The role of the deubiquitinating enzyme USP2 in circadian rhythms and behaviour

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ABSTRACT

Ubiquitination, a post-translational modification most often studied for its role in proteasomal degradation, is known to be crucial for protein function. Circadian rhythms are endogenous cycles of 24 h generated by a master clock located in the suprachiasmatic nucleus of the hypothalamus and clocks in other tissues. This clock relies on ubiquitination by ubiquitin ligases to regulate its activity. Additionally, evidence from various human neuropsychiatric diseases suggests that ubiquitination is important in the regulation of central nervous system function. However, the role of deubiquitinases, which reverse ubiquitin ligase activity, remains largely unexplored in the brain.

The aim of this project was to study the role of the deubiquitinating enzyme USP2 in the circadian clock and, more broadly, in the brain. We found that mice lacking USP2 have alterations in entrainment to an environmental light/dark cycle and in light responses by the suprachiasmatic nucleus. In addition to finding *Usp2* expression in several brain areas, we also discovered behavioural deficits in *Usp2* KO mice, which displayed reduced anxiety-like behaviour and certain motor coordination deficits. Our studies indicate that USP2 plays an important role in the circadian clock and its response to light cues, as well as in other brain functions.

RÉSUMÉ

L'ubitiquination, une modification post-traductionnelle ayant un rôle important dans la dégradation par le protéasome, est également essentielle pour la fonction des protéines. Les rythmes circadians sont des cycles endogènes de 24h générés par l'horloge centrale située dans le noyau suprachiasmatique de l'hypothalamus et des horloges dans d'autres tissus. Cette horloge dépend de l'ubiquitination par des ubiquitine ligases pour ajuster son activité. De plus, plusieurs études de maladies neuropsychiatriques chez les humains suggèrent que l'ubiquitination joue un rôle important dans le système nerveux central. Cependant, le rôle des déubiquitinases, qui ont une activité inverse de celle des ubiquitine ligases, reste largement inexploré dans le cerveau.

Le but de ce projet est d'étudier le rôle de la déubiquitinase USP2 dans l'horloge circadienne et, plus généralement, dans le cerveau. Nous avons démontré que les souris n'exprimant pas USP2 présentent des différences dans leur entraînement aux cycles jour-nuit et dans leurs réponses à la lumière dans le noyau suprachiasmatique. En plus de montrer que le gène *Usp2* est exprimé dans plusieurs régions du cerveau, nous avons découvert que les souris *Usp2* KO présentent un phénotype moins anxieux et un déficit de coordination motrice dans certains contextes. Nos études démontrent qu'USP2 joue un rôle important dans l'horloge circadienne et sa réponse à la lumière, ainsi que dans d'autres fonctions du cerveau.

CHAPTER 1: BACKGROUND

1.1 Ubiquitination and deubiquitination

Ubiquitination involves the addition of the small protein ubiquitin to a target protein. The number of ubiquitin moieties added to the protein, as well as the specific ubiquitin residue where the linkage occurs, dictate the effect that this post-translational modification will have on the target protein. Indeed, only polyubiquitin chains created by ubiquitin molecules internally linked via lysine 48 lead to proteasomal degradation, the most commonly discussed outcome of ubiquitination (Husnjak and Dikic, 2012). While the ubiquitin proteasome system is crucial for the proper functioning of many cellular systems, it is important not to disregard the other roles of ubiquitination which include signaling, endocytosis and DNA repair. Indeed, the diversity of signals that can be generated by ubiquitination allows this post-translational modification to be involved in nearly every cellular pathway (Strieter and Korasick, 2012). Ubiquitination is achieved through the activity of three types of enzymes, the E1, E2 and E3 enzymes, which together activate, conjugate and finally ligate the ubiquitin to a target protein (Varshavsky, 2012). The activity of these ubiquitinating enzymes is reversed by deubiquitinating enzymes (DUBs), a group of about 100 proteins, which have received much less attention so far but are likely to be just as important in the various aspects of cellular function (Wing, 2003). Indeed, DUBs have been involved in cellular function, protein stability, DNA repair, pathogenesis and kinase activation (Reyes-Turcu et al., 2009). Interestingly, there are only about 100 DUBs while there are around 600 E3 ubiquitin ligases (Komander et al., 2009). Accordingly, E3 ubiquitin ligases often have

fewer substrates compared to DUBs. While relatively few diseases have been linked to disruptions of deubiquitination, it is reasonable to believe that more will be discovered as DUBs become better studied.

1.2 Ubiquitination and the brain

Several neurodegenerative and neuropsychiatric illnesses have been linked to mutations affecting different aspects of the ubiquitin proteasome system. Mouse models have further suggested an important role of ubiquitination in normal brain function.

1.2.1 Neurodegenerative diseases: Alzheimer's disease, the most common neurodegenerative disorder, is characterized by an accumulation of amyloid plaques which has been suspected to result from disrupted protein degradation. This idea has been reinforced by the discovery of more than 10 protein mutations affecting the ubiquitin proteasome system in Alzheimer's (Riederer et al., 2011). The second most prevalent neurodegenerative disorder, Parkinson's disease, has also been linked to defects in the ubiquitination pathway. Indeed, the *parkin* gene, which was found to be mutated in several Japanese families affected by a specific type of Parkinson's disease known as autosomal-recessive juvenile parkinsonism, is an E3 ubiquitin ligase (Kitada et al., 1998). Since their discovery, mutations in *parkin* have become the second most known cause of Parkinson's and have been implicated in both familial and sporadic cases (Dawson and Dawson, 2010). It is of interest that for at least one DUB, UCH-L1, point mutations have been associated with familial forms of Parkinson's (Amerik and Hochstrasser, 2004).

1.2.2 Neuropsychiatric diseases: Several studies have linked genes involved in the ubiquitin pathway with schizophrenia (Altar et al., 2005; Bousman et al., 2010; Chen et

al., 2008; Kushima et al., 2010), anxiety (Kim et al., 2009), bipolar disorder (Bousman et al., 2010; Kushima et al., 2010; Ryan et al., 2006) and depression (Fukuo et al., 2011) in human patients. While most of these studies focus on the genes involved in ubiquitination, one group has identified an association between a deubiquitinating enzyme, ubiquitin-specific peptidase 46 (USP46) and major depressive disorder in Japanese populations in a haplotype analysis (Fukuo et al., 2011; Kushima et al., 2010). While none of these studies elucidated the mechanism by which the identified proteins contributed to the disease, they all identified a trend of decreased overall ubiquitination in the patients.

1.2.3 Mouse models: Despite the various associations between the ubiquitin system and neuropsychiatric disorders, surprisingly few studies have looked at the behaviour of mice with disrupted ubiquitination. However, as expected, a handful of studies looking at different E3 ubiquitin ligase mutants or knockouts were able to identify several behavioural abnormalities.

One such study focused on mice lacking *Kf-1*, a ubiquitin ligase whose gene transcripts were found to be more abundant in the brains of patients with Alzheimer's (Tsujimura et al., 2008; Yasojