05$.*

Further, to ascertain that the differences in DR do not stem from a change in locomotor activity of the mice, we quantified the average total distance covered by WT and KO mice in the NOR phase of the test. We found no difference in the total locomotion of the two genotypes (Welch corrected $t(15) = 0.341$, $p = 0.738$, Fig 4D). This is in line with the results of the OFT test (S1C Fig).

We also tested for spatial memory using the Morris water maze (MWM). Learning was observed in all the mice, over the first 4 days ($F(3, 45) = 12.71$, $p < 0.0001$) (Fig 5A). In the probe trial, both genotypes of mice spent more than 25% of the test time in the appropriate quadrant (WT: $t(8) = 2.55$, $p = 0.051$, KO: $t(10) = 2.17$, $p = 0.055$, Fig 5B), suggesting that the mice had learnt the position of the platform. In the cue trial, all the mice were equally adept at finding the platform at a new location ($t(15) = 0.09$, $p = 0.9235$, Fig 5C), confirming that performance factors unrelated to place learning were not involved

in the probe trial results. However, in all these procedures, no differences were found between the WT and *Usp2* KO mice.

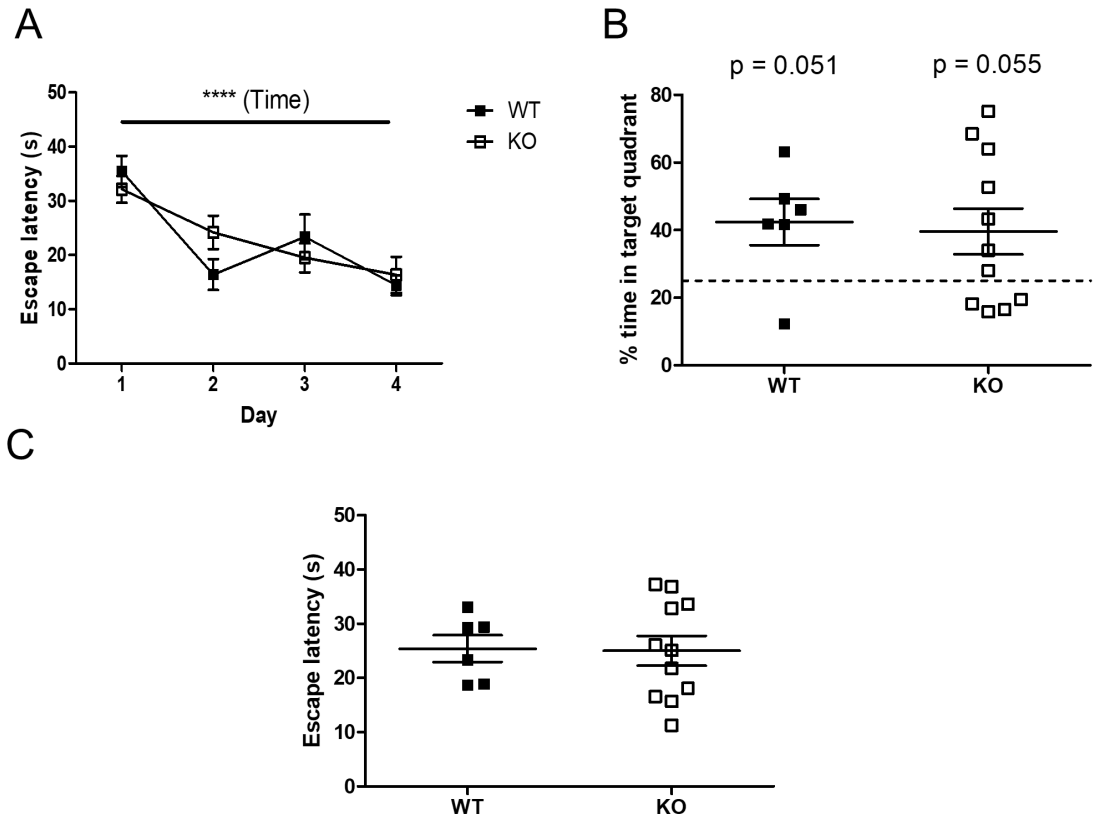

Fig 5. Unaltered spatial learning and memory of Usp2 KO mice. *(A) Time taken by the mice to find the platform on subsequent days, as a proxy for learning. (B) Probe trial: Proportion of time spent in the target quadrant in the absence of the platform on day 5. (C) Cue trial: Time taken by the mice to find the platform at a new location. Individual data points represent independent mice (n: WT = 6, KO = 11) and data are represented as mean ± SEM. Two-way ANOVA (A) or unpaired two-tailed t-tests and one sample t-test (B, C), ****: $p < 0.0001$ for effect of time in (A), interaction and effect of genotype n.s.*

2.4.5. Effects on sensorimotor gating in mice lacking USP2

PPI is known to be associated with the dopaminergic system, the limbic system, the olfactory bulb and the prefrontal cortex [35–38]. Among these regions, *Usp2* expression is high in the olfactory bulb, the striatum, the thalamus and the prefrontal cortex (Table 1, [20]). Hence, we tested for pre-pulse inhibition (PPI) of acoustic startle in *Usp2* KO mice. There was no difference between the genotypes in their baseline startle response to a 120 dB startle pulse ($t(20) = 0.45$, $p = 0.661$, Fig 6A). The % PPI averaged across the different pre-pulse intensities were also not different between the genotypes (Mann-Whitney $U = 41.0$, $p = 0.212$, Fig 6B). However, when the % PPI data were analyzed separately for each pre-pulse intensity, a Genotype x Pre-pulse intensity interaction was seen ($F(3, 63) = 2.79$, $p = 0.043$, Fig 6C). A simple main effect analysis revealed a trend for a difference between genotypes for a 12 dB pre-pulse intensity ($p = 0.06$). This suggested that *Usp2* may affect sensorimotor gating.

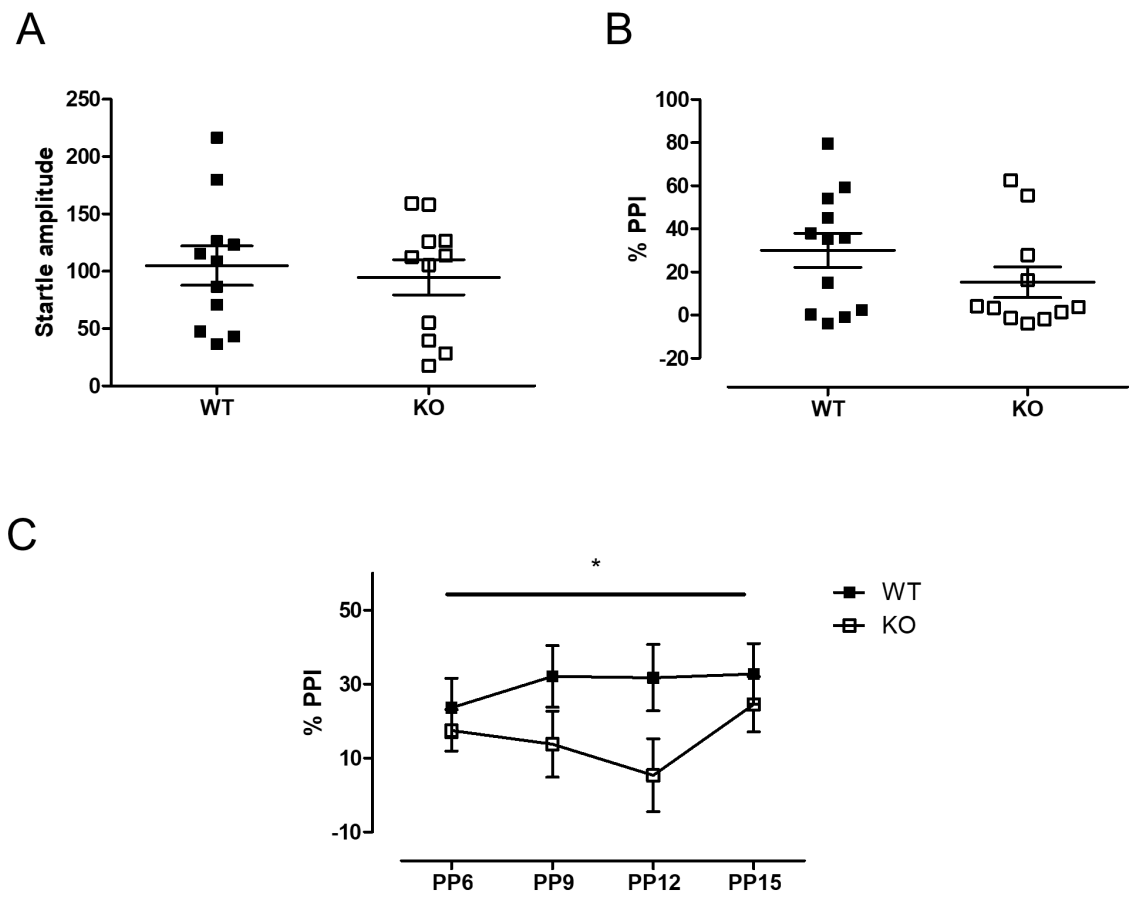

Fig 6. Reduced sensorimotor gating in Usp2 KO mice. (*A) Amplitude of baseline startle when a 120 dB pulse is given in the absence of any pre-pulse. (B) Average Percent pre-pulse inhibition (% PPI) of all of the non-zero pre-pulse intensities administered. (C) % PPI in response to each of the different intensities of pre-pulses administered. In (A) and (B), individual data points represent independent mice (n: WT = 11, KO = 11) and data are represented as mean ± SEM. In (C), each data point represents the average %PPI for the corresponding PPI. Two-way ANOVA (C) or two-tailed Mann-Whitney test (A, B), *: $p < 0.05$ for the interaction in (C).*

2.5. Discussion

In this report, we present evidence for a role of the deubiquitinating enzyme USP2 in the central nervous system. More specifically, we have uncovered alterations of anxiety-like behavior, learning and memory, and motor coordination in mice with a deletion in the *Usp2* gene. This work represents, to our knowledge, the first characterization of behaviors in the absence of USP2 function, beyond its established role in the regulation of circadian rhythms.

USP2 is a well-studied DUB with multiple established functions. The first known substrate of USP2 was the fatty acid synthase protein (FAS). In 2004, Graner and colleagues showed that USP2 binds to and stabilizes FAS [39], a protein known to be upregulated in many cancers including prostate cancer [40]. USP2 was shown to be involved in regulating the degradation of oncogene p53 by targeting the ubiquitin ligase MDM2 [41]. MDMX, another target of MDM2, is also deubiquitinated by USP2. Further, USP2 controls the cell cycle by deubiquitinating Aurora-A, a centrosomal kinase required for mitosis [42], as well as cyclins such as Cyclin A1 and Cyclin D [43–45], which are regulators of meiosis. Hence, unsurprisingly, the dysregulation of USP2 levels was associated with the development of various kinds of cancers, such as colorectal cancer [44], prostate cancer [43] and oral squamous cell carcinoma [46]. In the circadian system, USP2 regulates the ubiquitination of several clock proteins and contributes to the response of the circadian clock to light [11–14]. Apart from its functions within the clock, USP2 also mediates clock output. For instance, USP2 regulates the membrane scaffolding protein NHERF, in a circadian manner [47]. NHERF, in turn, regulates cellular

homeostasis of calcium absorption in a clock-dependent manner [47], hence making USP2 a clock output mediator.

While several functions of USP2 have been uncovered, little is known about its possible roles in the central nervous system. Based on data from the Allen Mouse Brain Atlas, *Usp2* gene is expressed in various brain regions (Table 1), which suggests that this DUB could be regulating behavioral processes. We addressed this using a battery of tests aimed at assaying various behaviors. This was initially prompted by our analyses of wheel-running behavior, in which we noted increased daily activity and a more consolidated activity pattern in *Usp2* KO mice. The reduction in activity fragmentation could be the result of an effect of the *Usp2* KO on the circadian system but our prior work using constant conditions, where limited effects on the endogenous period of the rhythms were found, argues against this. Thus, we used the rotarod to check a possible impact of *Usp2* gene deletion on motor coordination: the decrease in motor coordination of the KO mice suggests that this is also not the source of the altered activity patterns in mice lacking *Usp2*. Another possible source for this phenotype could be in the light-response pathways; however, the increased activity and consolidation is seen not only in the light phase of the 12:12LD cycle but also in the night, which suggests that an alteration of the light-response pathways (e.g. reduced masking of activity by light) is not involved. Finally, running wheel activity being a motivated behavior [48, 49], the phenotype in these assays could be due to changes in the reward pathways in the KO mice [50, 51]. Indeed, *Usp2* is highly expressed in the striatum (Table 1). Therefore, although we have not tested the *Usp2* KO mice for reward behaviors, this could represent an interesting avenue for future research.

The hippocampus and the cortex show the highest levels of *Usp2* expression. These are regions associated with the control of anxiety-like behavior. Accordingly, we found a decrease in anxiety-like behavior in mice lacking *Usp2*, both in the EPM and the NSF tests. In the former, the mice lacking *Usp2* spent more time in the open arms than their WT littermates, showing a reduction in anxiety-like behavior. In line with these findings, in the NSF test (which is a more sensitive measure of anxiety-like behavior, due to the pressure to feed), the mice lacking *Usp2* fed significantly quicker than the WT mice, reiterating the reduction in anxiety-like behavior in mice lacking USP2.

Further exploring the effects of cortical and hippocampal expression of *Usp2*, we looked at the effects its deletion might have on cognition. In the NOR test, the KOs showed no distinction in interacting with the novel and the familiar object (DR of 1 in both, the habituation and NOR phases), pointing to a deficit in either the learning of the two objects or to a deficit in working memory. On the other hand, the MWM test showed that spatial learning and memory were intact in the *Usp2* KO mice. Interestingly, a prior study has shown a correlation between a reduction of spatial memory in rats following stress with a downregulation of USP2 protein levels in the hippocampus, both reductions being concomitantly rescued by treatment with retigabine, an opener of Kv7 potassium channels [52]. This suggested that USP2 might play a role in spatial memory, at least under stress conditions, in rats. On the contrary, our data indicate no role for USP2 in spatial memory in mice under basal, unchallenged conditions.

Finally, using the PPI test, we looked at sensorimotor gating, or the ability of sensory inputs to guide motor responses in the organism. This behavior is guided by the striatum and olfactory bulbs, where the expression of *Usp2* is high as well. Also, PPI has

been known to be affected by the limbic and dopaminergic systems: it is especially affected by the dopaminergic cortico-striato-pallido-thalamic and limbic cortico-striato-pallido-pontine tracts [35]. Most of these regions also have a high expression of *Usp2*. Surprisingly, rather mild phenotypes were found in the PPI test. There seemed to be an effect of genotype on the startle response, but not at all pre-pulse intensities. Thus, further work will be required to delineate the effects of USP2 on sensorimotor gating.

In conclusion, our data indicate that *Usp2* plays a role not only in the circadian system, but also in controlling various other behaviors such as anxiety-like phenotypes, motor coordination and working memory. A limitation of our study is that it does not provide insights on what might be the substrates of USP2 in the brain regions where it is highly expressed. For the same reason, it is hard to speculate on whether the various phenotypes of the *Usp2* KO mice are due to shared vs. separate mechanisms within the brain. It will be an important future direction to find out what these substrates are, and how USP2 action on these proteins can lead to behavioral alterations like the ones we have observed in this study. Nevertheless, this report sets the stage for the study of the roles of USP2 in the central nervous system and shows that this deubiquitinase has many roles beyond those already described in peripheral organs.

2.6. Acknowledgements

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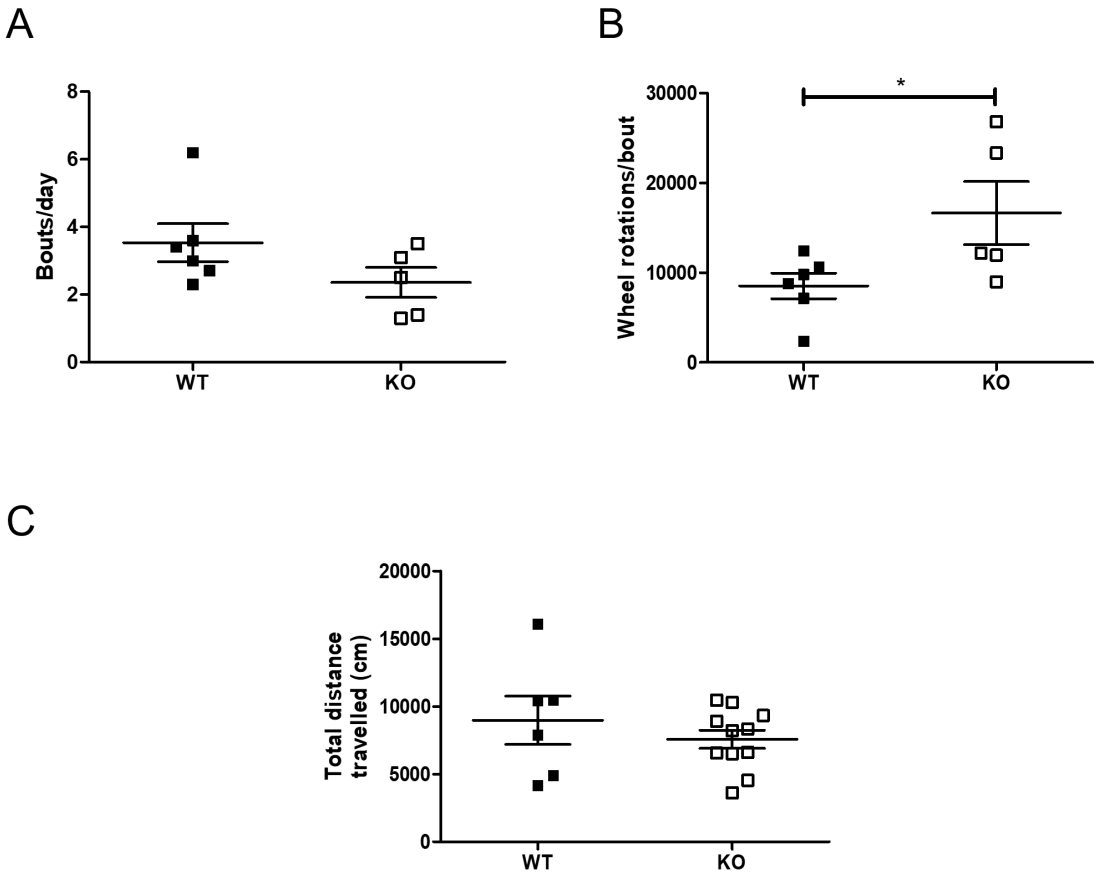
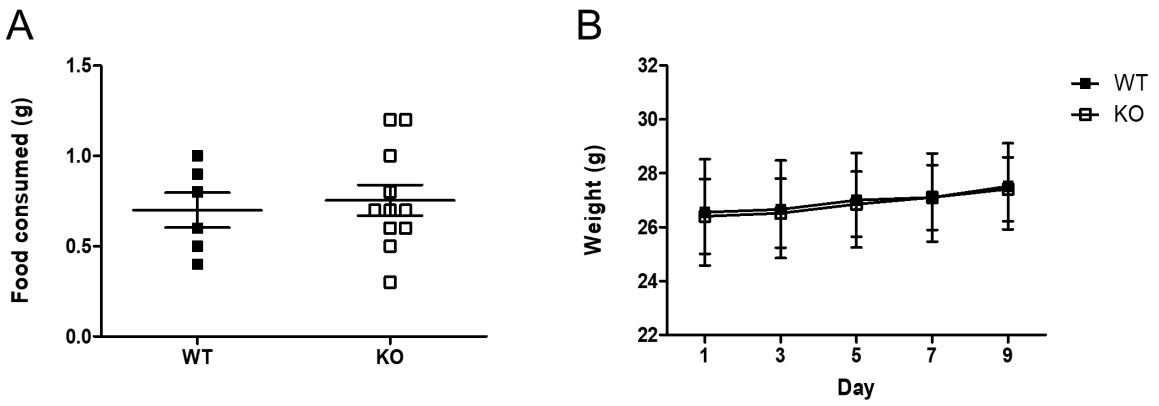
2.8. Supporting information



S1 Fig. Locomotor activity patterns of Usp2 KO mice. *(A,B) Quantification of averaged wheel running activity over 10 days: number of bouts of activity per day (A) and number of wheel rotations per activity bout (B). (C) Open field measurement of total distance traveled by WT and Usp2 KO mice as a measure of general locomotion. Individual data points represent independent mice (Wheel-running activity, n: WT = 6, KO = 5; Actimetry, n: WT = 6, KO = 11) and data are represented as mean ± SEM. Unpaired two-tailed t-tests, * p < 0.05.*



S2 Fig. Control measures of novelty-suppressed feeding (NSF) test. *(A) Quantity of food consumed within 10 minutes post-NSF test, in the home cage. (B) Weight of the mice over 9 days following the NSF test. Individual data points represent independent mice (n: WT = 6, KO = 11) and data are represented as mean ± SEM. Unpaired two-tailed t-tests (A) or two-way ANOVA (B), all n.s.*

Chapter 3: The deubiquitinase USP2 modulates early night photic entrainment of the circadian clock at the level of the SCN

The deubiquitinase USP2 modulates early night photic entrainment of the circadian clock at the level of the SCN

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Manuscript ready for submission

3.1 Preface

Our lab had shown a role of USP2 in the circadian clock, in papers published in 2012 and 2014. However, looking at the Allen brain atlas ISH data, which showed that *Usp2* expression was spread across the brain, it was essential that we looked at the effects of USP2 on the behaviors arising from these other brain regions to establish that the behaviors we observed in the earlier papers had a purely circadian basis.

Having shown that USP2's effects on circadian behavior were not related to the effects of USP2 observed on non-circadian behavior (chapter 2), in the following chapter we will take a closer look at the specific role of USP2 in the clock's response to light pulses at night.

The aim of the following study was to understand the specific role of USP2 in modulating photic entrainment of the clock. Investigating this question, we ended up with three major conclusions: 1) USP2 specifically affects photic entrainment in the early part of the night. 2) USP2 affects photic entrainment at the level of the SCN. 3) USP2 is essential for the induction of the light-responsive clock gene *Per1.* These results are described in detail, in the following chapter.

This chapter promotes a key role for USP2 in modulating early-night photic entrainment. The topics discussed in this chapter establish a foundation upon which the following chapter, the third scholarly work in this thesis, rests upon.

3.2. Abstract

Ubiquitin specific peptidase 2 (USP2) is a deubiquitinase with a diversity of functions in human physiology. One of the lesser studied functions of USP2 is its regulation of circadian rhythms, which are physiological rhythms with a period of about 24 hours. Previous studies have indicated a role for USP2 in photic entrainment, the process of the endogenous circadian rhythm synchronizing to environmental light information. In this study, we investigated the implication of USP2 in this process, using a *Usp2* knockout (KO) mouse model. We established that USP2 specifically controls entrainment of the clock to light in the early night. Further, we were able to show that the action of USP2 in modulating photic entrainment predominantly takes place in the suprachiasmatic nucleus, the seat of the central pacemaker in mammals. Finally, looking at the molecular basis of USP2 action, we found that induction of the light-responsive clock gene *Per1*, in response to light was diminished in *Usp2* KO mice. Thus, in this study we institute a key role for USP2 in mediating photic entrainment within the SCN.

3.3. Introduction

Circadian clocks are internal timing systems that enable organisms to anticipate and adapt their behavioral and physiological rhythms to the daily changes of their environment [1]. These clocks generate self-sustained oscillations at the cellular, tissue and behavioral levels. All organs in the body have their own circadian clocks to regulate local functions. The phase synchrony of all these clocks is maintained by a master pacemaker located in the suprachiasmatic nucleus (SCN), in the anterior hypothalamus of the brain [2]. Along with maintenance of bodily rhythms, the SCN is also able to integrate and adjust or entrain to variation in the external environment, such as changes in the light-dark cycle [2,3]. To entrain to photic stimuli, a subset of retinal ganglion cells, the intrinsically-photosensitive retinal ganglionic cells (ipRGCs), transmit photic information to the SCN by innervating the ventral part of the SCN via the retino-hypothalamic tract, leading to SCN firing and changes to downstream molecular pathways, which gives results in entrainment [3,4].

At the cellular level, circadian rhythms are maintained by a negative feedback loop of clock genes and proteins. Briefly, in the mammalian system, the clock proteins BMAL1 and CLOCK heterodimerize and activate the transcription of *Per1/2* and *Cry1/2* genes. The translated proteins themselves translocate to the nucleus, heterodimerize and repress the activity of the CLOCK/BMAL1. This entire process repeats with a periodicity of 24 hours [5–8]. A host of post-translational modifications (PTMs) on these clock proteins play a crucial role in determining the timing of their activity, as well as their localization [9,10]. Of these PTMs, ubiquitination and its counter process,

deubiquitination, play an especially important role in the circadian cycle, since the process of ubiquitination can affect both localization as well as the half-life of proteins [9–11].

Ubiquitin specific peptidase 2 (USP2) is a deubiquitinase (DUB) that has been shown to be involved in the circadian clock [12–14]. It is one of very few genes that is rhythmically expressed in all tissues [15–17], showing its tight regulation by the circadian clock. Further, USP2 has been shown to deubiquitinate the ubiquitinated forms of the clock proteins BMAL1 [18], PER1 [12,13] and CRY1 [19], thus showing its role in regulating the circadian clock. Earlier studies from our lab showed that it directly interacts with ubiquitinated PER1 and facilitates its localization to the nucleus [13]. In the absence of USP2 and normal nuclear translocation of PER1, mice showed an increase in period of endogenous rhythms as well as dysregulation of circadian light response [12]. More specifically, *Usp2* KO mice showed faster entrainment to phase delays and slower entrainment to phase advances, compared to WT mice [12]. However, despite having a diversity of functions within the circadian system, the specific role of USP2 in modulating circadian rhythms and photic entrainment haven't been studied. In this study, we investigated the spatio-temporal role of USP2 in the circadian system by understanding the time of USP2 action, as well, its role in modulating entrainment at the level of the retina and the SCN. We also looked at the molecular correlates of USP2 action within the circadian clock and in photic entrainment. We found that USP2 modulates early night photic entrainment of the circadian clock by promoting photic induction of *Per1* at the level of the SCN.

3.4. Methods

3.4.1. Animals

Usp2 KO mice as well as *Usp2* floxed mice [20] were obtained from Dr. Simon S. Wing and bred in house. While the *Usp2* floxed mice have LoxP sites before exon 3 and after exon 5, in the *Usp2* KO mice, exons 3, 4 and 5 are deleted[20]. The full body *Usp2* KO and their WT littermates (on a C57BL/6J background) were generated by breeding heterozygotes. Mice were weaned at 3 weeks of age, into cages containing no more than 5 mice per cage. They were maintained on a 12h:12h light: dark cycle (LD cycle) with light at ~300 lux. At 2 to 3 months of age, mice were designated to various experimental cohorts.

All procedures involving animals were carried out in accordance with guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committees of McGill University and Douglas Mental Health University Institute (protocol no. 2001-4586). For euthanasia at the end of the experiments, the animals were first anesthetized by isoflurane inhalation, followed by CO_2 euthanasia.

3.4.2. Wheel-running behavior

For running wheel experiments, 8- to 12-week-old WT and *Usp2* KO mice were singly housed in running wheel-equipped cages (Actimetrics, Wilmette, IL, USA), with ad-libitum access to food and water. All running-wheel data was analyzed using Clocklab software, version 6 (Actimetrics, Wilmette, IL, USA).

For the phase response curve (PRC), mice were maintained in a 12:12 LD cycle for 14 days, after which they were released into constant darkness (DD). On the first day

in DD, the mice were subjected to a 30-minute light pulse at one of the pre-determined timepoints. These time points were 10, 11, 12, 14, 16 and 20 hours after the start of the subjective day (denoted as CT 10, CT 11, CT 12, CT 14, CT 16 and CT 20). The wheel running activity was then recorded for 12 days. Phase shift of the rhythm was determined using regression analysis on activity onset data of the last 7 days of data and comparing it with the activity onset on the first day in DD. Advances were denoted as positive phase shifts and delays as negative phase shifts.

For the modified skeleton photoperiod protocol (mSPP), mice were maintained on a 12:12 LD cycle for 14 days. Then, the mice were exposed to a conventional skeleton photoperiod protocol [21–23] which consisted of one daily, 30-minute light pulse at the beginning of the day (M-light pulse) and one daily, 30-minute light pulse at the end of the day (E-light pulse). After 14 days in the skeleton photoperiod, the M-light pulse was eliminated, and mice were subjected to a 2-hour phase delay using only a daily, 30-minute E-light pulse. After 21 days, the mice were subjected to a 2-hour advance of the daily, 30-minute E-light pulse, for 21 days. The last 5 days of recorded data for each portion of the mSPP were used to assess two distinct entrainment parameters. The “Phase angle of entrainment” (PAE) or the amount of time it took the mouse to start running in the wheel after lights-off was averaged for each phase of the mSPP. Also, the “Interdaily stability” or the daily variability in time of activity onset was averaged for each phase of the mSPP. Since entrainment to E-light pulse was being quantified, mice that entrained to this light pulse as M-light pulse were excluded. Specifically, the exclusion criterion was as follows:

$$Avg\ PAE\ of\ last\ 5\ days\ in\ condition > Avg\ PAE\ of\ first\ 5\ days\ in\ condition * 1.1$$

3.4.3. Intra-cranial injections

The SCN of *Usp2* floxed mice were injected with an AAV vector containing a Cre recombinase enzyme in all cell types, to generate SCN specific *Usp2* KO mice. Briefly, the mice were anesthetized using isoflurane before being placed on the stereotaxic platform. The head was cleaned with iodine, the eyes were lubricated, carprofen (analgesic) solution was administered subcutaneously, and the head was then secured in place by ear-bars at the side, as well a nose piece at the front. The skin covering the skull was cut and the skull was cleaned. The nose piece was then adjusted to level the skull in the anterio-posterior axis. Starting at the bregma, the coordinates used for injections were X (Medio-lateral): ± 0.25, Y (anterio-posterior): -0.1, Z (dorso-ventral): -5.85. AAV solution (250 nl) was injected bilaterally, through holes drilled in the skull. The skin over the skull was closed using Vetbond, to allow for effective healing of the wound. Half of each *Usp2* flox litter used, was injected with AAV2/retro-CAG-Cre-eGFP (Canadian Neurophotonics Platform Viral Vector Core Facility (RRID:SCR_016477)) and the other half with AAV2/retro-CAG-Cre(δ)-eGFP (Canadian Neurophotonics Platform Viral Vector Core Facility (RRID:SCR_016477)), as a control (The δ-mutation is a loss of function mutation for Cre). After surgery, carprofen was injected for 3 days and mice were observed for any signs of pain, infection and/or post-surgical complications.

Three weeks after surgery, the mice were transferred into cages equipped with running wheels and they were subjected to the mSPP protocol. The average phase angle of entrainment was calculated for the last 5 days of data for each phase of the mSPP (as noted above, in the “Wheel running analysis” part of the Methods section). Mice with no GFP staining in the SCN were excluded from the analysis.

3.4.4. In-situ hybridization

Mice were group housed in regular housing cages and subjected to the mSPP protocol as described in the above “Wheel running behaviors” part of the Methods section. Ten days after the beginning of the phase advance, mice were anesthetized with a ketamine (100mg/kg)/xylazine (10mg/kg)/acepromazine (3mg/kg) cocktail 1 and 2 hours before the light pulse as well as 1 and 2 hours after the light pulse after which they were perfused with Z-fix (Anatech Ltd, Battle Creek, MI, USA). The perfused brains were dehydrated with 30% sucrose and frozen at -80ºC. These brains were sliced into 30µm sections encompassing the length of the SCN and subjected to in-situ hybridization.

Briefly, the slides were washed and then hybridized with the riboprobe (concentration: 400 ng/ml) diluted in the hybridization buffer (50% Formamide, 25% 20X SSC, 0.025% E. Coli tRNA, 10% Denhardt’s solution, 5% Salmon Sperm DNA, 10% DEPC-treated water) at 60ºC, overnight. For signal detection, after a series of wash steps using B1 solution (0.1 M Tris-HCl, 0.15 M NaCl in ddH_2O), the slides were incubated with blocking solution (10% FBS in B1 solution) for an hour at room temperature, followed by an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:7500 in blocking solution, Roche, Basel, Switzerland) for 2 hours at room temperature. After a series of washing steps using B1 solution (3x 20 minutes), followed by one wash with B3 solution (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2.5H_2O$ in ddH_2O), the sections were incubated with labelling mix (1.2 mg tetramisole, 11.25 µl nitroblue tetrazolium, and 17.5 µL 5-bromo-4-chloro-3-indolyl phosphate disodium salt per 5 ml of B3) for 48h in a dark, humidified chamber at room temperature. Once the color development was confirmed, the slides were washed with TE Buffer (10mM Tris-HCl and 1mM EDTA in ddH_2O) and

air dried. The slides were mounted with Fluoromount containing DAPI and covered with a glass coverslip. Images of the SCN were captured as stitches of 40x images, using a Zeiss Axio Observer Z1 Apotome microscope (Carl Zeiss microimaging GmbH, Gottingen, Germany).

The images were analyzed using QuPath software [24]. Briefly, subcellular detection was used to identify a cell as positive for the marker if staining was visible within 0.5 μm of the cell nucleus (which was identified using DAPI staining). This process was automated using a script written using the Groovy language in QuPath, to classify cells based on presence or absence of staining.

3.4.6. Pupillary light response

Mice were anesthetized using a ketamine (100 mg/kg)/xylazine (10 mg/kg) cocktail and their heads were stabilized using ear-bars. Using MVL7000 zoom lens (ThorLabs-MVL7000) with an Infra-Red (IR) sensor BlackFly S USB 3.0 (Model: BFS-U3-13Y3M-: 1.3MP, 170 FPS, ON Semi PYTHON 1300, Mono), the right eye was recorded. A 375nm LED (Thor labs, LED375L, USA) was placed 4 to 5mm away from the left eye, to provide photic stimulation to induce the pupillary constriction in response to light. The LED was controlled by using an Arduino Uno Rev3 (DigiKey-A000066) and a MATLAB script. The recording protocol was coded on MATLAB and involved 3 phases per trial and 3 trials per mouse. Each trial consisted of a 10s baseline phase with no light (baseline), followed by 5s of light stimulation (stim), followed by a 10s recovery period with no light (recovery). The size of the pupil, at each point in time, was quantified using a package on MATLAB called MEYE. This toolbox uses a Convolutional Neural Network (CNN) architecture

based on deep learning to track and measure the pupil size in real time [25]. The model has been trained with 11 897 grayscale images of human and mouse eyes, collected in dark environments using IR [25].

The quantified pupil sizes were normalized using the average pupil size during the baseline phase, for each mouse. Pupillary constriction was calculated as the percentage reduction of pupil size between the average pupil size during baseline and the lowest pupil size during stim.

$$\%\ Restriction = \frac{Average\ (normalized\ baseline)}{Minimum\ (normalized\ stim)} * 100$$

Also, the rate of recovery from pupillary constriction was calculated using a linear regression of the normalized pupil sizes during the stim phase. A larger slope value of the regression line shows faster recovery.

3.4.7. qRT-PCR

Mice were placed in 12:12 LD for 2 weeks, following which they were released into DD. After 24 hours in DD, the mice were sacrificed using cervical dislocation at different time points across the circadian day, specifically, CT 2, CT 8, CT 14 and CT 20. The mice were then enucleated, and their retinas were collected, and flash frozen using a 2-methyl butane bath in dry ice. Total RNA was extracted using TRIzol reagent (Invitrogen, Villebon sur Yvette, France). Total RNA was reverse transcribed using random primers and MMLV Reverse Transcriptase (Invitrogen, Villebon sur Yvette, France). Quantitative real-time reverse transcription PCR (qRT-PCR) was then performed on a LightCycler system (Roche Diagnostics) using the light Cycler-DNA Master SYBR Green I mix. The PCR

reaction was performed with 1 µl of cDNA diluted at 1 in 50, supplemented with 0.75 µl of LightCycler FastStart Enzyme and LightCycler FastStart Reaction Mix SYBR Green (Roche Diagnostic), 0.8 µl of forward and reverse primers (10 µM; Eurofins, Ebersberg, Germany), 1.2 µl of $MgCl_2$ (3 to 5 mM; Roche Diagnostic) in a total volume of 10 µl. The thermal cycler conditions were as follows: 8 min at 95ºC, and then 45 cycles of denaturation at 95ºC for 15 s, annealing at 68ºC for 15 s and the temperature was reduced by 0.5ºC each cycle until 60ºC. Hypoxanthine ribosyl-transferase (*Hprt*) was used for internal standardization of target gene expression. The efficiency and the specificity of the amplification were controlled by generating standard curves and carrying out melting curves. Relative transcript levels of each gene were calculated using the second derivative maximum values from the linear regression of cycle number versus log concentration of the amplified gene. Primer sequences are provided in Supplementary table 1.

3.4.9. Statistical analysis

Data were analyzed and plotted on GraphPad Prism 9. To compare within subject data in different lighting conditions (different shifts for mSPP, different light pulse timings in the PRC) and different genetic manipulations (*Usp2* KO vs WT or AAV-Cre vs AAV-Scrambled), mixed-model 2-way ANOVA tests with Greenhouse-Geisser correction were used. The correction was used to account for the violation of sphericity in the data sets. Holm-Sidak post-hoc tests were used to compare between genotypes. For the PLR data, two tailed t-tests were used for % Restriction and % Recovery. Cosinor analysis of timepoint data was used to check for rhythmicity of the different genes using the qRT-

PCR data of the retina, but the two genotypes themselves were not compared, if they were both rhythmic. For Cosinor analysis, the data points were fit to the following function:

$$Y = B + A * \cos\frac{2 * \pi * (X - P)}{24}$$

$$B = Baseline\ or\ MESOR$$

$$A = Amplitude\ of\ rhythm$$

$$P = Phase\ of\ rhythm$$

The data points were then fit to a straight line with a slope of 0 (to represent an undamped oscillation), $y = c$. The two fit functions were compared, and the data points were said to have a rhythm if the Cosinor fit significantly differed from the straight line fit. Differences were considered significant for all statistical tests if $p < 0.05$

3.5. Results

3.5.1. USP2 differentially affects photic delays at different times of night

To understand the role of USP2 in modulating the photic response of the clock, a PRC focussing on the early night was built (Figure 1A). A two-way ANOVA of this data showed a significant interaction between genotype and pulse timing ($F(7,77) = 3.568$, $p = 0.0022$). While there were no differences between WT and *Usp2* KO mice at CT 10 and CT 11, at CT 12, WT mice showed a delay (One sample $t(4) = 5.086$, $p = 0.0070$), as expected[21], while *Usp2* KO mice showed no delay (One sample t-test $t(3) = 0.4049$, $p = 0.7127$) (Figure 1B). Additionally, this difference in phase shifts between WT and *Usp2* KO mice was significant (post-hoc $p = 0.0302$). Later in the night, this trend reversed, with *Usp2* KO mice showing a significantly larger delay than WT mice at CT 14 (post-hoc $p = 0.0191$) (Figure 1B). The phase shifts following a light pulse at CT16, CT18 and CT20 showed no differences between genotypes. Thus, USP2 seems to differentially influence early night phase shifting of the murine circadian clock in response to light.

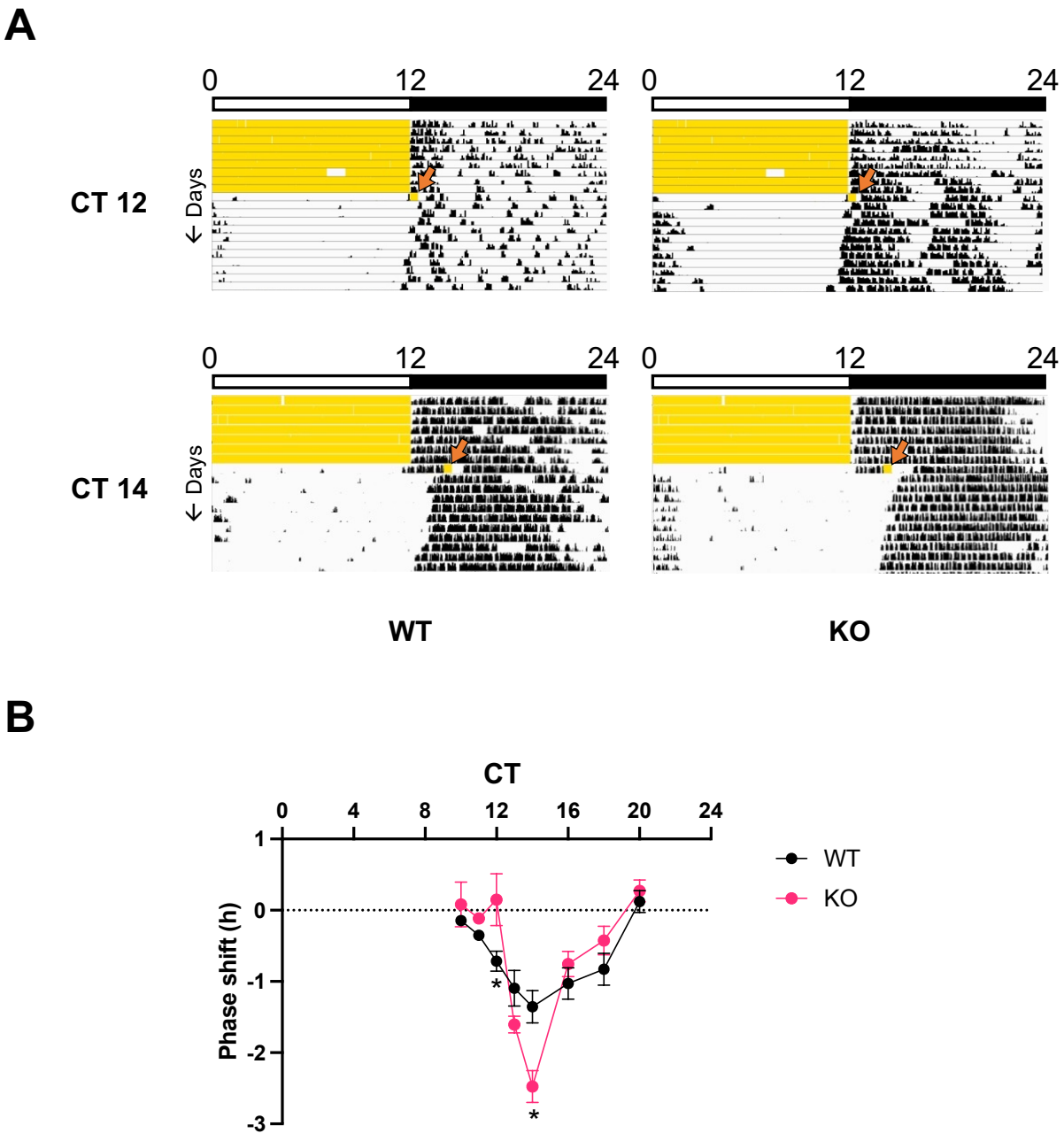

Figure 1: USP2 differentially affects entrainment at different times of night.

(A) *Representative actograms for wheel-running activity of WT and Usp2 KO mice light pulsed at CT 12 or CT 14, to make the phase response curve* ***(B)*** *for Usp2 KO mice. Individual data points represent individual mice (n = 3 to 8 mice per group) and data are represented as mean ± SEM. Two-way ANOVA, *: p < 0.05.*

3.5.2. USP2 affects entrainment to an early night light cue

We then studied the entrainment of *Usp2* KO and WT locomotor activity rhythms under a modified skeleton photoperiod (mSPP) paradigm (Figure 2A). In a regular skeleton photoperiod protocol (SPP), after a baseline 12:12 LD phase, mice are subjected to short light pulses, twice daily: once at the beginning and once at the end of the day [22,26,27]. However, previous studies have shown that the subjective dusk pulse (also called the entraining pulse) is sufficient to entrain the circadian clock of animals with an endogenous period < 24 hours to a 24-hour rhythm [28]. Hence, in order to look at entrainment exclusively in the early night, mice were subjected to phase shifts using a single, daily, 30-minute light pulse via the mSPP protocol.

The stability of entrainment was measured by the inter-daily stability (IS), which quantifies the variance in daily activity patterns [29,30]. There was no interaction between lighting and genotype for IS ($F(3,84) = 0.22$, $p = 0.8825$), but there was a strong main effect of lighting ($F(2.22, 62.3) = 12.8$, $p < 0.0001$), which is expected due to the reduced photic information being supplied, coupled with the phase shifting. Another measure of entrainment is its fidelity, assessed by the phase angle of entrainment (PAE), which measures the relation of the endogenous rhythm to the external zeitgeber, by quantifying the time difference between lights off and the start of activity of the mouse [26,31]. There was a significant interaction of lighting and genotype for this measure ($F(3,69) = 23$, $p < 0.0001$). There were no differences between genotypes in either of the measures, in either the baseline LD or the SPP condition (Figure 2B and 2C), showing that both genotypes can perceive light and synchronize well to a 24-hour rhythm. In the delay condition of the mSPP, however, there was a significant difference in the PAE (post-hoc

p = 0.0224) (Figure 2C), with *Usp2* KO mice showing lower PAE, but no difference in IS (Figure 2B). In the mSPP advance condition as well, there was a significant difference in PAE (post-hoc p = 0.0010) (Figure 2C), with *Usp2* KO mice showing a lower PAE, but no difference in IS were observed (Figure 2B). Hence, USP2 seems to affect fidelity of entrainment in the early night.

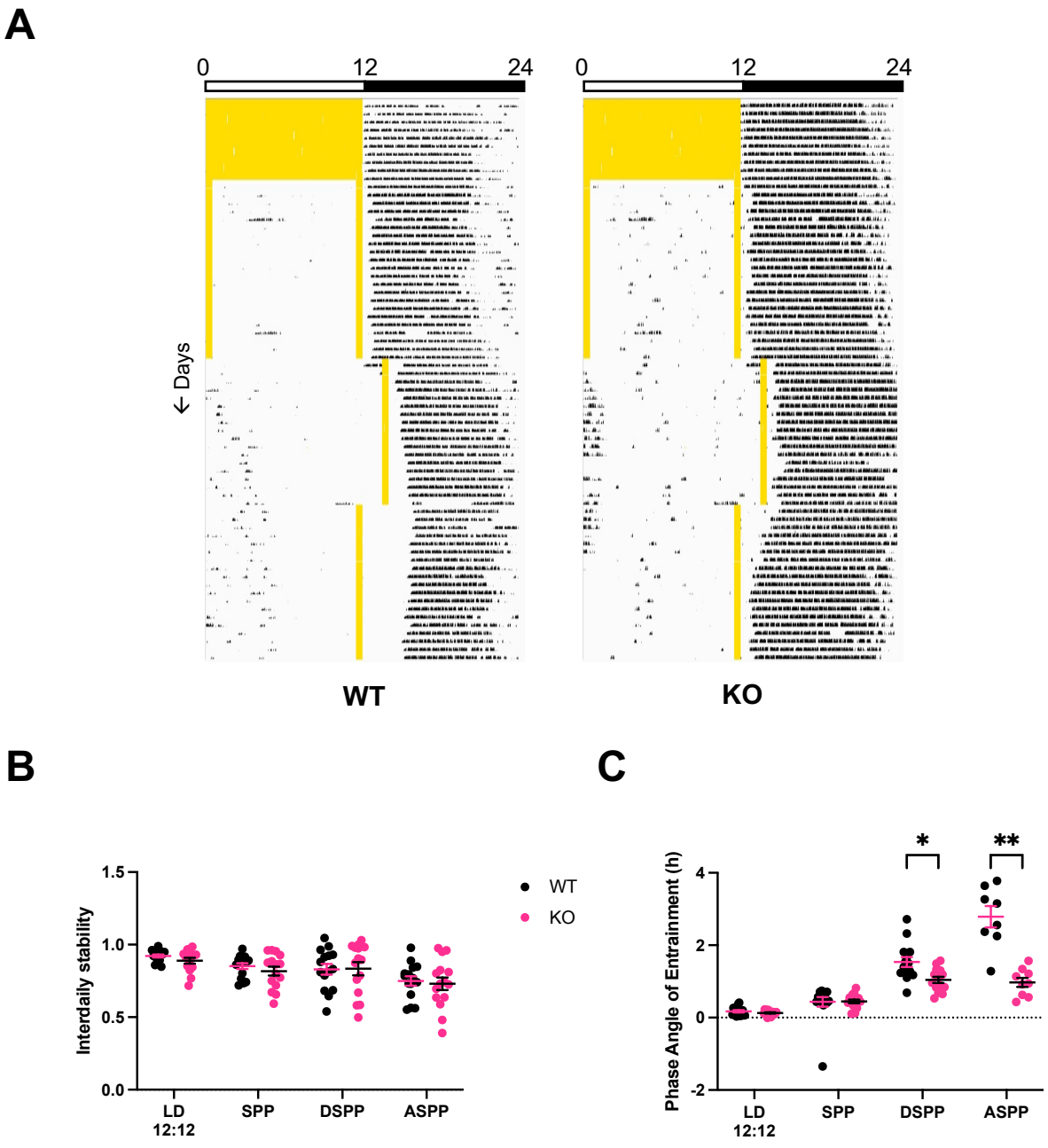

Figure 2: USP2 controls early night photic entrainment, independent of direction of phase shift. (A) *Representative wheel running actograms for WT and Usp2 KO mice going through the modified skeleton photoperiod protocol.* ***(B, C)*** *Quantification of two parameters of entrainment, Interdaily stability* ***(B)*** *and Phase angle of entrainment* ***(C)****. SPP = skeleton photoperiod, DSPP = phase delay with mSPP, ASPP = phase advance with mSPP. Individual data points represent individual mice (n = 8 to 15 mice per group) and data are represented as mean ± SEM. Two-way ANOVA, *: p < 0.05, **: p < 0.01*

3.5.3. USP2 regulates the photic induction of *Per1*

To assay the effect of USP2 on entrainment at the molecular level, *in-situ* hybridization of *Per1* was carried out during the advance phase of the mSPP. *Per1* is a light-responsive clock gene which is induced within the SCN, in response to light at night [32]. For this experiment, mice were sacrificed and perfused 10 days into the advance phase of the mSPP protocol, 2 hours before, 1 hour before, 1 hour after and 2 hours after the mSPP dusk light pulse. The SCNs of these mice were labelled with *Per1* riboprobes (Figure 4A). *Per1* was induced by the light pulse, with increased *Per1* labelling in the 1-hour post-light pulse SCNs, compared to the other samples, as expected [7,32,33] (Figure 4A, 4B). However, *Per1* induction was blunted in the *Usp2* KO SCNs, with the staining 1-hour post-light pulse being significantly lower than in WT SCNs. Thus, USP2 modulates photic entrainment by acting on the photo-responsive pathways of the clock.

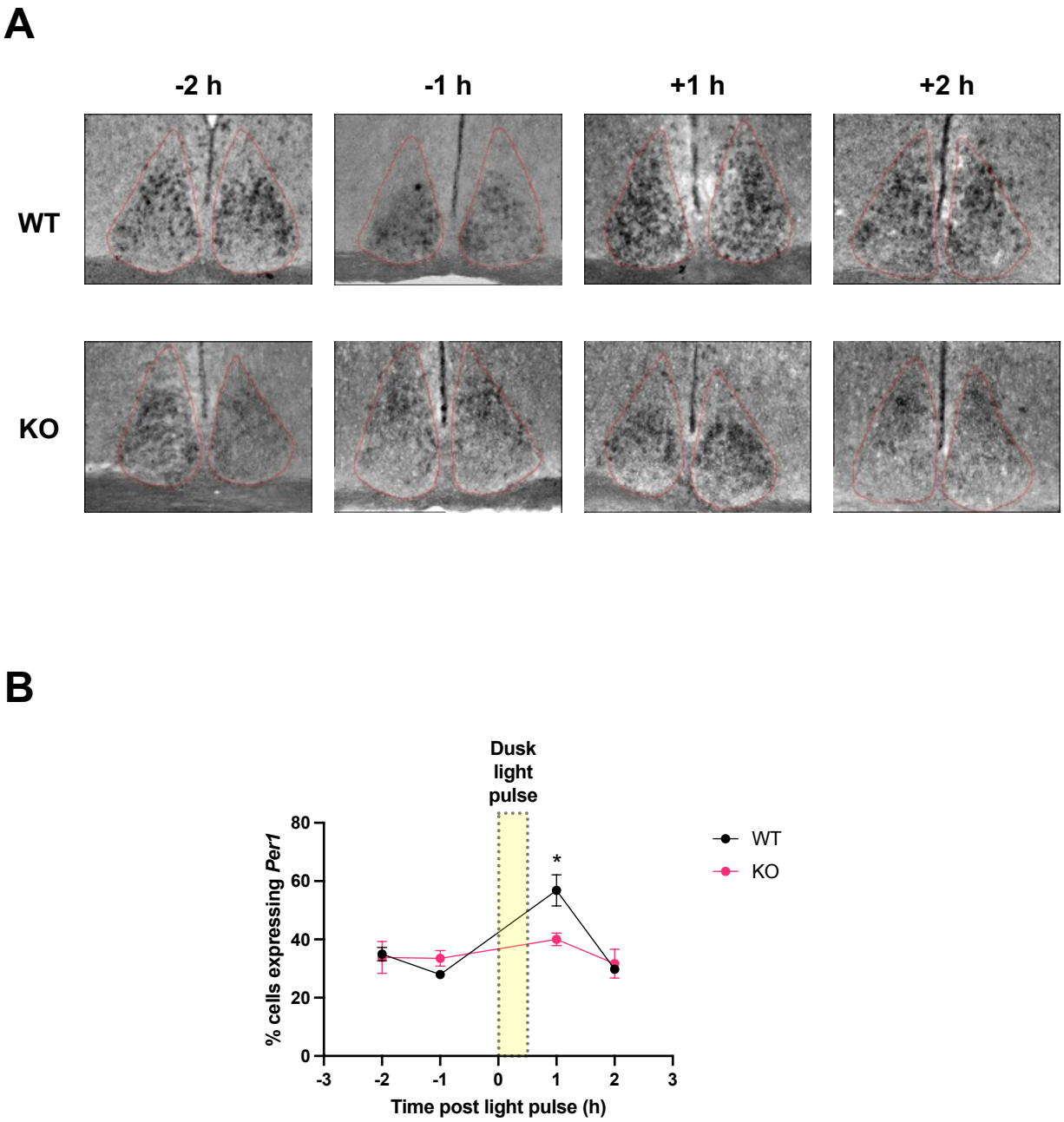

Figure 3: Photic induction of Per1 is abrogated in the absence of USP2. (A) *Representative images for DIG-labelled Per1 riboprobes at different time points around the modified skeleton photoperiod dusk light pulse. The red line represents SCN ROI* ***(B)*** *Quantification of Per1 staining. Individual datapoints represent*

*individual mice (n = 3 to 6 mice per group) and data are represented as mean ± SEM. Two-way ANOVA, *: p < 0.05.*

3.5.4. USP2 acts at the level of the SCN to modulate its effect on photic entrainment

Photic entrainment of circadian rhythms can be regulated at the level of the retina or at the level of the SCN. To investigate the location of USP2 action, *Usp2* was first knocked out specifically in the SCN (Figure 3A). Towards this aim, the SCNs of *Usp2* floxed mice were injected with a pan-cellular Cre recombinase containing adenovirus (AAV-Cre-GFP) (Figure 3B). When these mice were subjected to the mSPP protocol (Figure 3C), the phenotypes seen in the full-body KOs were replicated in the AAV-Cre mice. A 2-way ANOVA of this data showed no interaction, but main effects of lighting ($F(2.59, 44) = 23.9$, $p < 0.0001$) and injection ($F(1,17) = 5.22$, $p = 0.0354$) were observed. Compared to the Cre(δ) adenovirus injected controls (AAV-Sham-GFP), there were no differences in PAE in the AAV-Cre mice during the LD as well as the SPP conditions (Figure 3D). Despite not being significant, the average PAE was apparently lower in the delay phase (Figure 3D). Finally, in the advance phase, the SCN-Cre mice showed significantly lower PAE compared to the AAV-Sham mice (post-hoc $p = 0.0439$) (Figure 3D). Since the phenotypes of the whole body *Usp2* KO mice and SCN-specific *Usp2* KO mice are comparable, we can conclude that the USP2 in the SCN is necessary for normal photic entrainment.

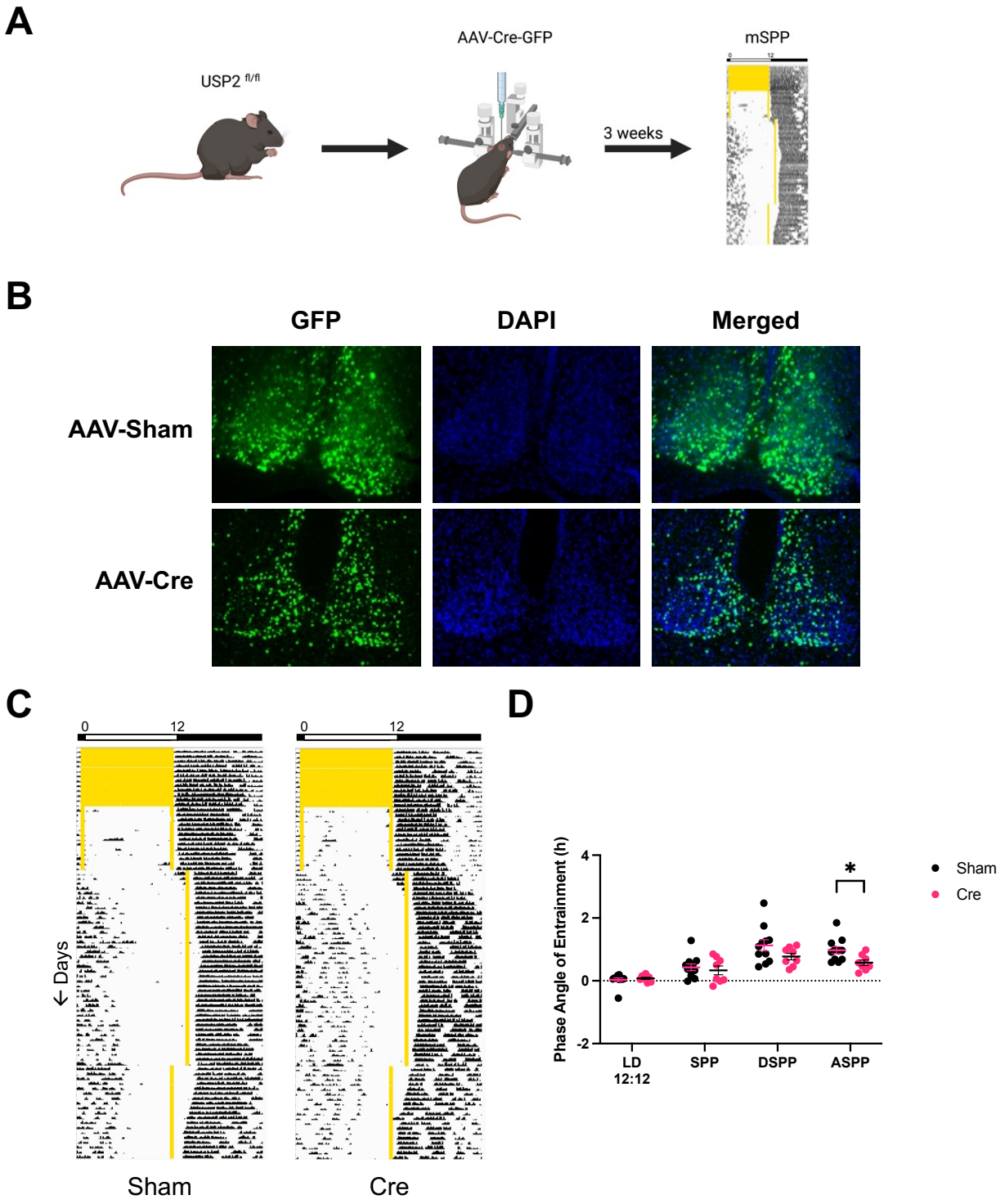

Figure 4: SCN-specific Usp2 KO mice mirror the photic entrainment phenotypes of full body Usp2 KO mice. (A) *Workflow to assess entrainment in*

SCN-specific Usp2 KO mice (image created using BioRender.com). ***(B)*** *Representative images showing SCN-specific targeting of AAV-Cre-GFP and AAV-Sham-GFP (green channel).* ***(C)*** *Representative wheel-running actograms of AAV-Sham and AAV-Cre injected mice subjected to the modified skeleton photoperiod protocol.* ***(D)*** *Quantification of phase angle of entrainment. SPP = skeleton photoperiod, DSPP = phase delay with mSPP, ASPP = phase advance with mSPP. Individual data points represent individual mice (n = 8 to 10 mice per group) and data are represented as mean ± SEM. Two-way ANOVA, *: p < 0.05.*

3.5.5. USP2 affects the rhythms of clock genes in the retina

While USP2 affects entrainment at the level of the SCN, it could also affect entrainment at the level of the retina. To find a basis for USP2 action in the retina, we looked at the effect of USP2 on retinal gene rhythms. To profile the patterns of gene expression in the retina, *Usp2* KO and WT mice were sacrificed at CT 2, 8, 14 and 20, and the RNA extracted from their retinas was subjected to qRT-PCR. Along with *Usp2*, we assayed the clock genes, *Per1, Per2, Bmal1, Clock, Cry1, Cry2, Dbp, Dec1, Dec2, Rev-erbα, Rorγ and E4bp*, as well, retinal function genes *Opn1sw, Opn1mw, Opn2* and *Opn4*. The clock genes *Per1, Bmal1, Cry1, Rev-erb*α and *Dec1* (Figure 5A), showed greater rhythmicity in retinas of *Usp2* KO mice compared to WT mice. Similar observations were made for the retinal function genes *Opn4* and *Opn1sw* as well (Figure 5A, Supplementary figure 1A). Additionally, while *Usp2* wasn't detected in *Usp2* KO retinas, a robust rhythm of *Usp2* was detected in WT retinas (Supplementary figure 1B), as expected, but with the opposite phase [15].

While most other genes assayed showed no significant rhythms, they all varied over time (Supplementary figures 1 and 2). Additionally, the genes were more rhythmic in KO retinas (lower p-value), compared to WT retinas (Supplementary figures 1 and 2), similar to the rhythmic genes in the KO retinas of Figure 5.

Similar to the trends of gene expression in our WT retinas, other studies [34,35] have also shown a lack of strong rhythms in whole retina qRT-PCR due to the presence of multiple oscillators within the retina, not in phase with each other. This points towards a role for USP2 in helping to maintain phase synchrony of the various oscillators in the retina.

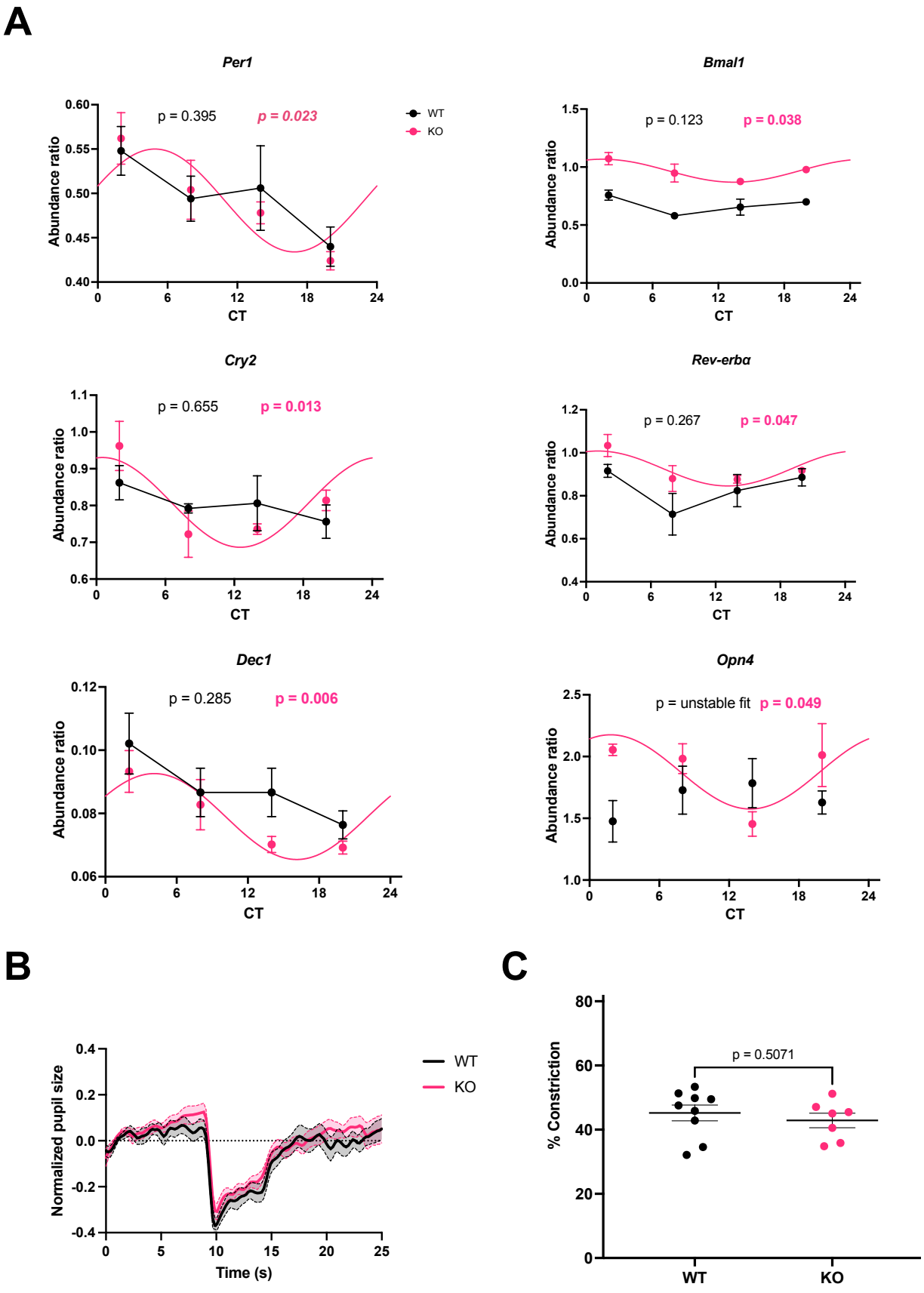
A
Per1
p = 0.395
p = 0.023
WT
KO
Abundance ratio
CT
Bmal1
p = 0.123
p = 0.038
Cry2
p = 0.655
p = 0.013
Rev-erbα
p = 0.267
p = 0.047
Dec1
p = 0.285
p = 0.006
Opn4
p = unstable fit
p = 0.049
B
Normalized pupil size
Time (s)
WT
KO
C
% Constriction
p = 0.5071
WT
KO

Figure 5: USP2 affects rhythmic gene expression in the retina, but not ipRGC functioning. (A) *Quantification of gene expression rhythms of some clock genes and some retina function genes in the retina. All data are represented as mean ± SEM (n = 4 to 5 retinas per group). Significant rhythms are represented by a continuous curve; non-significant rhythms are represented by dots connected by lines; unstable fits are represented by points not connected to each other.* ***(B)*** *Average pupillary constriction throughout the PLR experiment. The lighter umbra around the line represents SEM.* ***(C)*** *Quantification of percentage constriction of the pupil, in response to the light stimulation. Individual datapoints represent individual mice (n = 7 to 9 mice per group) and data are represented as mean ± SEM. Two tailed t-test, ns.*

3.5.6. USP2 does not affect ipRGC functioning

An interesting result in the above screen for gene rhythmicity in the retina is *Opn4*. *Opn4* is a marker for ipRGCs, which project to the SCN and convey photic information from the retina. The ipRGCs then further also project to the Olivary pretectal nucleus (OPN), which controls the Pupillary light constriction response (PLR) in response to light intensity. Thus, we used PLR as a proxy for ipRGC function. When subjected to a light stimulation, no differences were seen between the genotypes during constriction (Figure 5C, 5D) ($t(14) = 0.6808$, $p = 0.5071$), or in the process of recovery (Figure 5E) ($t(14) = 1.012$, $p = 0.3286$). Thus, USP2 doesn't seem to affect the functioning of ipRGCs in the retina.

3.6. Discussion

Our study is the first step towards understanding how the circadian DUB USP2 modulates entrainment of the circadian clock to photic stimuli. Our data shows a definitive role for USP2 in mediating entrainment of the circadian clock to photic phase shifts, especially in the early night. Further, USP2 acts at the level of the SCN, to modulate entrainment by affecting the transcription of the light-responsive clock gene *Per1*.

A 2012 study from our lab was among the first to indicate that USP2 played a role in the circadian clock and in photic entrainment [12]. To examine the effect of USP2 on entrainment, Yang and colleagues constructed a PRC, with more light pulses in the late-night phase than the early night phase. However, in response to the early night pulse at CT 14, they found a large phase shift [12]. Hence, in this study, we built a high-resolution PRC focussing on the early evening, between CT 10 and CT 16. Interestingly, we found that USP2 seemed to have different roles in entrainment at different times of the early night. While the phase delay was significantly higher in *Usp2* KO mice at CT 14, compared to WT mice, at CT 12 we observed a lack of phase shifting in *Usp2* KO mice, while WT mice showed a small phase delay, as expected [36,37].

With the knowledge that USP2 had different mechanisms of action in the early night, we created the mSPP protocol, to test entrainment to advances as well as delays using only a dusk zeitgeber. Studies have shown that a daily single dusk light pulse is sufficient to entrain a mouse to a 24-hour rhythm [22,37]. It directly follows that this daily single light pulse should also be able to phase shift mice like a 12:12 LD cycle. Since this is a modification of the conventional skeleton photoperiod (SPP), we termed it the modified SPP (mSPP). Unlike a single light pulse in a PRC, by sustaining the dusk light

pulse over multiple days, the light exposure of the clock is restricted to the dusk, thus ensuring entrainment to both advances and delays are both due to the light at dusk. This protocol allowed us to distinguish whether the effect of USP2 was on entrainment to phase delays or entrainment to a dusk light pulse, irrespective of direction of shift.

Most interestingly, irrespective of direction of shift, *Usp2* KO mice entrained better to light early at night. Additionally, entrainment to advances was faster in *Usp2* KOs subjected to mSPP unlike the result shown by Yang et al [12], which showed slowed entrainment to phase advances by *Usp2* KO mice. Thus, it could be argued that despite both studies phase advancing the mouse clock, USP2 affects entrainment differently to the dawn zeitgeber of Yang and colleagues [12] compared to the dusk zeitgeber in this study, due to which different phenotypes were observed. Crucially, these phenotypes were replicated in SCN-specific *Usp2* KO mice, showing that the SCN is essential for the role played by USP2 in photic entrainment.

In the context of the PRC results, the results obtained in the mSPP phase advance could be explained as follows: the light pulse might hit both genotypes in the dead zone of the PRC and does not elicit a reaction from the clock for the first few days. However, as the mice free run, the light pulse would soon correspond to ~CT 12 on the endogenous clock, leading to a slight phase delay in the WT mice, while the *Usp2* KOs are not affected. This process loops for the WT mice, leading to a maintained larger phase angle of entrainment, thus leading to an appearance of *Usp2* KO mice entraining with better fidelity to the light pulse (and mSPP in specific) than the WT mice.

What could the molecular basis of this entrainment response entail? *Per1* is a light-induced clock gene which has been shown to be essential for phase shifting [38–40].

Per1 induction directly correlates with phase shifts, especially in the early night [38,39]. Thus, we examined *Per1* accumulation in response to the mSPP phase advance light pulse. While WT mice showed robust photic induction of *Per1*, *Usp2* KO mice showed blunted *Per1* induction in response to light. This finding adds to the available literature of USP2's regulation of PER1. Previous studies have shown that PER1 is deubiquitinated by USP2, under homeostatic conditions [12]. While this deubiquitination does not affect stability of PER1, it promotes nuclear localization of PER1 [12,13], which is a key step in the negative arm of the circadian cycle. The blunted *Per1* induction in our experiments, along with a reduction in the nuclear localization of PER1 protein points towards a lack of photic feedback to the clock, which could account for the lack of phase shifting and better entrainment in the early night.

Better entrainment has been shown to be a hallmark of disruption in clock gene expression. For example, SIK1 is a protein involved in modulating the duration of *Per1* induction in response to light at night. SCN specific knockdown mouse models of *Sik1* have shown faster entrainment to advances than their WT counterparts [41]. Similarly, knockout models of Casein kinase 1ε (CK1ε), a kinase of PER1/2/3 [42], showed faster entrainment to phase shifts as well [43]. A KO model of GCN2 showed almost instantaneous entrainment to phase advances. GCN2 is a conserved kinase of eIF2α, which is involved in *Per1* and *Per2* induction [44]. The common factor in all these threads, unsurprisingly, is the dysregulation of the light responsive clock gene *Per1*. In the absence of normal levels of photic *Per1* induction, environmental light information does not reach the circadian feedback loop, thus preventing effective entrainment. Similarly, our phenotype of better photic entrainment of the clock is also correlated to the

dysregulation of the photic induction of *Per1*, pointing to deficits in photic induction in the absence of USP2.

This deficit could stem from faulty photoreception by the retina or due to impairments in reception of photic information at the level of the SCN. Thus, we asked if USP2 could be affecting the retina clock, and hence, photoreception in the retina. Clock genes in the whole retina do not show rhythmicity, due to different layers and cell-types having different circadian phases [34,45]. Compared to WT retinas, *Usp2* KO retinas showed robust rhythmicity of multiple clock and clock-controlled genes, as well as genes involved in retinal photoreception, suggesting a role for USP2 in regulating the phase relation between various oscillators in the retina.

Do deficits in the retinal clock translate to deficits in photoreception at the level of the retina? Intrinsically photosensitive retinal ganglionic cells (ipRGCs) make up about 1% of all retinal ganglionic cells [46,47]. These cells are responsible for carrying the photic information from the retina to the SCN via the retino-hypothalamic tract (RHT). The RHT then continues further to innervate the olivary peptic nucleus (OPN), which controls the constriction of the pupil in response to bright light [4,48,49]. Hence, the functioning of the ipRGCs can itself be used as a proxy for the efficiency of photic information transfer for non-image forming functions, including circadian photoreception at the level of the SCN. In the PLR test, the constriction of the pupil and its recovery after exposure to the light pulse did not vary between WT and *Usp2* KO mice. Thus, the lack of USP2 did not seem to affect ipRGC functioning, further solidifying the role of USP2 in photo-entrainment within the SCN.

In conclusion, USP2 affects circadian rhythms in multiple tissues. However, its role in modulating the photo-entrainment response seems to be localized to the SCN. Previous studies have shown that USP2 directly interacts with PER1 and affects its localization [13]. Our data suggests USP2 exerts an additional layer of control on PER1 by promoting *Per1* transcription in response to light. The mSPP data points towards increased fidelity of entrainment, which is generally a function of reduced robustness in the phase coupling within the SCN. Put together, we could posit that the absence of USP2 in the early night abrogates the phase synchronization of various oscillators within an organ, thus leading to unhindered entrainment.

Deubiquitinases tend to have multiple targets in the mammalian system, due to which USP2 could be acting on multiple players in the circadian light response pathway, which contributes to photic *Per1* induction. Thus, it would be important to investigate the role of USP2 in these pathways, to fully understand the modulation of photic response in the SCN by USP2. Additionally, while our data points to the necessity of the SCN for USP2's role in entrainment, it is interesting to note that the ipRGCs also innervate the intergeniculate leaflet (IGL), which has a role to play in entrainment to SPPs. Interestingly, the IGL is only region directly innervated by ipRGCs, which projects to the SCN. Hence, it would also be important to investigate the requirement of the IGL and the geniculothalamic tract for the modulation of photic entrainment by USP2.

3.7. Acknowledgements

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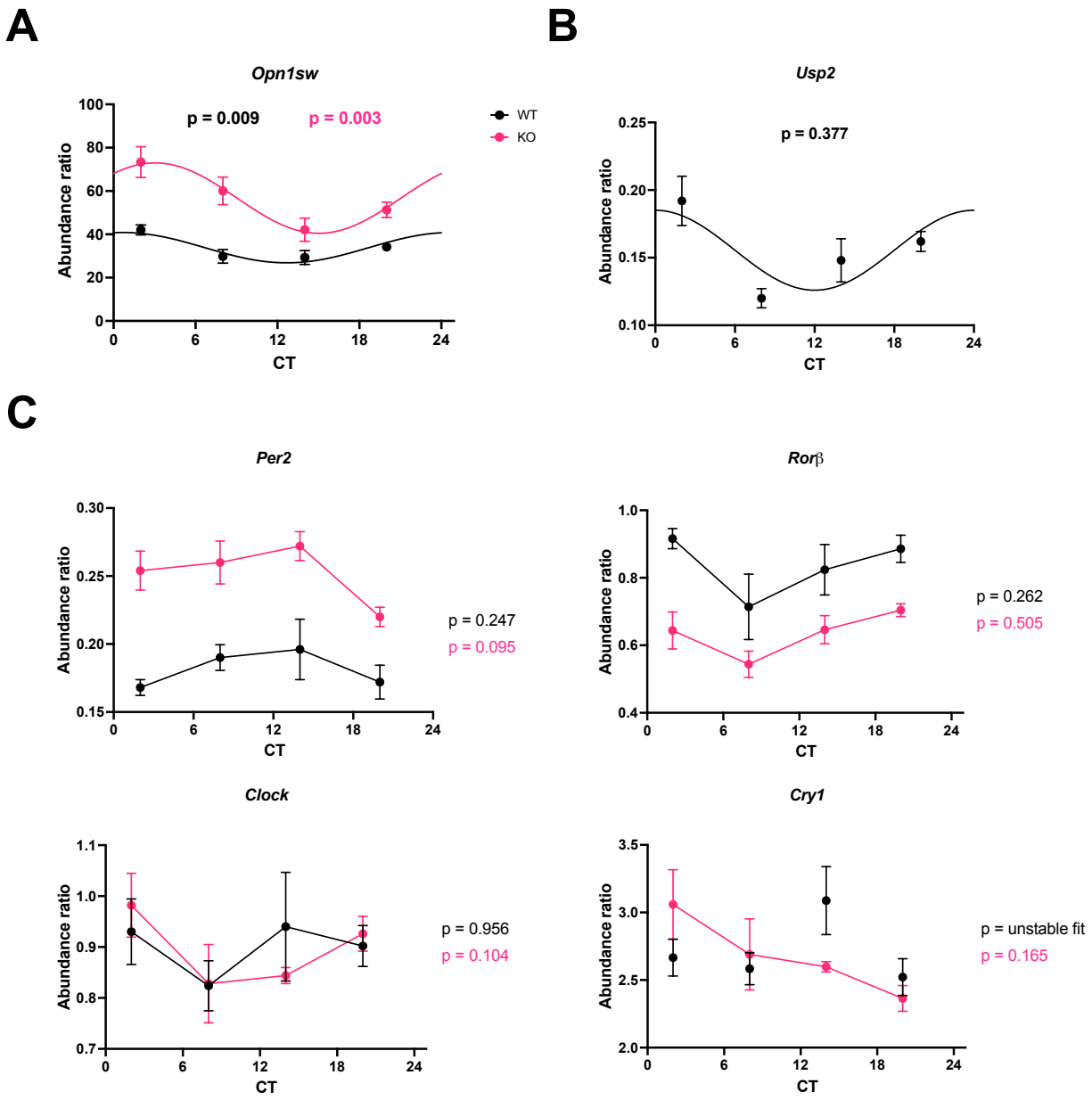
3.9. Supplementary data

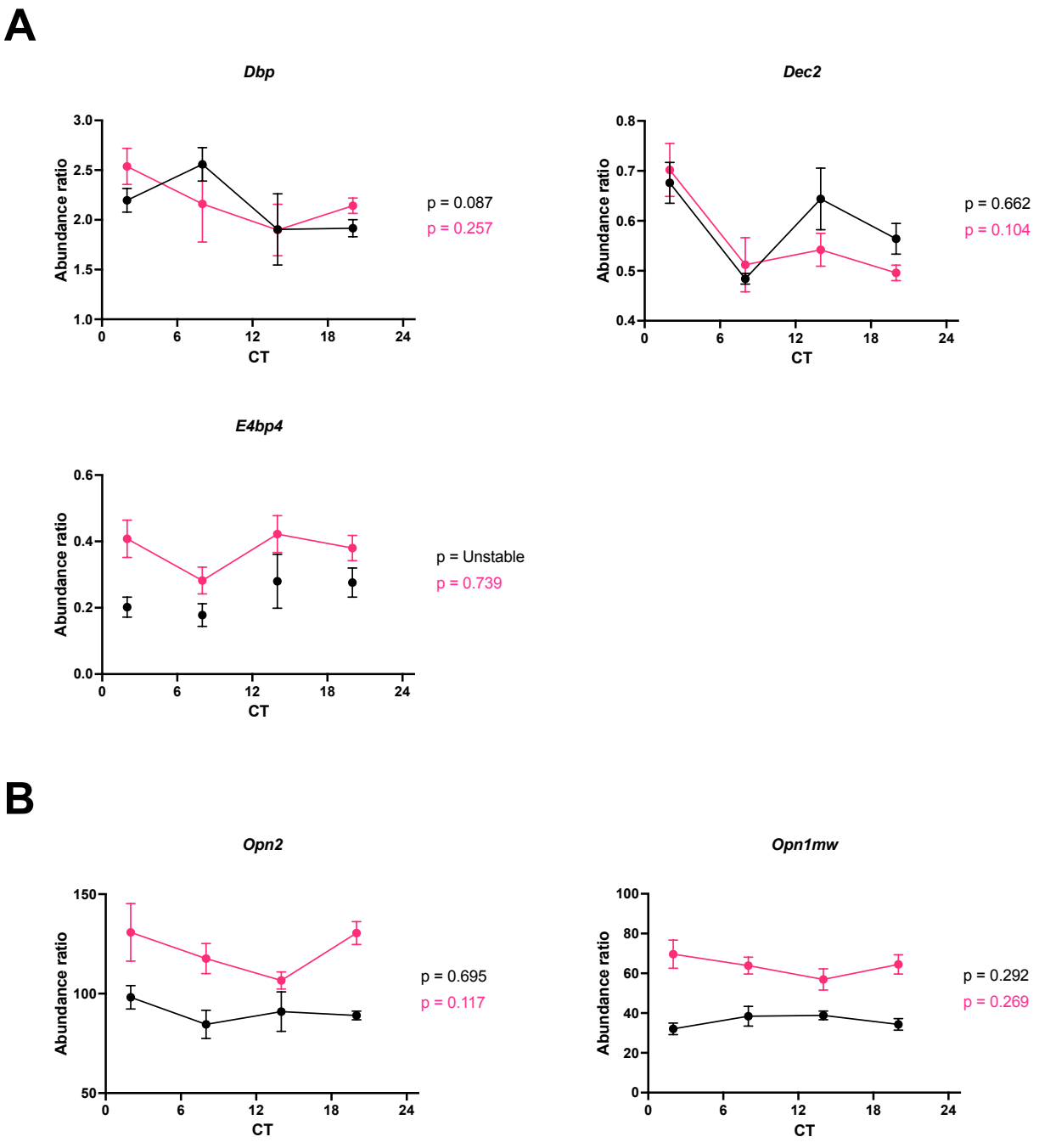
Primer	Forward	Reverse
Hprt	ATCAGTCAACGGGGGACATA	AGAGGTCCTTTTCACCAGCA
Clock	GTTTGATCACAGCCCAACTC	CTCCGCTGTGTCATCTTTTC
Bmal1	CTCAGCTGCCTCGTTGCAAT	GCTGTCGCCCTCTGATCTAC
Per1	GCGTTGCAAACGGGATGTGT	GAACCTGCAGAGGTGCCAG
Per2	CCACACTTGCCTCCGAAATA	ACTGCCTCTGGACTGGAAGA
Per3	CAGTGGCAGAGACGTGCGT	GACACTGTCGATACAGTTCAT
Cry1	GCCAGCTGATGTATTTCCCAG	CGCCAGCCTCAGTAGCCAG
Cry2	GAGAGACCTCGGATGAATGC	CTCGCCACAGGAGTTGTCCA
Rev-erbα	GCTCCATCGTTCGCATCAAT	CTAGAGGGCACAGGCTGCT
Rorβ	GCGAGCACAAATTGAAGTGA	AACGGTTCCTGTTGGTTCTG
E_4BP_4	CGGAAGTTGCATCTCAGTCA	GCAAAGCTCTCCAACTCCAC
Dbp	CGTGGAGGTGCTAATGACCT	CGGCTCCAGTACTTCTCATC
Dec1	AAAAGAGACGTGACCGGATTAA	AGTCTGGAAACCTGAGCAGAAC
Dec2	CGAGACGATACCAAGGATACC	GGC TGT TAG CGT TTC AAGT
Opn4	TGCGAGTTCTATGCCTTCTG	GGCACGTAGGCACTCCAAC
Opn1mw	GCTGCATCTTCCCACTCAG	GACCATCACCACCACCAT
Opn1sw	CAGCCTTCATGGGATTTG	GTGCATGCTTGGAGTTGA
Opn2	GCCACCACTCAGAAGGCAG	GATGGAAGAGCTCTTAGCAAAG
Usp2	CTCCACCCTGAAGCGCTATA	CCCCTGTCACAGTCCAGAAT

Supplementary table 1: List of primers used for qRT-PCR of genes in the retina.



Supplementary figure 1: Quantification of more gene expression rhythms in the retina. ***(A)*** *Short wave opsin, Opn4sw is rhythmic in Usp2 KO retinas but not WT retinas.* ***(B)*** *Usp2 is rhythmic in WT retinas.* ***(C)*** *Quantification of more clock gene rhythms in the retina. All data are represented as mean ± SEM (n = 4 to 5 retinas per group). Significant rhythms are represented by a continuous curve;*

non-significant rhythms are represented by dots connected by lines; unstable fits are represented by points not connected to each other.



Supplementary figure 2: Quantification of gene expression rhythms of circadian and retina function genes in the retina. *Gene expression rhythms of* ***(A)*** *circadian genes and* ***(B)*** *opsins of rods (Opn2) and cones (Opn1mw) were quantified. All data are represented as mean ± SEM (n = 4 to 5 retinas per group). Non-significant rhythms are represented by dots connected by lines; unstable fits are represented by points not connected to each other.*

Chapter 4: The deubiquitinase USP2 modulates photic entrainment by affecting photic response pathways in the SCN

The deubiquitinase USP2 modulates photic entrainment by affecting photic response pathways in the SCN

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Manuscript ready for submission

4.1. Preface (Bridging text before proteomics paper)

In the previous chapter, we were able to establish the spatial and temporal role of USP2 in the photic entrainment of circadian rhythms. We showed that USP2 affects early-night photic induction of the light-responsive clock gene *Per1*, within the SCN. In the following chapter, we attempted to tease out the molecular basis for this action of USP2.

In this third manuscript of the thesis, we aimed to assay for rhythms of USP2 in different neuron types in the SCN and to look for specific effects of USP2 on the neurons receiving photic information from the retina. Further, since USP2 is a DUB which modifies proteins post-translationally, we aimed to study the specific pathways affected acutely by USP2 in response to light in the early night.

Our results pointed to three main conclusions: 1) *Usp2* expression varies over time in the SCN, and the pattern of this variation is different in different sets of neurons. 2) The absence of USP2 in VIP-expressing neurons, which are innervated by the retina, doesn't replicate the phenotypes of the whole SCN *Usp2* knockout. 3) The activation of the MAPK/ERK cascade and EIF2-mediated protein translation pathways, which are induced in response to light, are abrogated in the absence of USP2.

This work not only shows a role for USP2 in modulating photic entrainment upstream of the circadian clock but is also the first to show the regulation of entrainment pathways upstream of the clock by DUBs. This chapter connects the dots between existing literature and emerging hypotheses, to elucidate the role of USP2 in entrainment pathways.

4.2. Abstract

The synchronization of circadian rhythms to the external light-dark cycle is a complex process involving the activation of multiple molecular cascades in the neurons of the suprachiasmatic nucleus. Ubiquitin specific peptidase 2 (USP2) is a deubiquitinase that interacts with multiple clock proteins, and we previously showed that it is involved in circadian photic entrainment as well. However, how USP2 regulates entrainment is yet to be understood. In this study, using *Usp2* knockout (KO) mouse models we investigated the specific mechanisms affected by USP2 in modulating entrainment. We established that USP2 action is not localized to a specific neuron subset within the SCN. Then using a proteomic screen, we found photic entrainment pathways such as MAPK/ERK, EIF2 signalling and mTOR signaling to be differentially regulated in the absence of USP2. Finally, we showed that USP2 affects the phosphorylation of proteins in the MAPK/ERK and EIF2 signalling cascades upstream of the clock to affect photic entrainment in the SCN. Hence, our data adds to the molecular understanding of the function of USP2 in photic entrainment of the clock.

4.3. Introduction

Circadian rhythms, with a period of approximately 24 hours, regulate most aspects of our physiology. At the molecular level, circadian rhythms emerge from feedback loops of clock genes and proteins [1,2]. Post-translational mechanisms like phosphorylation, acetylation, and ubiquitination are crucial in maintaining the precision of this oscillation [3–5]. Ubiquitination involves tagging proteins with ubiquitin, a 76 kDa protein that controls protein localization and degradation. Deubiquitination, on the other hand, removes these tags and leads to protein accumulation and differential localization. USP2 is one such deubiquitinase (DUB) which was shown to interact with the clock proteins PER1 [6], CRY1 [7] and BMAL1 [8]. Knockout models of USP2 have shown an increase in period length, as well as altered patterns of photic entrainment [6].

Photic entrainment is the ability to of the clock to adapt to changes in the external light-dark cycle. Since the endogenous circadian period in mammals is slightly different from 24 hours, the process of entrainment allows organisms to adapt to the environmental 24-hour light-dark cycle, daily [9,10]. This process begins with photic information gathered by the retina being transferred to the suprachiasmatic nucleus (SCN), the master-pacemaker of the body, via glutamatergic projections [11]. These projections innervate the ventro-lateral part of the SCN, which primarily consists of neurons expressing the neuropeptide vasoactive intestinal peptide (VIP). The activation of NMDA receptors in these neurons triggers multiple protein phosphorylation cascades [12]. These, in turn, result in changes in the transcription of clock genes such as *Per1* and *Per2*, leading to entrainment of the SCN to the new light-dark cycle [13].

In our previous studies, we showed that USP2 affects early-night entrainment at the level of the SCN and that photic induction of *Per1* is reduced in the absence of USP2 [6,14]. In this study, we continue this investigation of the role of USP2 in light response. We probe the patterns of *Usp2* expression within different subpopulations of neurons within the SCN and correlate it with the functional relevance of USP2. We then use a proteomic screen to further scrutinize pathways upstream of the clock being affected by USP2, hence affecting light response in *Usp2* KO mice. We found that USP2 action is not restricted to VIP-expressing neurons. Finally, using a proteomic screen, we found that USP2 affects the levels of multiple photic-response-related proteins and pathways within the SCN, which we validated using IHC. Thus, we established that USP2 mediates entrainment by affecting photic-response pathways within the SCN.

4.4. Materials and methods

4.4.1. Animals

Usp2 KO mice and *Usp2* floxed mice [15] were obtained from Dr. Simon S. Wing and bred in house. The full body *Usp2* KO and their WT littermates (on a C57BL/6J background) were generated by breeding heterozygotes. VIP-IRES-Cre mice (STOCK $Vip^{tm1(cre)Zjh/J}$; stock number 010908; The Jackson Laboratory) were obtained from Dr. Mark Brandon and crossed with the *Usp2* floxed mice to generate the VIP-cell-specific *Usp2* KO mouse line. Mice were weaned at 3 weeks of age, into cages containing no more than 5 mice per cage. They were maintained on a 12h:12h light: dark cycle (12:12 LD cycle) with light at ~300 lux. At 2 to 3 months of age, mice were designated to various experimental cohorts.

All procedures involving animals were carried out in accordance with guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committees of McGill University and Douglas Mental Health University Institute (protocol no. 2001-4586). For euthanasia at the end of the experiments, the animals were first anesthetized by isoflurane inhalation, followed by CO_2 euthanasia. All efforts were made to minimize suffering.

4.4.2. Wheel-running behavior

For running wheel experiments, 8- to 12-week-old WT and *Usp2* KO mice were singly housed in running wheel-equipped cages (Actimetrics, Wilmette, IL, USA), with ad-libitum access to food and water.

The modified skeleton photoperiod protocol (mSPP) was carried out as previously described[14]. Briefly, after 14 days in a baseline 12:12 LD cycle, the mice were subjected to a conventional skeleton photoperiod protocol for 14 days. Then, the mice were subjected to a 2-hour phase delay using only a single, daily, 30-minute dusk light pulse, for 21 days. After this, the mice were subjected to a 2-hour phase advance using the single, daily, dusk pulse for 21 days. The last 5 days of recorded data for each portion of the mSPP were analysed on the Clocklab software Version 6 (Actimetrics, Wilmette, IL, USA). The phase angle of entrainment or the amount of time it took the mouse to start running in the wheel after lights-off was averaged for each lighting condition of the mSPP.

4.4.3. Tissue preparation for RNAscope

To obtain tissue over the course of 24 hours, perfusion was carried out at time points that were 6 hours apart. Specifically, mice were maintained in a 12:12 LD cycle for 14 days, after which they were released into DD. On the first day in DD, mice were perfused 1, 7, 13 and 19 hours after the start of the day (denoted as circadian time or CT 1, 7, 13 and 19). At the appropriate time points, mice were perfused transcardially with ice-cold 1X PBS for 4 minutes followed by 4 minutes of freshly prepared 4% paraformaldehyde (PFA). The brains were post-fixed in 4% PFA for 24 hours, followed by an increasing concentration gradient of sucrose solutions (10%, 20%, and 30%) for 24 hours each, at 4ºC. The brains were then frozen in isopentane over dry ice and stored in an airtight container at -80°C. For the experiment, 10 µm thick SCN containing, coronal sections were collected directly on positively charged slides, using a cryostat, and stored in an airtight container at -80ºC until staining.

4.4.4. RNAscope: fluorescent in-situ hybridization assay.

Fixed-frozen slides were washed in 1X PBS for 5 minutes before being baked at 60ºC for 30 minutes in a HybEZ oven. The slides were then post-fixed for 15 minutes in 4% PFA at 4ºC. Tissues were dehydrated using an increasing ethanol solution concentration gradient at room temperature (RT) (50%, 70%, and twice in 100% EtOH), then air dried for 5 minutes at RT. The solutions were then incubated with hydrogen peroxide for 10 minutes at RT, followed by two washes in distilled water. RNAscope Multiplex Fluorescent Assay v2 and RNAscope 4-plex Ancillary Kits from Advanced Cell Diagnostic (Newark, CA, USA) was used to perform the assay. Target retrieval was

carried out by placing the sections in a beaker containing 1X "RNAscope target retrieval reagent" at 100ºC for 5 minutes. The slides were washed with 100% ethanol and then distilled water, and a hydrophobic barrier was drawn around the sections using an ImmEdge hydrophobic barrier pen. Next, Protease III was applied to each section and the sections were placed in a humidity tray in the HybEZ oven for 30 minutes at 40ºC. The slides were then washed with distilled water. The probes used are listed in supplementary Table 1. The sections were hybridized with probe mix for 2 hours at 40ºC, followed by washing with 1X wash buffer and incubation in 5X SCC overnight at RT.

Amplification was performed using RNAscope Multiplex FL v2 Amp1 followed by Amp2 for 30 minutes each at 40ºC in the HybEZ oven, and then for 15 minutes at 40ºC for the third amplification. Each channel was developed by adding their respective HRP (C1, C2, C3) to each slide for 15 minutes at 40ºC. Specific fluorescent “opals” (see supplementary table 2 for opals used) were then applied for 30 minutes at 40ºC, followed by blocking the channel using HRP blocker for 15 minutes at 40ºC. Once all channels were developed, the sections were counterstained with DAPI for 30 seconds at RT. The slides were dried for 30 minutes in darkness, cover slipped using Fluoromount-G, and stored at 4ºC. Finally, the sections were imaged using a Zeiss LSM 710 confocal microscope (Montreal Clinical Research Institute, QC, Canada).

4.4.5. RNAscope multiplex analysis

Each Z-stack of confocal images was subjected to a max intensity Z projection using ImageJ. Subsequently, QuPath [16] was used to perform multiplexed analysis on these images, with three classifiers trained for *Avp*, *Vip*, and *Usp2*. These classifiers were

trained to identify a cell as positive for a marker if staining for that specific marker was visible within a 0.3 µm radius around the cell nucleus, using DAPI to identify the cell nuclei. Once trained, these classifiers were applied to another set of co-stained images to make four different quantifications: *Avp* with *Usp2*, *Vip* with *Usp2*, double positive (expressing *Avp*, *Vip* and *Usp2*), and double negative (expressing only *Usp2*). For RNAscope images, subcellular detection was used to detect each probe separately, and scripts were written using the Groovy language in QuPath, to classify cells based on their respective probe amounts.

4.4.6. Proteomic analysis

After maintaining mice in a standard 12:12 LD cycle for 14 days, they were released into DD. At CT 14 on the first day of DD the mice were exposed to a 30-minute light pulse (or not). At CT 16 the mice were sacrificed, and their brains were sliced into 300 µm-thick slices on a vibrating blade microtome (Leica biosystems, Ontario, CA). From the appropriate slice, the SCN tissue was micro-dissected under a simple light microscope. The SCN containing tissue sections were lysed in of 50 µl of lysis buffer containing 5% SDS in 100 mM TRIS pH 8.5 and the samples were heated at 95ºC for 10 minutes. The samples were consequently homogenized with a probe ultrasonicator (Thermo Sonic Dismembrator) and centrifuged at 21,000 x g for 5 minutes. 10% of the resulting extract was reserved for the determination of protein concentration using bicinchonic acid assay (BCA) (Thermo/Pierce). The remainder of the sample was reduced in 20 mM tris(2-carboxyethyl)phosphine (TCEP) for 30 minutes at 60°C and alkylated in 25 mM iodoacetamide (IAA) for 30 minutes in the dark. Then, 20 µg of protein lysate was

digested using trypsin (Promega, sequencing grade) overnight at 37ºC in S-TRAP micro cartridges (Protifi). Following digestion, peptides were extracted from the S-TRAP micro cartridges with 50 mM ammonium bicarbonate, 0.2% formic acid, and 50% acetonitrile respectively. Peptide samples were frozen at -80ºC and lyophilized prior to analysis by nLC-MS/MS.

4.4.7. LC-MS/MS data acquisition and analysis

Peptide samples were reconstituted in 0.1% formic acid, to a final concentration of 500 ng/µL and an equivalent of 1 µg was analyzed using an Easy-nLC 1200 system coupled to a Q Exactive Plus (Thermo Fisher Scientific). The peptides were first loaded onto a pre-column (Acclaim PepMap 100 C18, 3 µm, 75 µm ID x 2 cm length, Thermo Fisher Scientific), and then separated using a 100 minute gradient with an Acclaim PepMap 100 C18 analytical column (2 µm particle, 75 µm ID x 250 mm, Thermo Fisher Scientific) using water with 0.1% formic acid (mobile phase A) and 84% acetonitrile with 0.1% formic acid (mobile phase B) at 300 nL/min from 3-40 %B. MS analysis used data dependent acquisition mode (DDA), based on the top 15 most intense precursor ions (+2 to +4 charge state). Full MS scans were acquired at 70K resolution from 350 – 1500 m/z (AGC 1E6, 50 ms max injection time), and MS2 spectra were collected at 17.5K resolution (AGC 2E4, 64 ms max injection time) using a normalized collision energy (NCE) of 28. Dynamic exclusion was set to 40 seconds.

Acquired MS/MS data was analyzed using Proteome Discoverer (PD) version 2.5 (Thermo Fisher Scientific). Database searching was conducted using the Sequest HT node, using a reference mouse proteome FASTA file containing only reviewed canonical

sequences downloaded from Uniprot (downloaded August 25th, 2021). Label free quantitation was conducted using the Minora feature detection node. The proteins were quantified based on unique peptides, scaled based on total peptide abundance, and missing values were imputed using the low abundance resampling method. The protein expression ratios were calculated based on protein abundance, and p-values were calculated using t-test (background based) within PD 2.5. Only proteins quantified with at least 1 protein unique peptide in at least 3 out of 5 biological replicates from at least one sample group were retained. Statistical significance of differentially expressed proteins was based on a 2-sigma fold-change cut-off (effectively 2-fold change), as well as having an FDR adjusted p-value less than 0.05. Statistically differentially expressed proteins were used as the input for a canonical pathway enrichment analysis using Ingenuity Pathway Analysis software (Qiagen). Volcano plots, principal component analysis, and hierarchical clustering were performed using Instant Clue (v0.11.2) [17].

4.4.8. Immunohistochemistry (IHC)

Mice were maintained in standard 12:12 LD for 14 days following which they were released into DD. On the first day in DD, mice were light pulsed (or not) for 30 minutes at CT 14. One or two hours after the light pulse (or at CT15 for the no light pulse condition), the mice were anesthetized with a ketamine (100mg/kg)/xylazine (10 mg/kg)/acepromazine (3 mg/kg) cocktail. Following this, the mice were transcardially perfused using ice-cold 1X PBS for 4 minutes followed by 4 minutes of ice-cold fixative Z-Fix (Anatech Ltd, Battle Creek, MI). Brains were post-fixed for 24 hours in Z-fix followed by 48 hours in 30% sucrose at 4ºC. They were then frozen in isopentane over dry ice and

stored at -80ºC. The day prior to the IHC, 30 µm thick coronal sections of the brain, containing the SCN were directly collected on gold-charged slides, in a cryostat.

Brain sections were cleared using Xylene (2 x 3 minutes) followed by rehydration in a decreasing ethanol (EtOH) concentration gradient (3 minutes 1:1 100% EtOH:Xylene, 2 x 3 minutes 100% EtOH, 3 minutes 95% EtOH, 3 minutes 80% EtOH, 3 minutes 60% EtOH, 3 minutes in cold water). Antigen retrieval was then carried out by placing the slides in a container with boiling 10 mM Sodium acetate solution, which was placed in a 100ºC water bath for 20 minutes. The slides were then quenched for 10 minutes in cold, running water. A hydrophobic barrier was drawn on the slides, around the sections using an ImmEdge hydrophobic (H-4000, Vector laboratories) barrier pen. The sections were then permeabilized using permeabilization solution (0.4% Triton X-100 and 1% BSA in 1X TBS) for 10 minutes at room temperature (RT). The sections were then blocked using blocking solution (0.4% Triton-X 100, 5% donkey serum and 1% BSA in 1X TBS) for 1 hour at RT. The sections were then incubated for 48 hours in primary antibody at 4ºC (primary antibody and 1% BSA and 5% Donkey serum in 1X TBS. Primary antibodies are listed in Supplementary table 1).

After three washes of five minutes each in TBS-T (0.025% TritonX-100 in 1X TBS), the sections were incubated for 1 hour at RT in secondary antibody solution (secondary antibodies and 1% BSA in 1X TBS. Secondary antibodies listed in the table below). After three more washes of five minutes each in TBS-T, the slides were incubated for 3 minutes in DAPI solution (1:6000 DAPI in 1X TBS; D9542, Millipore Sigma) and then washed again with TBS-T. The slides were dried for 30 minutes in darkness, cover slipped using Fluoromount-G, and stored at 4°C. Images were taken using a Zeiss Observer Z1

fluorescence microscope. Using ImageJ, ROIs were drawn around the SCN on the DAPI channel and the mean intensity of staining was measured in the red channel using the same ROI. To obtain the final mean intensity, the mean intensity of control images (sections stained with secondary antibody only) was subtracted from the mean intensity of the image of interest. This value was then plotted using Graphpad Prism.

4.4.9. Statistical analysis

All the data were plotted on GraphPad Prism 9 using the appropriate statistical tests. To compare within-subject data in different photic-conditions for mSPP a mixed-model 2-way ANOVA tests with Greenhouse-Geisser correction were used. The correction was used to account for the violation of sphericity in the data sets. To compare between-subject data in the IHCs, 2-way ANOVA tests with Greenhouse-Geisser correction were used. Holm-Sidak post-hoc tests were used to compare between conditions. To assess rhythmicity of *Usp2* in the SCN, Cosinor analysis was used. First, the data was fit to the following function:

$$Y = B + A * \cos\frac{2 * \pi * (X - P)}{24}$$

$$B = Baseline\ or\ MESOR$$

$$A = Amplitude\ of\ rhythm$$

$$P = Phase\ of\ rhythm$$

The data points were then fit to a straight line with a slope of 0 (to represent an undamped oscillation), $y = c$. The two fit functions were compared, and the data points

were considered to be rhythmic if the Cosinor fit significantly differed from the straight line fit.

For all statistical tests, differences were considered significant if $p < 0.05$.

4.5. Results

4.5.1. The role of USP2 in photic entrainment within the SCN is not exclusively mediated at the level of VIP-expressing neurons.

Since the ventro-lateral, VIP-expressing “core” of the SCN is retinorecipient, we wanted to investigate if the role of USP2 in modulating entrainment was localized to this region of the SCN.

By crossing VIP-IRES-Cre mice with *Usp2* floxed mice, we generated mice that lacked USP2 specifically in the core of the SCN. Previous studies [18,19] found that mice expressing Cre on both alleles of VIP showed an overall reduction in VIP levels, as well, altered circadian behaviors. However, one of these studies also showed that mice expressing a single Cre allele showed no differences in circadian behavior compared to wildtype mice [18]. Hence, for our experiments, we exclusively used mice heterozygous for the VIP-IRES-Cre allele. These mice were subjected to the modified skeleton photoperiod protocol (mSPP) (Figure 1A). To measure fidelity of entrainment, phase angle of entrainment to the different phases of mSPP was assessed, as we did in our previous study [14]. There was a main effect of lighting condition ($F(1.398,13.98) = 23.73$, $p < 0.0001$), similar to our previous results, showing that the overall pattern of entrainment to this light cycle is comparable to our previous experiments [14]. However, we found no differences in entrainment between the control groups and the VIP-expressing-neuron-specific *Usp2* KO mice ($F(6,30) = 0.7061$, $p = 0.641$) (Figure 1B), unlike the results we found in whole SCN *Usp2* KO mice [14].

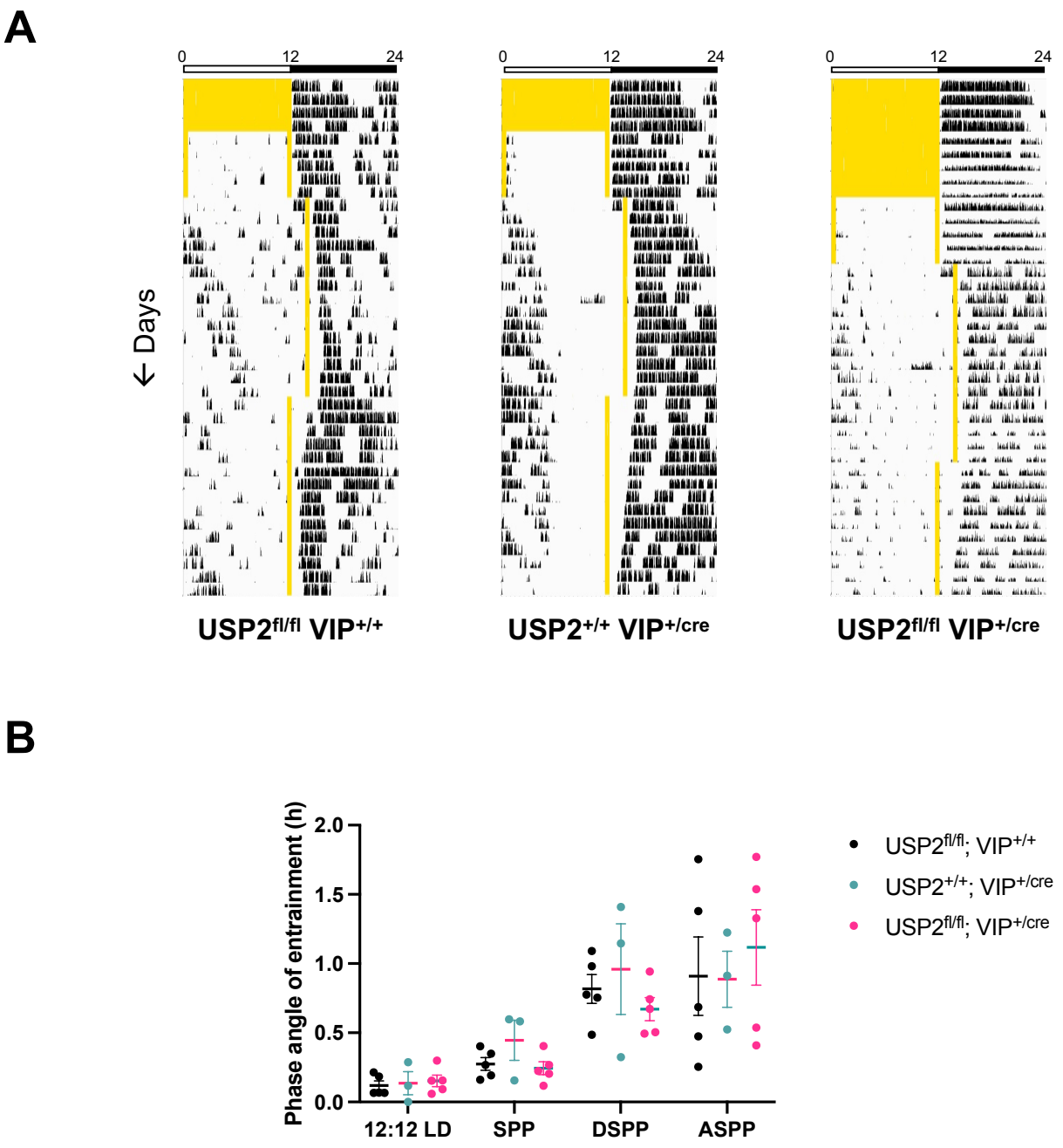


Figure 1: Unaltered entrainment in VIP-specific Usp2 KO mice. (A) *Representative actograms for the wheel running activity of the three different genotypes being tested, under the modified skeleton photoperiod lighting protocol.* ***(B)*** *Quantification of the phase angle of entrainment averaged over the last 5 days*

in each phase of the mSPP. SPP = skeleton photoperiod, DSPP = phase delay with mSPP, ASPP = phase advance with mSPP. Individual data points represent individual mice (n: $USP2^{fl/fl}$ $VIP^{+/+}$ = 5, $USP2^{+/+}$ $VIP^{+/cre}$ = 3, $USP2^{fl/fl}$ $VIP^{+/cre}$ = 5). All data are represented as Mean ± SEM (n = 3 to 5 mice per group). Mixed model 2-way ANOVA, n.s.

4.5.2. *Usp2* expression is rhythmic in AVP-ergic neurons, but not in the whole SCN

Since the role of USP2 doesn't seem to be localized exclusively to VIP-expressing neurons, we wanted to examine expression patterns of *Usp2* in different neuronal subpopulations within the SCN. Towards this end, mice were perfused at CT 1, 7, 13 and 19 and their SCNs were subjected to an RNAscope fluorescent in-situ hybridization assay, with probes against *Avp*, *Vip* and *Usp2*. In these SCNs, most cells expressed *Avp* or *Vip*. However, some cells expressed both, while others expressed neither. We looked at the overall *Usp2* expression in the SCN, as well as its expression patterns in these different cell groups (Figure 2A).

There was a trending difference in *Usp2* expression levels between the time points in the whole SCN ($F(3,22) = 0.9329$, $p = 0.0615$) (Supplementary figure 1A), but there was no significant rhythm (Figure 2B). Further, investigating the localization of *Usp2* with *Avp* and *Vip* in the SCN, we found a significant interaction between time-points and cell groups ($F(9.87) = 5.862$, $p < 0.0001$). While there was variation of *Usp2,* across time, in all the cell groups (Supplementary figure 1B), *Usp2* expression showed significant rhythmicity only in the *Avp* expressing cells ($p = 0.0022$), in contrast to the other cell groups (Figure 2C). Additionally, the difference in the average amount of *Usp2* expressed

in each of these neuronal groups was minimal. This points towards USP2's role potentially being spread over the different neuronal groups in the SCN.

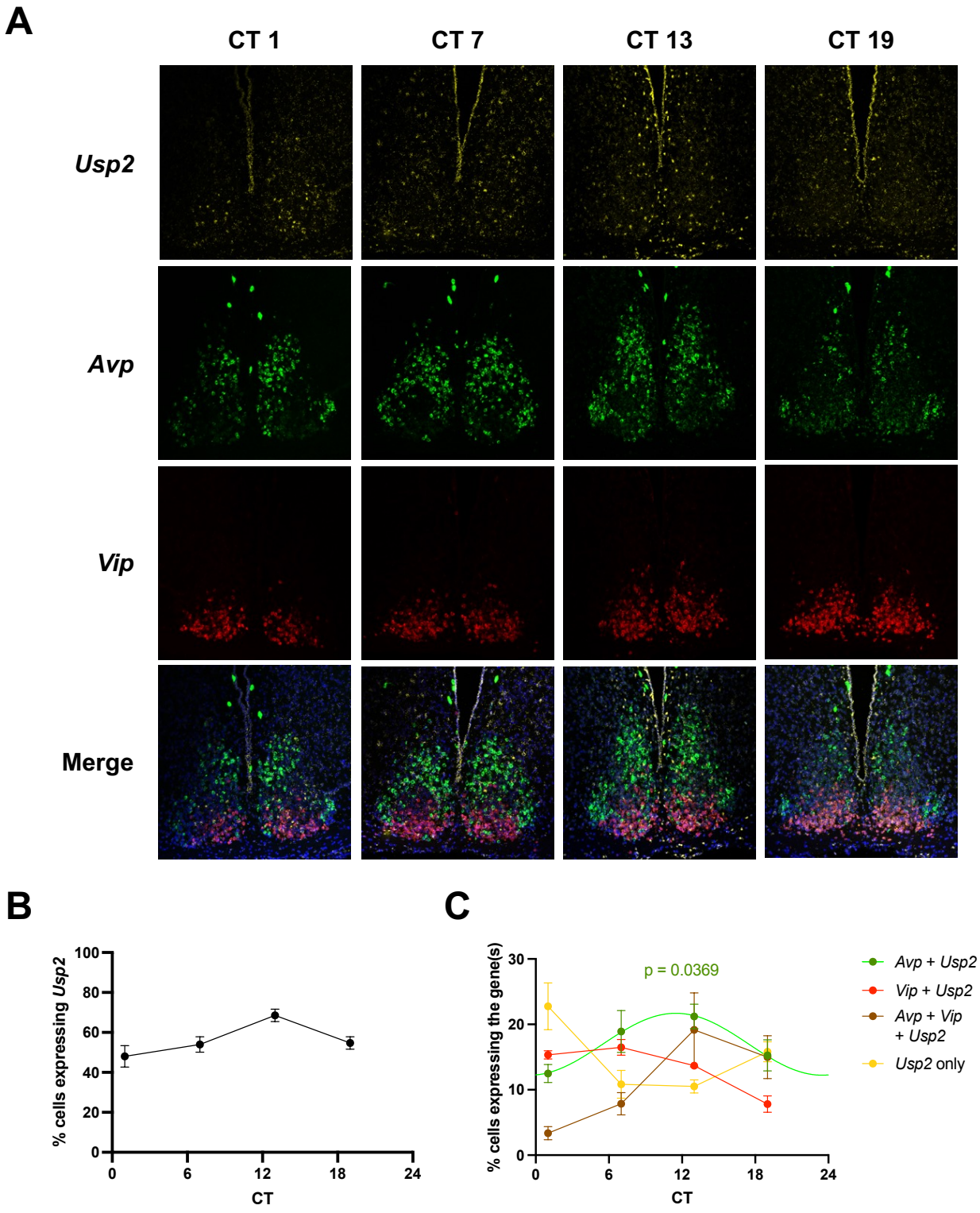


Figure 2: Usp2 expression is rhythmic in AVP-expressing neurons. (A) *Representative RNAscope images of Usp2, Avp, Vip and merged with DAPI at 4 different time points (circadian time 1, 7, 13 and 19).* ***(B)*** *Quantification of percentage of SCN cells that express Usp2 across time points.* ***(C)*** *Quantification of percentage of cells that express Usp2 along with the specific neuropeptide(s). Individual points represent individual SCN lobes. All data are represented as Mean ± SEM (n: CT 1 = 6, CT 7 = 8, CT 13 = 5, CT 19 = 7). Significant rhythms are represented by a smooth Cosinor fit line.*

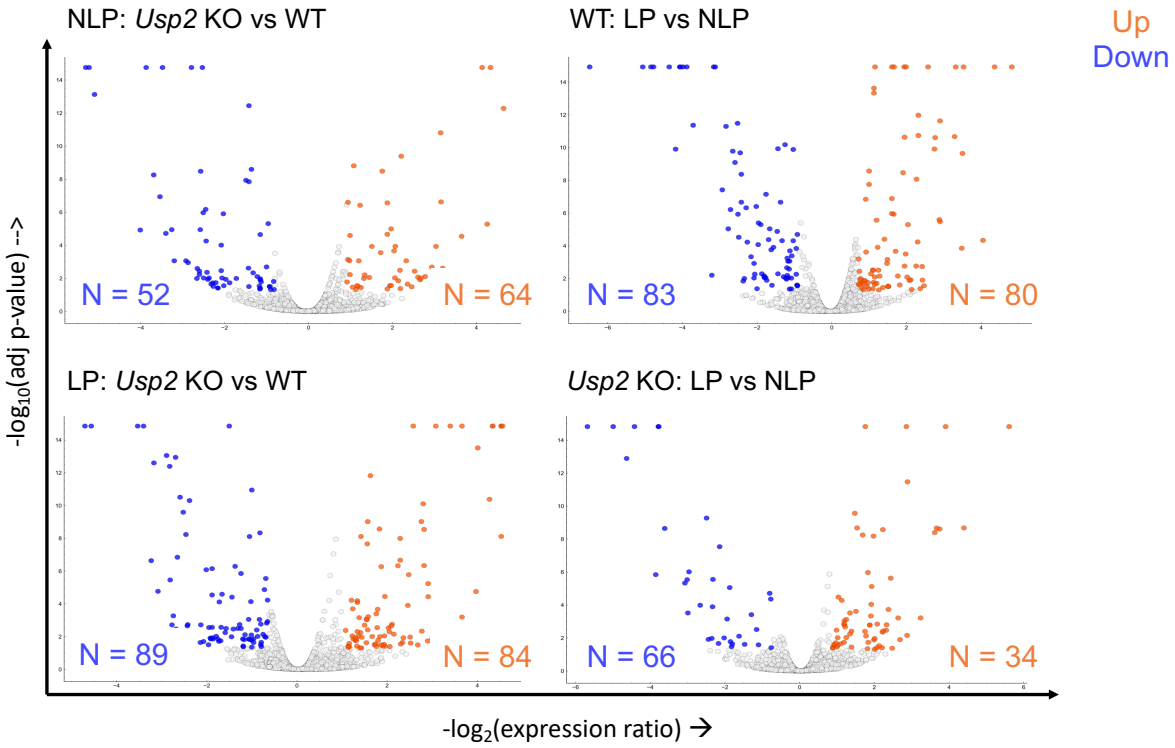
4.5.3. Proteins affected by USP2 in the SCN

To further understand the role of USP2 in SCN light response, we looked at the proteomic profile of the whole SCN tissue. In previous studies from the lab, the largest difference between genotypes in response to light was at CT 14, with *Usp2* KO mice showing a larger phase delay in response to a light pulse than WT mice [6,14]. Hence, we used nLC-MS/MS-based label-free quantitative proteomics to look for proteomic alterations between SCNs from WT and *Usp2* KO mice micro-dissected at CT 16 after being subjected to a light pulse (LP) or no light pulse (NLP) at CT 14 (n = 5 per group). Performing principal component analysis (Supplementary figure 2A) showed that one sample (*Usp2* KO, NLP #3) was a strong outlier. Hence, this point was excluded from further analysis.

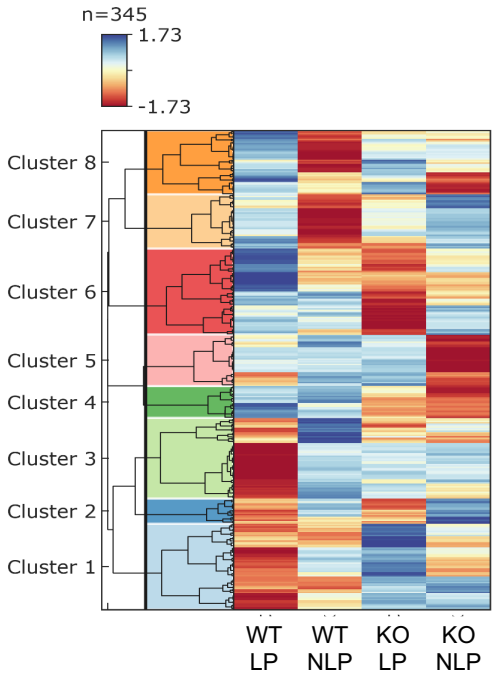
From this analysis, 3063 proteins were quantified, of which 3053 were identified with at least two protein-unique peptides. Only proteins that were quantified in at least

half of the replicates of at least one group were kept and missing values were imputed (Supplementary figure 2B). The differentially expressed proteins in each pair of conditions were plotted on volcano plots. To control for false positives, only proteins detected with certainty of more than 95% ($p < 0.05$) and with a fold-change calculated as *Expression > 2*SD or Expression < -2*SD (SD = standard deviation)*, were selected (colored dots in Figure 3A). Of these identified peptides, 345 peptides were differentially expressed in at least two conditions (Figure 3B). Specifically comparing the proteins differentially expressed in response to a LP (compared to NLP), 135 proteins were differentially expressed only in the WT SCNs, 72 were differentially expressed only in the *Usp2* KO SCNs and 28 proteins were differentially expressed in SCNs of both genotypes (Figure 3C). Similarly, looking at KO (compared to WT) (Supplementary figure 3), we again found that the compared sets of proteins were largely distinct from each other, showing that both, the genotype and lighting condition played a significant role in regulating proteins within the SCN.

A


NLP: *Usp2* KO vs WT
WT: LP vs NLP
Up
Down
N = 52
N = 64
N = 83
N = 80
LP: *Usp2* KO vs WT
Usp2 KO: LP vs NLP
N = 89
N = 84
N = 66
N = 34
-log10(adj p-value) -->
-log2(expression ratio) →

B


n=345
1.73
-1.73
Cluster 8
Cluster 7
Cluster 6
Cluster 5
Cluster 4
Cluster 3
Cluster 2
Cluster 1
WT LP
WT NLP
KO LP
KO NLP

C

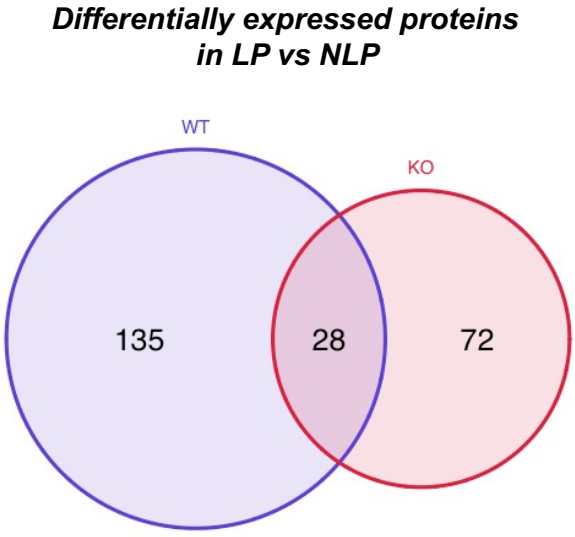

Differentially expressed proteins in LP vs NLP
WT
KO
135
28
72

Figure 3: Differentially expressed proteins in the shotgun proteomic screen. ***(A)*** *Volcano plots showing the differential expression of proteins in various pairs of conditions. NLP or WT were considered as the reference conditions for all the comparisons. A:B vs C as the header implies the plotted values are for the ratio B/C, when they were both in A condition. The significance on the X axis is determined as expression > 2*SD (orange dots) or expression < -2*SD (blue dots) (SD = standard deviation). On the Y-axis, significance is determined as p < 0.05. "N" represents the number of proteins upregulated (blue) or downregulated (orange) in that comparison.* ***(B)*** *Heatmap of all the proteins that were differentially regulated in at least two conditions.* ***(C)*** *Venn diagram of proteins differentially expressed in each genotype, in the LP as compared to the NLP condition.*

4.5.4. Pathways affected by USP2 in the SCN

Passing the differentially expressed proteins for each pair of conditions through Ingenuity Pathway Analysis (IPA), we obtained many functionally enriched pathways (Figure 4, Supplementary figures 4 and 5). The premise of our experiment was to look at differences between pathways of WT and *Usp2* KO mice in response to a light pulse. Thus, we first looked at the IPA for pathways enriched in *Usp2* KO mice, compared to WT mice, when they were subjected to a light pulse (LP: KO/WT) (Figure 4A, Supplementary figure 4A). Of the top 15 significantly enriched pathways, there was strong positive enrichment of the EIF2 signalling pathway, as well as negative enrichment of the mTOR pathway and the ERK/MAPK pathway (Figure 4A), which are all pathways involved in the initial response to light within the SCN [20–22]. To verify that this enrichment was indeed

an effect of light, we looked the pathways enriched in *Usp2* KO mice compared to WT mice when they were both not subjected to a light pulse (NLP: KO/WT) (Figure 4B, Supplementary figure 4B). All three above given pathways were not enriched in the NLP: KO/WT condition, showing that the enrichment in LP: KO/WT was indeed an effect of light. Put together, pathways involved in light response are differentially enriched in *Usp2* KO and WT SCNs, in response to light.

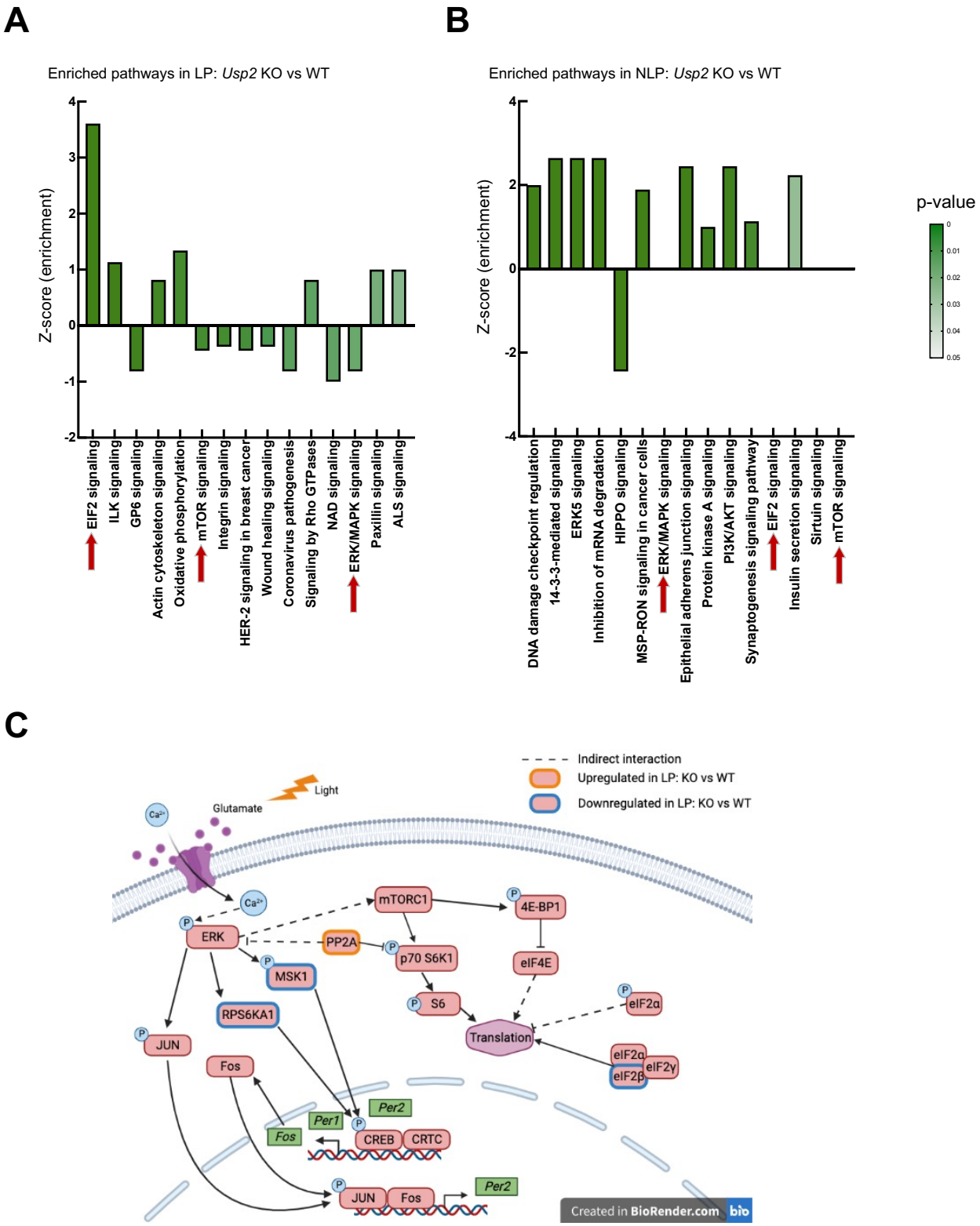

A
Enriched pathways in LP: Usp2 KO vs WT
Z-score (enrichment)
EIF2 signaling
ILK signaling
GP6 signaling
Actin cytoskeleton signaling
Oxidative phosphorylation
mTOR signaling
Integrin signaling
HER-2 signaling in breast cancer
Wound healing signaling
Coronavirus pathogenesis
Signaling by Rho GTPases
NAD signaling
ERK/MAPK signaling
Paxillin signaling
ALS signaling
B
Enriched pathways in NLP: Usp2 KO vs WT
Z-score (enrichment)
DNA damage checkpoint regulation
14-3-3-mediated signaling
ERK5 signaling
Inhibition of mRNA degradation
HIPPO signaling
MSP-RON signaling in cancer cells
ERK/MAPK signaling
Epithelial adherens junction signaling
Protein kinase A signaling
PI3K/AKT signaling
Synaptogenesis signaling pathway
EIF2 signaling
Insulin secretion signaling
Sirtuin signaling
mTOR signaling
p-value
C
Indirect interaction
Upregulated in LP: KO vs WT
Downregulated in LP: KO vs WT
Light
Glutamate
Ca2+
mTORC1
4E-BP1
ERK
PP2A
p70 S6K1
MSK1
eIF4E
S6
eIF2α
RPS6KA1
JUN
Translation
Fos
eIF2α
eIF2β
eIF2γ
Per2
Per1
Fos
CREB
CRTC
JUN
Fos
Per2
Created in BioRender.com

Figure 4: Pathway analysis of the differentially expressed proteins in the proteomic screen. (A, B) *Differential enrichment of pathways in Usp2 KO SCNs compared to WT SCNs, when* ***(A)*** *they are exposed to light (LP) or* ***(B)*** *they are not exposed to light (NLP). The red arrows show pathways of interest explored further, in the text* ***(C)*** *Simplified schematic of specific pathways involved in photic entrainment response of the SCN, immediately after a light pulse. Specific proteins differentially expressed in the proteomic screen, which are also involved in the enriched pathways are highlighted in the schematic.*

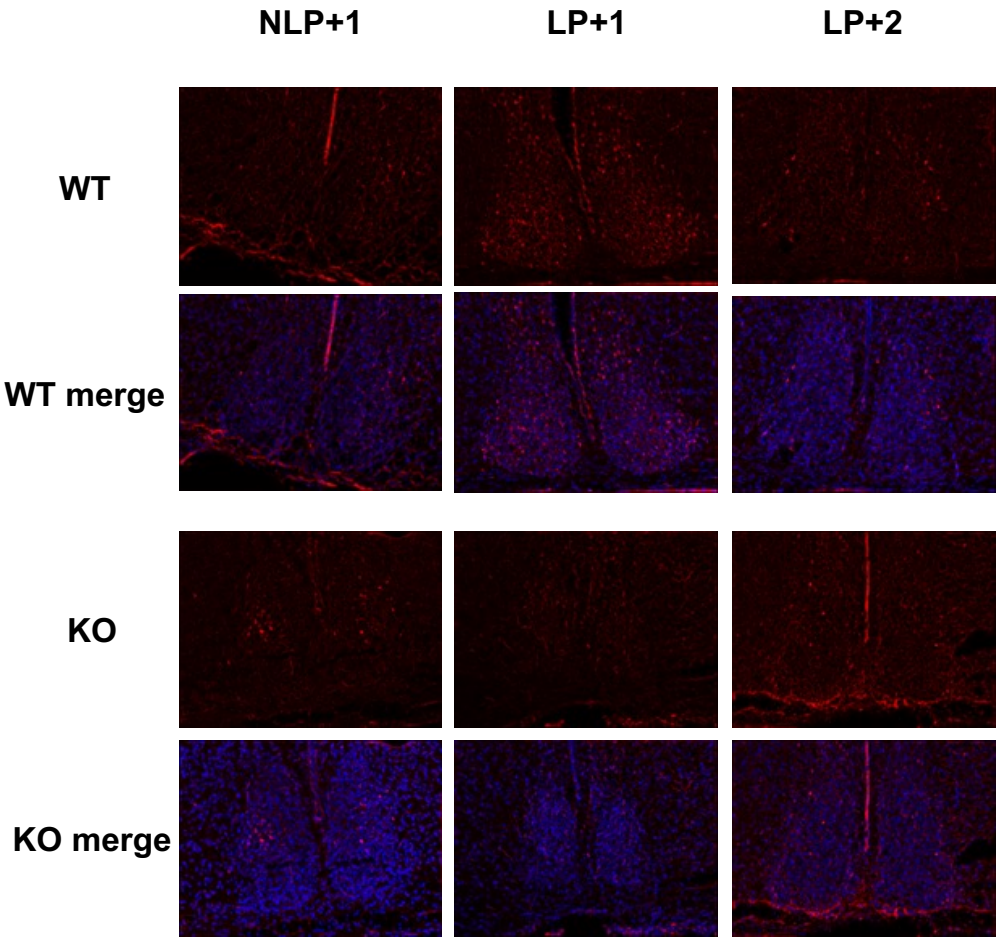
4.5.5. Validation of top light-response pathways enriched in the absence of USP2

To validate the findings of our proteomic analysis, we conducted immunohistochemistry (IHC) for specific proteins of the enriched pathways affected by USP2. To replicate the conditions of the proteomic screen, we exposed WT and *Usp2* KO mice to LP or NLP on the first day in DD, at CT 14, following 14 days in 12:12 LD. Since we wanted to assess the acute effects of USP2 on circadian light response, brains were perfused and collected 1 hour (CT 15: LP+1; NLP+1) or 2 hours (CT 16: LP+2) after the light pulse. SCN-containing slices were stained for phosphorylated forms of ERK 1/2 (a component of the ERK/MAPK cascade), EIF2α (a subunit of EIF2, and hence, the EIF2-mediated translation initiation pathway), and S6 (a component of the mTOR cascade).

There was an interaction between lighting condition and genotype in the phosphorylation of ERK ($F(2,16) = 12.75$, $p = 0.0005$) (Figure 5A). One hour after LP, pERK levels were significantly higher in WT mice compared to those subjected to NLP (post-hoc $p = 0.0495$), whereas this elevation was not observed in *Usp2* KO SCNs (Figure

5A, 5B). Additionally, pERK levels in WT SCNs were significantly higher than in *Usp2* KO SCNs ($p = 0.0008$), at LP+1 (Figure 5B). Further, at LP+2, pERK levels were reduced ($p = 0.0223$), compared to LP+1, in WT SCNs (Figure 5B), unlike in the *Usp2* KO SCNs. The increase in pERK levels, in response to light, observed in WT SCNs is consistent with previous work [23].

A


NLP+1
LP+1
LP+2
WT
WT merge
KO
KO merge

B

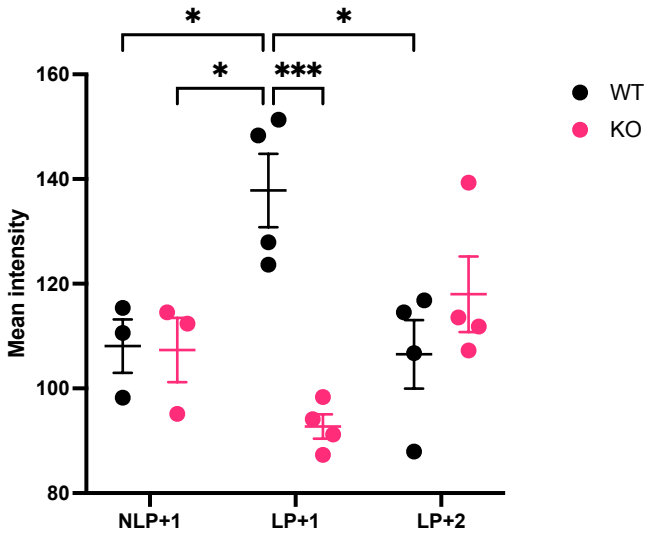
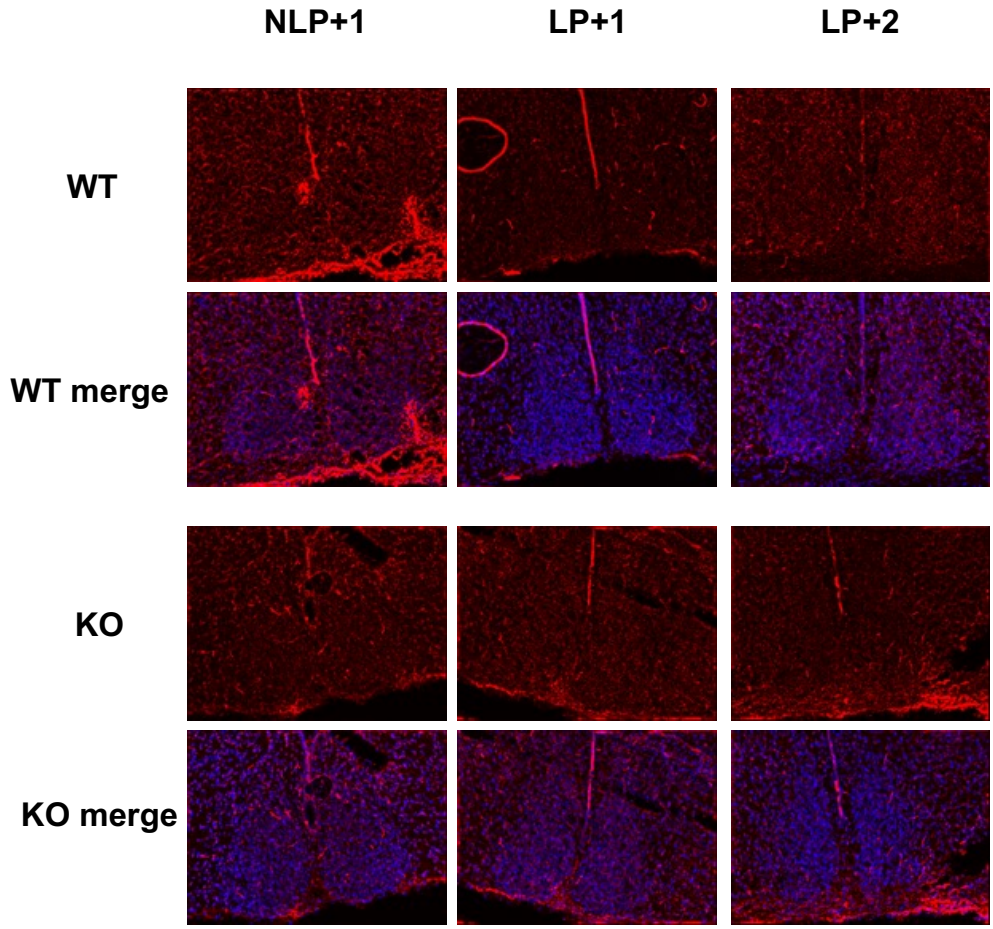

*
*
*
***
160
140
120
100
80
Mean intensity
NLP+1
LP+1
LP+2
WT
KO

Figure 5: ERK phosphorylation is increased in response to light in WT but not in Usp2 KO mice. *(A) Representative fluorescence microscopy images of pERK staining 1 hour (LP+1) or 2 hours (LP+2) after a light pulse or 1 hour after no light pulse (NLP+1) and merged images with DAPI staining (B) Quantification of pERK staining averaged across the SCN. Individual data points represent individual mice (n: NLP+1,WT = 3; NLP+1,KO = 3; LP+1,WT = 4; LP+1,KO = 4; LP+2,WT = 4; LP+2,KO = 4). All data are represented as mean ± SEM. Two-way ANOVA, with Holm-Sidak post-hoc test. *: p < 0.05, ***: p < 0.001*

Analysis of the phosphorylation of EIF2α showed that there is an interaction between lighting condition and genotype ($F(2,17) = 11.28$, $p = 0.0008$) (Figure 6A). At LP+1, WT mice exhibited a significant decrease in pEIF2α compared to NLP+1 (post-hoc $p = 0.0277$), whereas this effect was not observed in *Usp2* KO mice (Figure 6B). Furthermore, in the NLP condition, WT mice exhibited a significantly higher amount of EIF2α phosphorylation than *Usp2* KO mice (post-hoc $p = 0.0233$) (Figure 6B).

Surprisingly, no differences in pS6 levels were observed between the different genotypes and lighting conditions (Figure 7A, 7B).

A


NLP+1
LP+1
LP+2
WT
WT merge
KO
KO merge

B

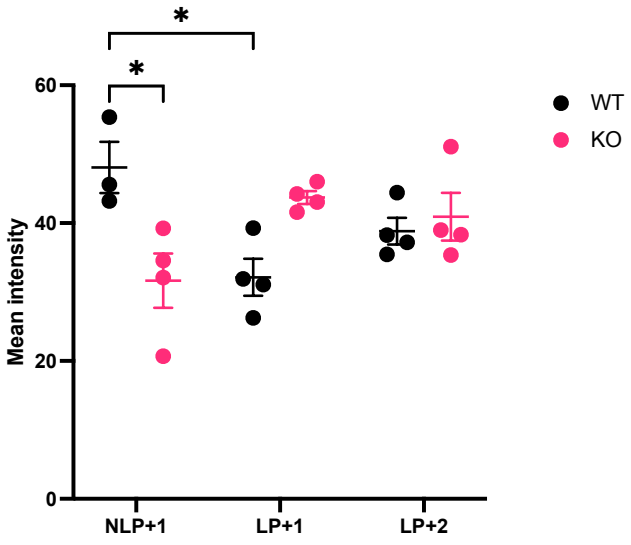
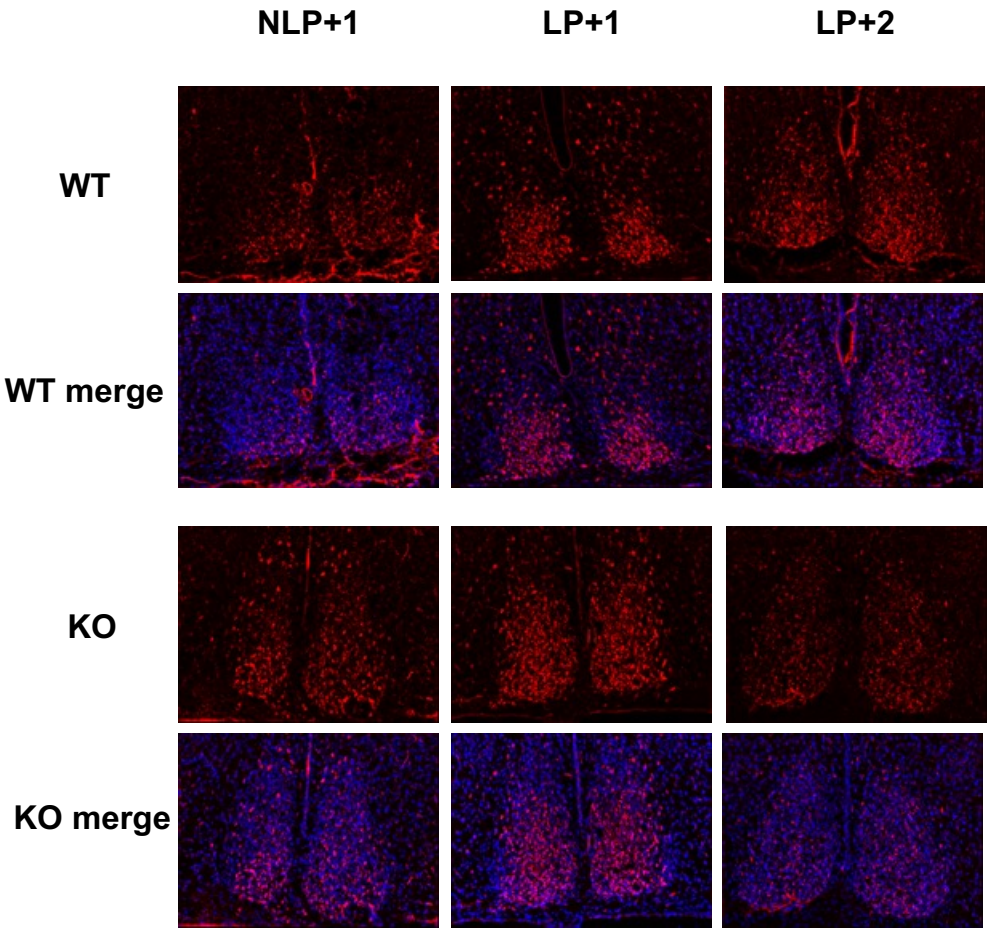

*
*
60
40
20
0
Mean intensity
NLP+1
LP+1
LP+2
WT
KO

Figure 6: EIF2α phosphorylation is decreased in response to light in WT but not Usp2 KO mice. *(A) Representative fluorescence microscopy images of pEIF2α staining 1 hour (LP+1) or 2 hours (LP+2) after a light pulse or 1 hour after no light pulse (NLP+1) and merged images with DAPI staining (B) Quantification of pEIF2α staining averaged over the entire SCN. Individual data points represent individual mice (n: NLP+1,WT = 3; NLP+1,KO = 4; LP+1,WT = 4; LP+1,KO = 4; LP+2,WT = 4; LP+2,KO = 4). All data are represented as mean ± SEM. Two-way ANOVA, with Holm-Sidak post-hoc test. *: $p < 0.05$*

A


NLP+1
LP+1
LP+2
WT
WT merge
KO
KO merge

B

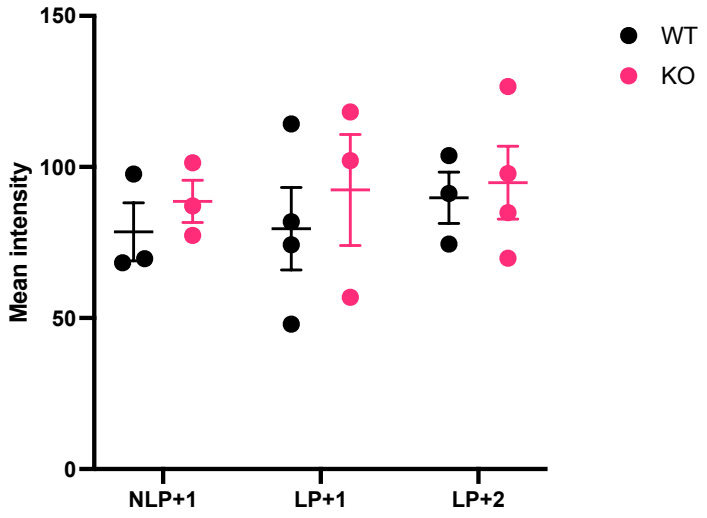

150
100
50
0
Mean intensity
NLP+1
LP+1
LP+2
WT
KO

Figure 7: S6 phosphorylation is unchanged in response to light in WT and Usp2 KO mice. *(A) Representative fluorescence microscopy images of pS6 staining 1 hour (LP+1) or 2 hours (LP+2) after a light pulse or 1 hour after no light pulse (NLP+1) and merged images with DAPI staining (B) Quantification of pS6 staining averaged across the whole SCN. Individual data points represent individual mice (n: NLP+1,WT = 3; NLP+1,KO = 3; LP+1,WT = 4; LP+1,KO = 3; LP+2,WT = 3; LP+2,KO = 4). All data are represented as mean ± SEM. Two-way ANOVA, n.s.*

4.6. Discussion

In this study, we aimed to investigate the molecular basis of USP2 action in the photic response of the SCN clock. We present evidence for rhythmicity of *Usp2* in specific parts of the SCN. Further, we also present possible avenues for the action of USP2 in modulating circadian light response, by showing that normal signalling by ERK and EIF2 pathways is disrupted in the absence of USP2. While we proposed a role for USP2 in circadian light response in our previous article [14], the current study is the first to point towards a mechanism of USP2 action in light response of the circadian clock.

USP2 is a multifaceted DUB in mammals, with established roles in the regulation of cell cycle progression [24–29], maintenance of kidney [30,31] and liver [32] health, glucose metabolism, and muscular differentiation [33] in a homeostatic body context [34,35]. However, USP2's contribution to circadian rhythms has been less studied, despite its involvement in the regulation of multiple clock proteins [4–8,36] and circadian light response [6,14], as well as the modulation of clock outputs [30–32].

The circadian light response is critical in our daily lives, as our internal clock's period is slightly different from 24 hours, while the day-night cycle is 24-hours long. Thus, the internal clock needs to adjust or entrain to the external cycle every day. Previous studies from our lab have demonstrated the importance of USP2 in this process of entrainment [6,14]. The SCN is predominantly divided into two parts: the dorso-medial, “shell”, predominantly populated by neurons expressing the neuropeptide Arginine vasopressin peptide (AVP) and the ventro-lateral “core”, populated predominantly by neurons expressing the peptide Vasoactive intestinal peptide (VIP) [37]. Additionally, some cells in the SCN also express other neuropeptides such as GRP, CCK, GABA and

NMS [38,39]. While the VIP cells are tasked with assimilating light information from the retina, AVP-expressing neurons are involved in maintaining the endogenous rhythmicity of the organism [40]. Since we wanted to check if USP2 specifically acted on the core of the SCN which received inputs from the retina, we knocked out *Usp2* specifically in VIP-expressing cells. We found no differences in entrainment in these KOs, compared to controls. However, this doesn't rule out the possibility of USP2 affecting entrainability of these cells. Rather, it indicates that multiple cell types in the SCN work in conjunction to modulate the photic-entrainment response of the animal. Thus, we explored *Usp2* expression and rhythmicity in different parts of the SCN, to gain clues about its action in the SCN. Using RNAscope we found strong rhythmicity of *Usp2* specifically in AVP-expressing cells with a peak around CT 11.5, but not in other cell types. However, *Usp2* showed a trend towards rhythmicity in the whole SCN. Since RNAscope is a semiquantitative method, further exploration with a more sensitive technique like qRT-PCR could provide a more accurate assessment of the rhythmicity of *Usp2* in the whole SCN. However, it was evident that *Usp2* was expressed throughout the SCN, pointing towards a larger potential area of action for USP2 in light response.

Then, to explore the specific pathways affected by USP2, we performed a proteomic screen on whole SCN protein lysates from mice subjected to a light pulse in the early night (CT 14). Our previous studies have shown that the largest effects are seen in the early night [6,14], due to which an early-night time-point was chosen for the proteomics screen as well. Subjecting these data to Ingenuity Pathway Analysis showed that multiple pathways were enriched in the different pairs of conditions. Other studies have also looked at the SCN proteome in response to light in the early night [41]. In their

study, Tian and colleagues looked at the differences in protein expression in the SCN, in response to a light pulse at CT 15. Tian and colleagues aimed to study the proteins expressed in response to the light pulse. Thus, they collected SCN tissue later than us (at CT 19, 4 hours after the LP at CT 15). However, based on our previous results showing a lack of *Per1* induction in response to light [14], our proteomic screen aimed to capture the differences in pathway enrichment much closer to the time of light pulse (at CT16, 2 hours after the LP at CT 14), rather than assay protein expression in response to the LP. Thus, the pathways enriched, and proteins differentially expressed are different in the two studies, due to fundamental differences in their design.

Concentrating on the pathways enriched in *Usp2* KO animals compared to WT animals in response to the LP, we found that pathways upstream of the circadian clock, crucial to circadian photic response such as the ERK/MAPK transcription cascade [20], the mTOR cascade [42] and the EIF2 pathway, which is essential for translation initiation [22], were enriched. Briefly, in response to light, glutamatergic neurons from the retina innervate the SCN and activate NMDARs, which promote Ca^{2+} influx into the cell. This influx results in ERK activation and phosphorylation, leading to further activation of the ERK/MAPK cascade [23]. This cascade results in the phosphorylation of CREB [43], which is involved in the transcription of *Per1*, a clock gene, as well as an early immediate gene in the photic response pathway of the SCN [44], as well as *Per2*, sometime later [45]. Another downstream target of the ERK/MAPK cascade is the mTOR complex 1 (MTORC1) [42]. This complex facilitates the activation of the mTOR cascade, which initiates translational pathways within the cell, in response to light [46]. The EIF2 pathway is involved in translation initiation, by assisting the assembly of the ribosomal complex

required for the translation of mRNAs synthesized in response to light [22]. The phosphorylation of the alpha subunit of EIF2 (EIF2α) however, blocks the attachment of t-RNA to the translation initiation complex [47]. Hence, pEIF2α represses translation [48].

Using IHC, we validated the results of the screen and found evidence for the involvement of USP2 in modulating the ERK/MAPK and EIF2 signaling cascades, providing insight into the specific pathways affected by USP2 and its role in photic entrainment.

Another interesting hit in the proteomic screen is Phosphoinositide 3-kinase (PI3K). This protein is also upregulated in *Usp2* KO mice, as compared to WTs, in response to light. While it is well known that the PI3K is involved in the activation of mTORC1, a study has shown that PI3K is not activated in response to light, though mTOR is activated by light [42]. Other studies show that PI3K/AKT signalling is important to modulate gene transcription downstream of the clock [49], as well as their feedback into the clock [50]. Put together, USP2 not only regulates processes upstream of the clock (phosphorylation of ERK and EIF2α) and within the clock (binds and affects localization of PER1[51]), but also downstream of the clock, in response to light.

Additionally, the upregulation of PI3K in *Usp2* KO mice could upregulate mTORC1 activation. This could be a possible mechanism to counteract the downregulation of mTORC1 due to a reduction in photic pERK induction. The opposing effects of these two pathways could be an explanation for the lack of differences in pS6 in the IHC validation, despite pS6 being intricately intertwined in the photic response pathway within the SCN.

In conclusion, we show that *Usp2* shows strong rhythmicity in certain parts of the SCN, and we provide evidence for the specific mechanisms of USP2 action in the

circadian light response pathway. However, the specific binding partners of USP2 in this process still need to be characterized. With only 110 DUBs in the mammalian proteome, it is very likely that USP2 interacts with multiple proteins within the whole photic-response pathway, to regulate their half-lives and/or localization. Moreover, it is also possible that multiple DUBs work in conjunction to regulate this process. Nevertheless, our study is the first in-depth characterization of a DUB in the context of circadian light response in a mammalian system. This study shows potential roles for DUBs in regulating pathways upstream of the clock to modulate photic entrainment of the clock itself.

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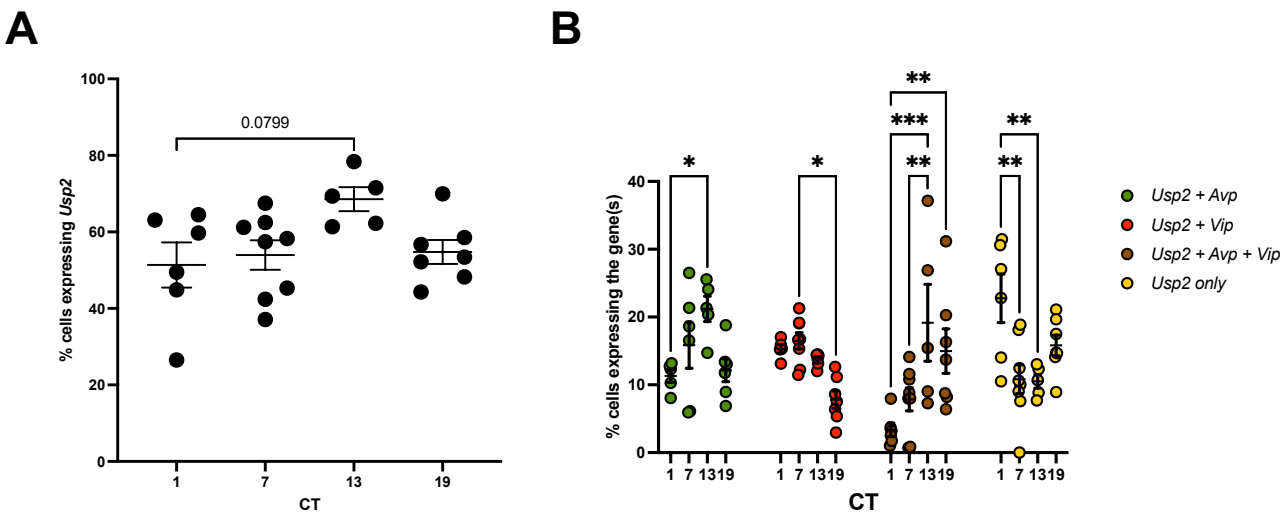
4.9. Supplementary materials

Probe	Fluorescent Opal
RNAscope © Probe-Mm-*Usp2*; transcript variant 1 (471321, Akoya Bioscience)	Opal 570 (PN FP1488001KT, Akoya Biosciences)
RNAscope © Probe-Mm-*Vip*-C3; mRNA (415961-C3, Akoya Bioscience)	Opal 690 (PN FP1497001KT, Akoya Biosciences)
RNAscope © Probe-Mm-*Avp*-C4; mRNA (401391-C4, Advanced Cell Diagnostics)	Opal 520 (PN FP1487001KT, Akoya Biosciences)

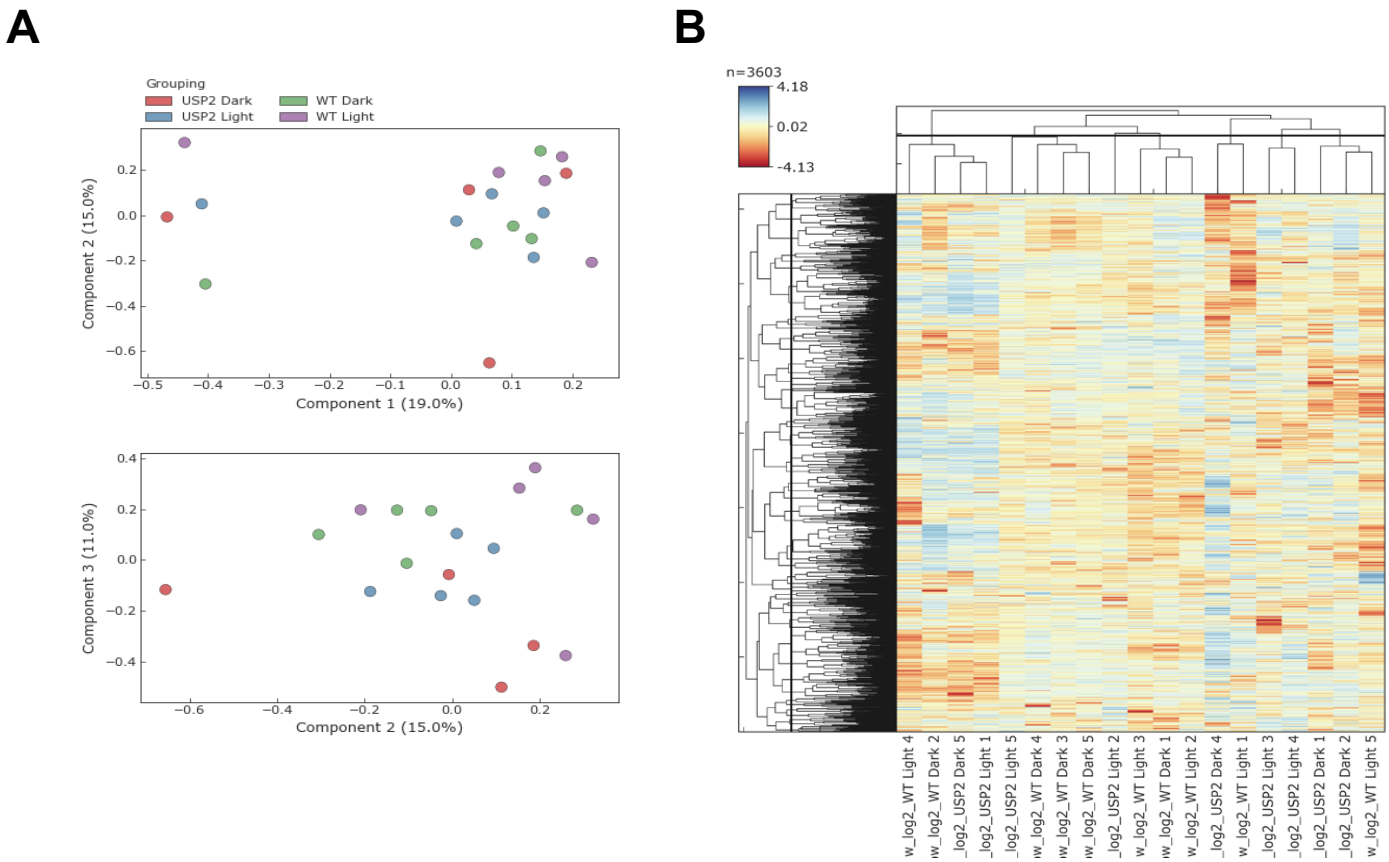
Supplementary table 1: Probes and Opals used for RNAscope © fluorescent in-situ hybridization assay

Primary antibody	Primary antibody concentration	Secondary antibody	Secondary antibody concentration
Rabbit anti-p-ERK (4370S; Cell signalling technologies)	1:750	Donkey-anti-rabbit Alexa Fluor 594 (711-585-152, Jackson ImmunoResearch Inc)	1:1000
Rabbit anti-p-EIF2α (3398S; Cell signalling technologies)	1:150	Donkey-anti-rabbit Alexa Fluor 594 (711-585-152, Jackson ImmunoResearch Inc)	1:400
Rabbit anti-p-S6 (5364S; Cell signalling technologies)	1:1000	Donkey-anti-rabbit Alexa Fluor 594 (711-585-152, Jackson ImmunoResearch Inc)	1:1000

Supplementary table 2: Antibodies used for immunohistochemistry and their concentrations



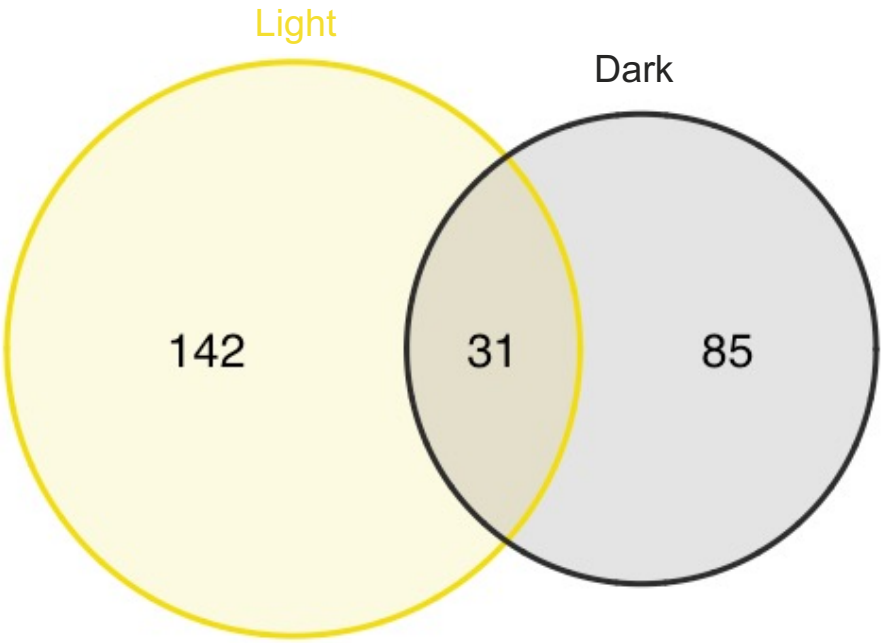
Supplementary figure 1: RNAscope fluorescent in-situ hybridization analysis. (A) Variation of *Usp2* levels in the whole SCN, over time. **(B)** Variation of *Usp2* in the different cell groups within the whole SCN, over time. *Individual points represent individual SCN lobes. All data are represented as mean ± SEM (n: CT 1 = 6, CT 7 = 8, CT 13 = 5, CT 19 = 7). One-way ANOVA with Holm-Sidak post-hoc test. Two-way ANOVA, with Holm-Sidak post-hoc test. *: p < 0.05, **: p < 0.01, ***: p < 0.001*



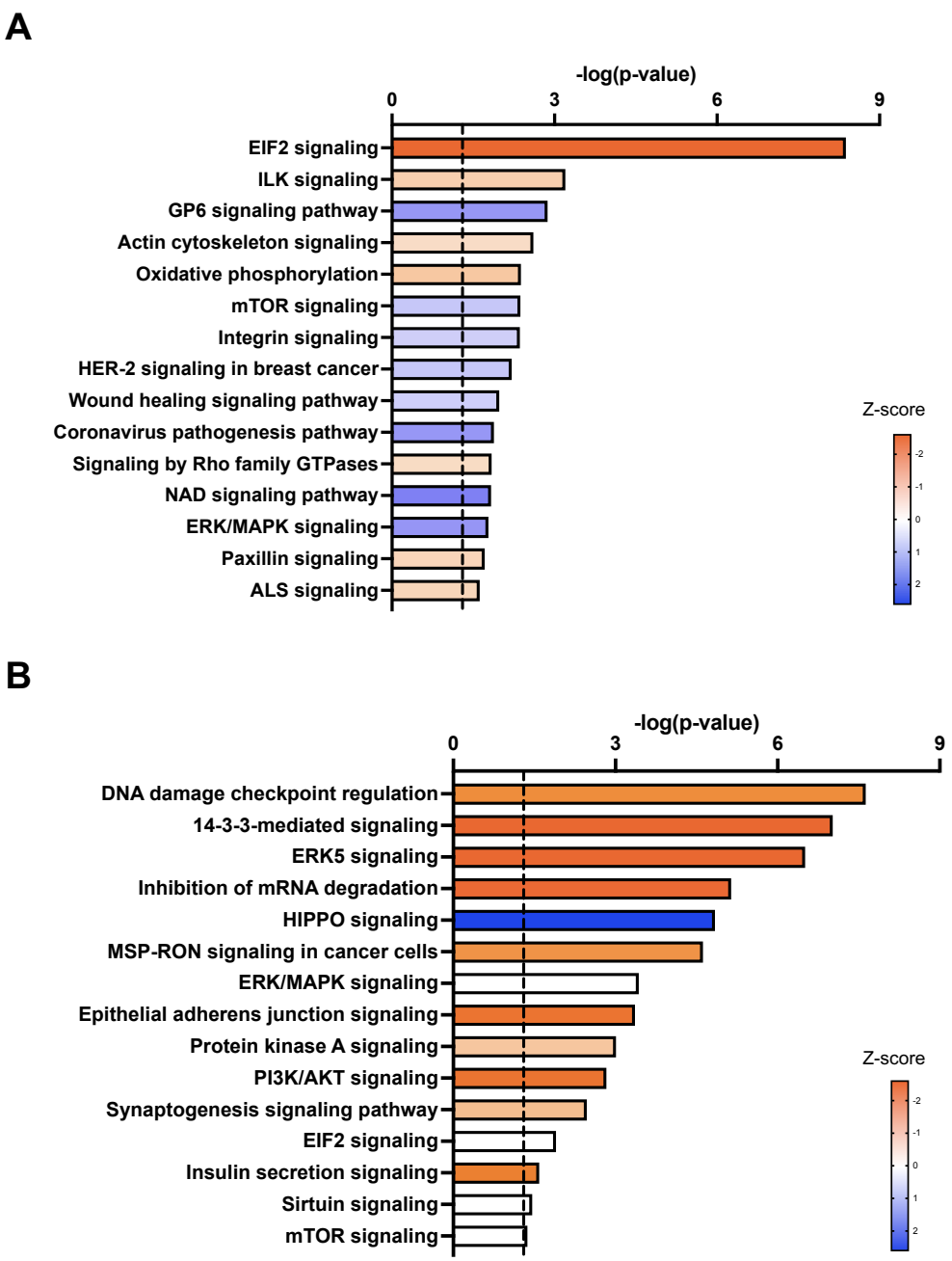
Supplementary figure 2: Basic analysis of proteomic screen. (A) *Principal component analysis of proteomic screen groups.* ***(B)*** *Dendrogam of the 3055 proteins detected in the screen.*

A

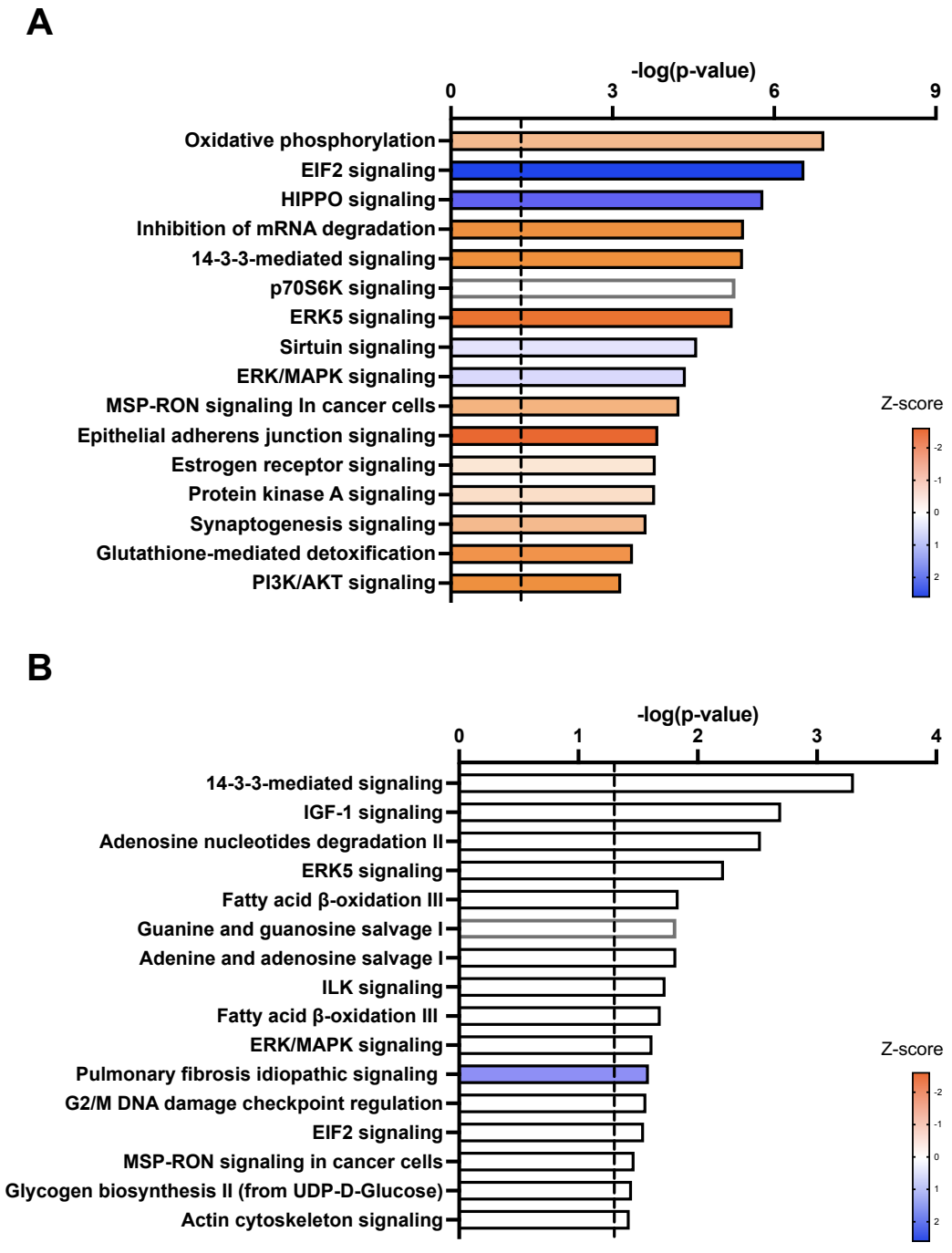
Differentially expressed proteins in KO vs WT



Supplementary figure 3: Venn diagram of proteins differentially expressed in each lighting condition, in the Usp2 KO as compared to the WT SCNs.



Supplementary figure 4: Pathway analysis of the differentially expressed proteins in the proteomic screen; KO/WT. (A, B) *Differential enrichment of pathways in USP2 KO SCNs compared to WT SCNs, when* ***(A)*** *they are exposed to light (LP) or* ***(B)*** *they are not exposed to light (NLP). These data are same as Figure 4, except they are plotted according to p-value (Y-axis) here.*



Supplementary figure 5: ***Pathway analysis of the differentially expressed proteins in the proteomic screen; LP/NLP. (A, B)*** *Differential enrichment of pathways in LP SCNs compared to NLP SCNs, when* ***(A)*** *they are in WT mice or* ***(B)*** *they are in Usp2 KO mice.*

Chapter 5: Summary, discussion and future directions

Summary, discussion and future directions

USP2 is a deubiquitinase (DUB) found in mammals that removes or shortens ubiquitin tags from proteins, affecting their half-life and/or location [106,123,132]. It has 7 different isoforms, of which USP2-45 and USP2-69 are the predominant forms [123]. USP2 interacts with multiple proteins, regulating various physiological functions such as renal sodium balance [133], lipid metabolism, hepatic gluconeogenesis[134] and muscular differentiation [135], as well as in immunity and inflammation-related pathways [108,119]. Dysregulation of USP2's interaction with cell-cycle proteins is involved in the pathogenesis of multiple types of cancers [108,109,113,136–140]. USP2-45 is also expressed in the brain and affects the circadian system in mammals. The following discussion is a deep dive into the roles of USP2 in the brain and in circadian rhythms, using a top-down experimental approach. Let’s take a look!

5.1. USP2 and non-circadian behaviors

The Allen brain institute's in-situ hybridization screen showed that *Usp2* is expressed throughout the brain (chapter 2, table 1). Despite its widespread expression, it was unclear whether USP2 had any effects on behavioral outputs of the brain. Studies have suggested a correlation between stress-induced downregulation of USP2 and impaired memory retrieval [126], a correlation between USP2 levels and psychotic disorder severity in patients with schizophrenia and bipolar disorder [127] and a correlation between USP2 mutations and developmental delays and seizures [141]. However, these studies provided no direct evidence of USP2 causing behavioral changes.

In our first paper, we aimed to find evidence of the role of USP2 in modulating behavior. While previous studies correlated behavioral changes to changes in USP2 levels, we directly knocked out *Usp2* (*Usp2* KO) in mice and evaluated their behavior.

5.1.1. The role of USP2 in memory

According to data from the Allen Brain Atlas, *Usp2* has the highest expression in the cortex and hippocampus. This is unsurprising as the hippocampus is a region in the brain with known USP2-associated functions, such as the stress-related signaling pathway [126] and modulation of blood glucose via sympathetic neuron activation [125]. Given the close link between these brain regions and memory, we investigated the effect of *Usp2* knockout on memory, using the Morris water maze test (MWM) to examine spatial memory. While another study correlated a reduction in spatial memory with a decrease in USP2 expression in the hippocampus [126], our results in chapter 2 showed that *Usp2* KO had no effect on spatial memory in mice [122]. A possible explanation for this discrepancy is that stress-induced pathways could affect both *Usp2* transcription and spatial memory, without the two being interlinked. Next, using the novel object recognition test (NOR), we found that *Usp2* KO does affect recognition memory [122]. Thereafter, we investigated another hippocampus-mediated behavior: anxiety-like behavior using the novelty-suppressed feeding test and the elevated plus maze and found that knocking out *Usp2* reduces anxiety-like behavior in mice [122].

It would be interesting to understand the differences in roles of USP2 in specific brain regions, leading to deficits in recognition memory but not in spatial memory. While neocortical structures are more involved in encoding recognition memory [142,143],

hippocampal structures play a larger role in spatial memory [144–146]. While we have looked at NOR here, the novel place recognition (NPR) test assays recognition memory while manipulating spatial information in the experiment [147]. Studies have shown a larger hippocampal component for NPR compared to NOR [148,149]. Thus, NPR could provide clues about the specific brain regions and pathways affected by the loss of USP2. Additionally, knocking out *Usp2* in specific brain regions could also pinpoint the regions and brain pathways modulated by USP2 to control anxiety-like behavior, as well as memory, in mice.

5.1.2. The role of USP2 in sensorimotor gating

Data from the Allen brain atlas also showed high *Usp2* expression in the olfactory lobe and striatum, which are involved in sensorimotor gating. Additionally, prepulse inhibition (PPI), the primary test for sensorimotor gating is also affected by the limbic and dopaminergic systems: it is especially affected by the dopaminergic cortico-striato-pallido-thalamic and limbic cortico-striato-pallido-pontine tracts [150], all regions with high *Usp2* expression. However, the PPI test showed a very mild effect of *Usp2* KO, with the KO mice exhibiting a lower level of PPI overall but reaching significance only at specific prepulse intensities. Thus, further study is required to define how and the extent to which USP2 modulates sensorimotor gating in mice. It could also be interesting to look at the role of USP2 in the specific brain regions modulating PPI, to understand the lower sensorimotor gating phenotype observed in the absence of USP2.

5.1.3. The role of USP2 in wheel running behavior

The dopaminergic system controls motivated behaviors such as wheel running in mice [151,152] and when we examined this behavior, our study found that *Usp2* KO mice exhibited increased wheel running and running bout length compared to WT mice in a 12:12 LD cycle (Although not presented in this thesis, this pattern has held true for a majority of *Usp2* KO mice that have gone through wheel-running during all the circadian experiments of chapter 3 as well). While increased bout length could be attributed to improved motor coordination without USP2, the rotarod test revealed the opposite, with *Usp2* KO mice exhibiting a decreased latency to fall off the rod and hence, reduced motor coordination. Thus, the wheel running phenotypes aren't related to motor coordination. However, they could be related to reward pathways, and this represents an interesting avenue for future research. Additionally, wheel running behaviors also inform the circadian system and USP2 has a role in modulating circadian rhythms.

5.2. USP2 and why we hypothesized that it has a role in circadian rhythms and photic entrainment

Studies by Scoma [120], Tong [119], and our lab [117], were the first to report the interaction of USP2 with key circadian proteins BMAL1, CRY1, and PER1, respectively. Our lab also found that *Usp2* KO mice showed differences in entrainment patterns when subjected to photic phase shifts, implicating USP2 in photic entrainment [117]. We also demonstrated that USP2 directly deubiquitinates PER1, promoting its nuclear localization [118]. Further, Zhang and colleagues found that among 12 mouse tissue types, only 10 genes showed rhythmicity in all sampled tissues, including *Usp2* [21]. This finding was

also echoed by other studies [130,153,154]. Finally, there is a report of *Usp2* expression in the liver itself being under BMAL1/CLOCK transcriptional control [131]. Therefore, we hypothesized that USP2 plays a crucial role in regulating photic entrainment due to its rhythmicity, direct interactions with clock proteins, and involvement in light response in mice.

5.3. USP2 in photic entrainment

Our lab's paper characterizing the role of USP2 in the circadian clock had a phase response curve (PRC) to examine the role of USP2 in photic entrainment. While *Usp2* KO mice showed a reduced phase shift in response to a light pulse at CT 22, in response to the only early night light pulse (CT 14) time point, *Usp2* KO mice showed a significant, almost two-fold increase in phase delay, compared to WT mice [117]. Thus, to better understand how USP2 critically affects phase delays in mice, we constructed a high-resolution PRC, with data points every hour between CT 10 and 16. In our PRC we replicated the two-fold increase in delay at CT14. Surprisingly, at CT 12, *Usp2* KO mice showed no delaying response, while WT mice did show a small phase delay to the light pulse at CT 12. In essence, USP2 had different effects on light response at different times within the early night itself.

To probe these different effects of USP2 on early-night light-response, we developed the modified skeleton photoperiod (mSPP) protocol. The skeleton photoperiod (SPP) with two daily light pulses flanking the subjective night is a sufficient zeitgeber to maintain a 24-hour daily rhythm in rodents [155]. Studies have shown that a daily, single dusk [67,156–158] pulse is also sufficient to entrain rodents to a 24-hour rhythm.

However, the power of a one-pulse SPP to assess phase shifting without any masking effects hasn't been exploited in the circadian field, with Olde Engberink and colleagues who subjected mice to a Aschoff type 2 shift after a conventional SPP [69] being the only group looking at any format of phase shifting after an SPP protocol after the original papers by Pittendrigh and Daan [67,159]. The biggest USP (forgive the pun) of the mSPP, however, is the ability to assess both phase delays and advances, with the light exposure being limited only to the early night, thus delineating phase shifting of the clock from the timing of light exposure. As shown in chapter 3, *Usp2* KO mice entrained better to both advances and delays, compared to WT mice, thus establishing a specific role for USP2 in early evening entrainment, irrespective of the direction of phase shift.

5.4. USP2 in the SCN

5.4.1. USP2 in the whole SCN

Remarkably, knocking out *Usp2* specifically in the SCN and subjecting these KO mice to the mSPP produced very similar phenotypes to that of the whole body *Usp2* KO. The fact that the phenotypes weren't the same as the whole body *Usp2* KO model could stem from multiple factors. The most likely explanation is the difference in strain backgrounds between the two lines. While the *Usp2* KO line has a strong C57BL6/J background, the *Usp2* floxed line although originating from the same line, has been heavily inbred. This could explain the differences in entrainment between WT and *Usp2* floxed mice. Second, the SCN is not the only region of the brain receiving inputs from the retina, with the intergeniculate leaflet (IGL) and the dorsal raphae nucleus (DRN) being two other retinorecipient regions which innervate the SCN. The role of IGL in circadian

rhythms has been widely studied [160], with lesion studies showing its importance in the masking response of the clock [161], sensing light intensity [162], maintenance of period [163,164] and locomotor activity amplitude [164], in conjunction with the SCN [165]. Additionally, the IGL has shown to have an important role in entraining to skeleton photoperiods [166]. With the thalamus showing high *Usp2* expression as well, it is possible that the IGL also contributes to the mSPP phenotype. It would be interesting to knockout *Usp2* in the different retinorecipient regions that innervate the SCN and investigate mSPP entrainment. Another way to distinguish the specific role of the SCN in the process of USP2-dependent photo-entrainment is using ex-vivo SCN explant cultures. These ex-vivo explants could be subjected to skeleton photoperiods using light-inducible channels and their rhythms could be used as a proxy to understand the role of USP2 in modulating SCN photoentrainment. While this would not inform us if the role of USP2 is SCN-specific, it would provide accurate information about how USP2 specifically affects the SCN.

Eventually, however, the striking similarity between entrainment in the SCN specific *Usp2* KO and the full body *Usp2* KO clearly shows that the role of USP2 in entrainment is primarily in the SCN.

5.4.2. USP in specific neuron clusters of the SCN

To further narrow down the zone of USP2 action within the SCN, we knocked out *Usp2* specifically in the VIP-expressing neurons. The ventro-lateral “core” of the SCN, predominantly populated by VIP-expressing neurons is the retinorecipient region of the SCN, making it the most likely region of the SCN to be affected by USP2, to modulate

photic entrainment. However, no differences were seen between the controls and the VIP cell specific *Usp2* KOs when subjected to the mSPP. This phenotype doesn't rule out a role for USP2 in VIP-expressing cells. However, it does show that the role of USP2 is not restricted exclusively to the VIP-expressing core region of the SCN. Probing for *Usp2* localization in the SCN with *Avp* and *Vip*, the two most abundant neuropeptides in the SCN [31] using RNAscope showed no differences in average expression levels of *Usp2* within cells expressing either of these peptides. Moreover, the same average amount of *Usp2* was also expressed in cells not expressing either *Avp* or *Vip*. On one hand, these cells not expressing *Avp* or *Vip* could be neurons expressing one or a combination of other neuropeptides such as GRP, CCK, NMS or GABA [31,33,36]. On the other hand, it is possible that these cells are not neurons but rather, astrocytes, which cross talk with neurons and play a key role in maintaining circadian rhythms and mediating photic entrainment in the SCN [167–169].

5.4.3. Looking past neurons for the potential roles of USP2 in entrainment

The viral construct we used in chapter 3, for the SCN-specific *Usp2* KO contained a Cre gene transcribed under a CAG promoter, which is not cell-type specific [170] and is also robustly expressed in all the glial cell types [171]. Additionally, our proteomic screen in chapter 4 showed an upregulation of astrocyte function associated proteins, GFAP (Glial fibrillary astrocyte associated protein) and PEA15 (phosphoprotein-enriched in astrocytes 15) [172], in response to light, in WT mice, but not in *Usp2* KO mice. Previous studies have already established that GFAP is increased in the light [173,174] and that PEA15 modulates photic *Per1* induction via the MAPK/ERK cascade [175]. In

chapter 3 we showed that *Per1* induction is blunted in *Usp2* KO mice and in chapter 4 we saw that phosphorylation and activation of ERK was reduced in *Usp2* KOs, in line with the lack of photic induction of PEA15 in *Usp2* KO mice. At the cellular level, while it is known that the SCN neurons drive astrocyte rhythms [176], recent studies also show that astrocytes are able to entrain neuronal rhythms [177]. Although a specific role for astrocytes in photic entrainment isn't known, studying the role of USP2 in astrocytes, in the context of entrainment could be a promising window into this process, as well as to understand the specific cell-types in the SCN where USP2 affects entrainment. Interestingly, single cell RNAseq studies of the SCN have shown that USP2 is expressed in the astrocytes at similar levels as in neurons [33,36], further supporting a potential role for USP2 in astrocytes, to modulate photic entrainment of the SCN.

Could this potential role for USP2 in astrocytes be localized to a specific region of the SCN? While the role of glutamate in photic entrainment is well known in neurons [73,86,178,179], Brancaccio and colleagues showed that glutamate released by astrocytes had an inhibitory effect on neurons in the night [180]. Additionally, they also showed a localization of this astrocyte signalling in the dorsal part of the SCN, which is also primarily populated by AVP-expressing neurons. Interestingly, in our RNAscope analysis, we found that *Usp2* levels showed significant rhythmicity only in AVP-expressing cells. AVP-expressing cells of the SCN are known to maintain the strongly rhythmic endogenous rhythms of animals [34], as well as a limiting factor for the speed of photic resetting during entrainment [24]. The entrainment to mSPP in chapter 3, with *Usp2* KO mice showing better entrainment than WT mice points towards a reduction in resistance to entrainment. Moreover, in our proteomic screen, proteins like SEC22B, VTI1B, VAMP2

and SYAP1, which are involved in vesicle fusion are upregulated in response to light in WT but not *Usp2* KO mice, while GRIK3 and GRIA1, glutamatergic receptor subunits, are downregulated. This points towards potential defects in cell-cell communication within the SCN. Put together, it would be interesting to investigate whether USP2 plays a key role in the cross talk between astrocytes and AVP-expressing neurons in the dorsal SCN, to modulate photic entrainment in the SCN.

5.5. USP2 in the retina

Mammalian photic entrainment begins in the eye, with retinal glutamatergic projections, called the retinohypothalamic tract (RHT) innervating multiple brain regions including the SCN, the IGL and the OPN (olivary pretectal nucleus) [181]. The OPN is a primary visual center which is detects changes in incoming luminance information, as well, modulates saccadic motions of the eye [54]. It is also involved in the constriction of the pupil in response to bright lights [54]. In the retina, 1% of all the cells are ipRGCs [182]. These cells transmit information about light presence and intensity to the brain. Thus, pupillary light-constriction response (PLR) in response to bright light can be used as a proxy for ipRGC functioning [47,183]. PLR was not different in *Usp2* KO mice compared to WT mice. However, ipRGCs aren't a homogenous population, with different subsets involved with PLR and with SCN photoreception [55]. Thus, we could argue that *Usp2* signalling could be selectively affecting one subset of ipRGCs.

Additionally, retinal clock and retinal function gene rhythms in *Usp2* KO retinas showed a more robust rhythm for the clock genes *Bmal1*, *Per1*, *Cry2*, *Rev-erbα* and the retinal function genes *Opn1sw* and *Opn4,* compared to WT retinas. The damped rhythms

of clock genes in WT whole-retinas has been reported in earlier studies, to be a result of different retinal layers having different phases of clock genes [38,39] or the result of some cells in the retina just not having rhythms [40]. Of note, among the genes gaining rhythmicity in the absence of USP2, is *Opn4*, which is a marker of ipRGCs. Thus, while PLR is unaffected, the effects of *Usp2* on retinal gene expression is undeniable.

5.5.1. Future outlook on the potential roles of USP2 in the retina

The implications of these findings in photoreception and in information transfer to the SCN warrant a closer look. An obvious first step is to generate a retina-specific *Usp2* KO, similar to the SCN-specific *Usp2* KO, to investigate if USP2 in the retina plays any role in photic entrainment at all. Further, an ipRGC-specific *Usp2* KO model could be subjected to mSPP to explore the role of USP2 in transferring photic information to the SCN. Unlike the PLR assay which informed us about ipRGC functioning, this would specifically assay photic information transfer from the retina to the SCN via the RHT, hence specifically informing us about the role of USP2 in mediating this process. Moreover, Scoma and colleagues had found that *Usp2* KO mice had increased light sensitivity [120]. Using PLR, this phenotype could also be further investigated, to understand if the basis for it lies within the ipRGCs.

Finally, it is hard to ignore the striking results of *Usp2* KO on retinal gene rhythms. Studying the rhythms of *Usp2* in the different layers of the retina, as well, assessing the interaction of USP2 with clock proteins in the retina (compared to those in the SCN), could point towards the specific pathways affected by USP2 in the process of retinal

photoreception. This could also provide further information about the increased light sensitivity observed in *Usp2* KO mice, by Scoma and colleagues [120].

5.6. USP2 in photic entrainment pathways

From all the results in chapter 3, we identified the SCN as the main site of USP2 action for photic entrainment of the clock. Hence, we then asked how this process takes place. Exposing *Usp2* KO (and WT) mice to a CT 14 light pulse (or not), we subjected the protein lysates of their SCNs to shotgun proteomics. Of the 3055 identified peptides, 345 peptides were differentially expressed in at least one condition. Of these, comparing the 235 proteins differentially expressed between genotypes in LP vs NLP, 72 were differentially regulated only in KO mice and 135 were differentially regulated only in the WT mice. Hence, knocking out *Usp2* extensively affects proteins and pathways involved in and downstream of photic entrainment of the SCN.

5.6.1. USP2 affects the MAPK/ERK cascade

Looking specifically for pathways involved in light response, we found differential expression of proteins involved in the MAPK/ERK cascade (RPS6KA1, MSK1, PEA15, PP2A and PI3K), which is one of the first cascades activated in response to light [84]. We confirmed this using IHC, where the phosphorylated or activated form of ERK was at baseline levels and hence significantly lower in *Usp2* KO mice compared to WT mice, in response to a light pulse. The ERK cascade culminates in the phosphorylation of cAMP response element binding protein (CREB) [84], which leads to the induction of *Per1*, hence coupling the photoreception pathway to the SCN clock, leading to entrainment

[84,87]. Assaying *Per1* levels in response to a light pulse in chapter 3 showed an abrogation in photic induction of *Per1*, further verifying the differential regulation of proteins in the MAPK/ERK, in *Usp2* KO mice. This makes the MAPK/ERK pathway a strong target pathway for USP2 action. In addition, differential expression of proteins upstream of this pathway, such as glutamatergic receptor subunits (GRIK3 and GRIA1), as well, Ca^{2+}/Calmodulin dependent kinases (CAMK2 and CAMK1D), in *Usp2* KO mice when exposed to a light pulse, point towards Ca^{2+} regulation being a potential target pathway for USP2 action in photic entrainment. While roles of GRIK3, GRIA1 and CAMK2 are known in the circadian system, the role of CAMK1D in this system is unknown. However, in-situ hybridization data from the Allen brain atlas shows significant expression of *Camk1d* in the SCN, pointing towards a potential role for the protein in photic entrainment.

5.6.2. USP2 affects the EIF2 translation initiation pathway

The EIF2 pathway, which is involved in initiating protein translation[96], was one of the most differentially enriched pathways between the genotypes in response to light, with PI3K (upregulated), 40S ribosomal subunit (upregulated), 60S ribosomal subunit (upregulated) and EIF2β (downregulated) being differentially regulated in *Usp2* KO mice, in response to light. The inhibitory phosphorylation of EIF2α [96] in WT mice was reduced in response to light. Reduction in pEIF2α has been associated with an increase in PER1 [184]. *Usp2* KO mice showed no differences in pEIF2α levels, in response to light. Additionally, pEIF2α levels were lower in *Usp2* KO mice compared to WT mice, in the no light pulse condition. Studies showed that pEIF2α cycling is essential for rhythmic protein

translation, with pEIF2α levels peaking around the day night transition [185]. The reduced pEIF2α in the absence of light point towards a different phase for pEIF2α rhythms or deficits in circadian translation in *Usp2* KO mice. Additionally, Pathak and colleagues showed that reduced pEIF2α led to rapid entrainment to phase shifts, damped PER1 rhythms and an increase in circadian period, all of which we observe in *Usp2* KO mice (chapter 3, [117]). Put together, the EIF2 pathway is a strong potential pathway being affected by USP2 to modulate entrainment.

5.6.3. USP2 has no effects on the mTOR pathway

The mTOR pathway is a pathway between the MAPK/ERK pathway and the EIF2 pathway, in photoreception [95,186]. The ERK cascade activates the mTOR cascade [93], which in turn feeds into translation pathways in the SCN [95]. The mTOR pathway is negatively enriched in *Usp2* KO mice, in response to light. One of the intermediate steps in this cascade is the phosphorylation of the S6 kinase [94]. Unlike the pERK phenotype, there is no difference between pS6 in WT and *Usp2* KO, in response to the light pulse. This is surprising, considering that pathways upstream and downstream of this pathway are affected in the absence of USP2. Further studies will be required to understand this phenotype. One possible explanation for this could involve, the upregulation of PI3K in *Usp2* KO mice, which could upregulate mTORC1 activity [60]. Since pERK leads to activation of the mTORC1 complex [93], a reduction in ERK phosphorylation in *Usp2* KO mice, in response to light, could lead to downregulation of mTORC1. The opposing effects of these two pathways could explain the overall lack of differences in pS6 levels (between genotypes and lighting conditions) found using the

IHC validation, despite the mTOR pathway being intricately intertwined in the photic response pathway within the SCN [187].

5.6.4. A unified model of USP2 action on entrainment

While my thesis, as well as previous studies from our lab, firmly establish a role for USP2 in early night photic entrainment of the SCN, we don't yet have an in-depth understanding of the mechanistic basis of USP2 action in this process. Based on all our results, I have constructed a model of USP2 action in photic entrainment and the circadian clock (Figure 1), which I will further explain below.

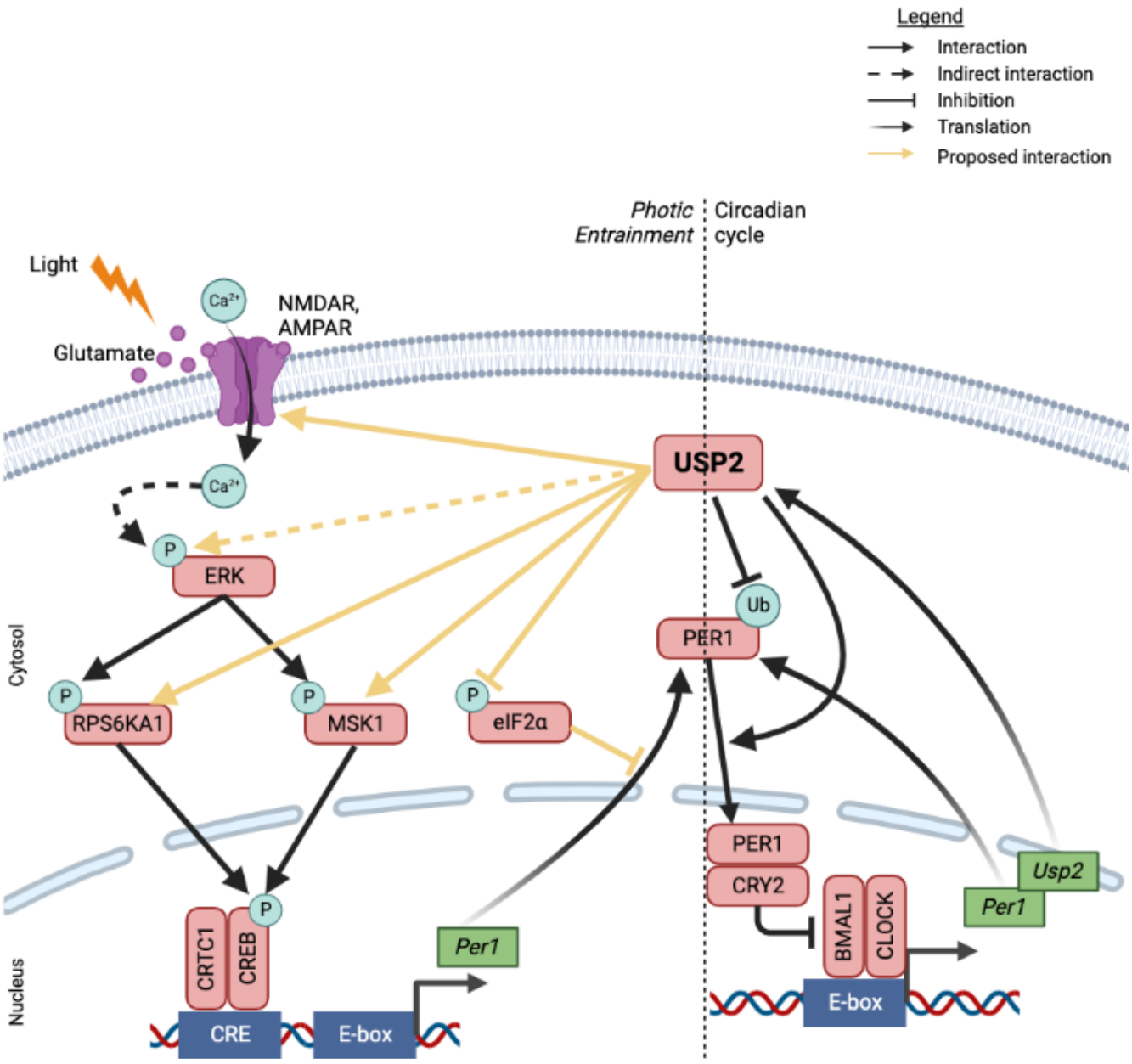


Figure 1: A cohesive view of known and proposed interactions of USP2 in modulating responses in photic entrainment, as well as the circadian clock.

(Created with BioRender.com)

Beginning with a light pulse, we can assume that USP2, a deubiquitinase, increases the abundance of NMDA and AMPA receptors either by binding and

deubiquitinating them or by acting on vesicle fusion pathways to promote an increase in these receptors in the post-synaptic membrane or by acting on a completely distinct mechanism, to potentially promote Ca^{2+} influx into the cell. Ca^{2+} influx into the neuron upon glutamate application could be measured electrophysiologically in *Usp2* KO SCNs to investigate if USP2 indeed affects Ca^{2+} signalling in the SCN. Additionally, Ca^{2+} rhythmicity, which is essential for effective SCN resetting, could also be studied in *Usp2* KO mice. Further, co-immunoprecipitation assays could be used to find out what are the specific binding partners of USP2 in this phase.

Either way, USP2 eventually promotes ERK phosphorylation, which leads to the phosphorylation of downstream targets such as RPS6KA1 and MSK1 [82], which phosphorylate CREB to mediate the photic induction of *Per1* [85–88]. In the proteomic screen, RPS6KA1 and MSK1 were both not upregulated in response to light, in *Usp2* KO mice. Thus, USP2 might deubiquitinate and stabilize these proteins, which can be verified by co-immunoprecipitation of these proteins with USP2 (to check for binding of proteins) and using a CHX assay (to assay protein stability).

The *Per1* mRNA is then translated into PER1 protein. Phosphorylated EIF2α inhibits translation and USP2 inhibits EIF2α phosphorylation. Thus, it is possible that USP2 might indirectly be promoting PER1 translation by blocking phosphorylation of EIF2α. Further, the deubiquitination of PER1 by USP2 promotes its translocation to the nuclear compartment [118], where it binds CRY2 and suppresses the activity of the CLOCK/BMAL1 complex [7,8]. The CLOCK/BMAL1 complex is involved in the transcription of genes such as *Per1* [8] and *Usp2* [131]. Hence, by suppressing this complex, PER1 not only passes on photic information to the clock by changing the phase

of the TTFL, but also, it represses USP2 production, thus inhibiting the light-response pathways activated by USP2 action.

Thus, USP2 and PER1 could be key components of a photic entrainment-circadian cycle feedback loop, with USP2 acting as the positive regulator of this loop, while PER1 is the negative regulator. While further work would be required to establish all molecular players of this loop, my work establishes USP2 as a key mediator between the photic entrainment pathway and the circadian feedback loop.

5.7 Other pathways affected by USP2, in the SCN

5.7.1. The ubiquitination pathway

Apart from these known light response pathways, there were also other interesting hits in the screen. Very interestingly, the “ubiquitination of proteins” pathway was downregulated in WT SCNs, in response to light and this pathway was represented by UBFD1, UBE2H, UFD1 and UBL3. With the induction of multiple genes in response to light, it is only logical that protein translation is upregulated in response to light. Thus, it would make sense that this pathway is downregulated in response to the light. Tian and colleagues also found a downregulation of ubiquitin ligases in their light-response proteomic screen, specifically UBE3A [188], which we were also able to detect, but it didn’t pass our significance cut-off. They also found an upregulation of DUBs USP9X and USP7, which again we were able to detect, without significant changes between conditions. However, we detected the DUB VCPIP1 which was significantly upregulated in WT but downregulated in *Usp2* KO, in response to light. One major reason for the discrepancy between our results is explained by the experimental design: while our light

pulse was at CT 14 with tissues collected 2 hours after, the light pulse in their study was at CT 15, with tissues collected 4 hours after [188]. It follows, therefore, that the two screens would not detect the same proteins being differentially expressed. However, though we didn't find differential expression of the same proteins, the fact that similar pathways were enriched in both experiments gives further credence to the results of both experiments.

5.7.2. Neurological disease-related pathways

Another Interesting set of differentially regulated proteins are those involved with neurological disorders. APLP2 (Alzheimer's disease-related protein) [189] is upregulated in *Usp2* KO mice. APOE (Alzheimer's disease-related protein) [190], SNCA (Alzheimer's and Parkinson's related protein) [191–193] and HIP1 (Huntington's disease-related protein) [194] are all differentially regulated in *Usp2* KO mice, as compared to WT mice, in response to light. It is well known that all these diseases are associated with circadian disturbances [195–200], with some studies suggesting a role for circadian disruption in the pathogenesis of these diseases [196,199,200]. In our modern lifestyles with a high prevalence of artificial lights at night, we are in a state of consistent circadian disruption [201]. Multiple studies have shown the detrimental effects of continuous light exposure at night [201–204]. Hence, in the modern context, it is vital to further probe the links between circadian disruptions and the pathogenesis of these neurological diseases to maintain better brain health in modern society. In recent studies from our lab that use a circadian disruption along with maternal immune activation (MIA) as a model for neurodevelopmental disorders [205], a transcriptomic screen showed that *Usp2*

expression was increased by 1.5 times in MIA offspring that were exposed to circadian disruption as compared to those that weren't (unpublished data). Thus, the role of USP2 in neurological disorders and its potential role in this process by mediation of daily entrainment to the modern environment of artificial lights, would make for an interesting study spanning across the multiple themes of the lab.

5.8. Concluding remarks

Our studies open the door to understanding the role of USP2 in mouse behavior. In this thesis, I have provided experimental proof of specific non-circadian behavioral deficits elicited in mouse behavior due to a loss of USP2. I have also carefully assessed the role of USP2 in the process of circadian photic entrainment. This thesis establishes that USP2 acts predominantly within the SCN to modulate photic entrainment of the clock. Further, within the clock, USP2 affects the MAPK/ERK cascade and the EIF2 pathway to acutely affect light perception in the SCN, thus providing an understanding of the specific pathways USP2 acts on, to modulate entrainment.

While previous studies from our lab specifically focussed on the role of USP2 within the clock [117,118], in my thesis, I looked at the whole entrainment pathway and was able to find that USP2 has a role upstream of the clock as well. This is the first study to decipher the specific pathways being affected by a DUB in circadian photic entrainment. These studies open the doors for further studies of DUBs and ubiquitin ligases as an additional layer of control on the photic entrainment pathway. Put together, I have provided evidence for USP2 being a key player in a feedback loop involving both, the photic entrainment pathway and the circadian transcription-translation feedback loop. While USP2 promotes

CREB-mediated *Per1* transcription and PER1 protein translocation to the nucleus, PER1 acts as the negative arm of this feedback loop, to potentially repress *Usp2* transcription, thus closing the loop.

Additionally, along with a deep understanding of the role of USP2 in photic entrainment, my thesis provides avenues for future research specifically with respect to USP2 as well as more generally on the topic of photic entrainment. How does USP2 affect the functioning of the retina? Can USP2 be localized to a specific part of the SCN? A specific cell type? What roles can astrocytes play in the process of entrainment? What are the specific binding partners of USP2? What are the pathways enriched acutely, in response to a light pulse and how do they fit into light response pathways? What are the implications of these findings to human health?

Understanding entrainment is essential to tackle human-exclusive problems like jetlag and shiftwork, both of which have many detrimental effects on human health [206–212]. Current strategies to manage circadian-environmental effects are either impractical in the modern world (such as reduced light exposure at specific times) or are pharmacological strategies that affect pathways downstream of the clock, without actually addressing clock desynchrony itself (administration of melatonin or alertness-promoting agents) [213]. Comprehensively understanding the molecular basis for entrainment, and more specifically the role of USP2 in entrainment, could overcome these limitations. Specifically, discovering therapeutic targets within the circadian light response pathway itself to modulate entrainment allows us to correct the basic problem of clock desynchrony in jetlag and shiftwork conditions. Additionally, this kind of therapeutic could be extremely beneficial to many patients suffering from specific sleep and circadian disorders [214],

which forces them to live in a state of constant desynchrony, compared to the external environment and world.

In conclusion, over the course of my thesis, I have found that USP2 modulates early night photic entrainment of the clock at the level of the SCN and is involved in the integration of these signals into the circadian system. Thus, along with previous data, we hypothesize that USP2 is a key player in an additional feedback loop that integrates signals between the circadian clock and the photic entrainment pathway. Further study of these pathways and targets of USP2 could provide additional insight into entrainment mechanisms and might unearth therapeutic targets to modulate circadian photic entrainment of the clock.

To bring this thesis full circle, I would like to quote the song, “Loop”, by DallasK, Martin Garrix, and Sasha Alex Sloan, to sum up my project in a few lines,

“It's like I'm stuck in a loop.

I swear I want something new.

But everything that I do

Just brings me right back to you.”

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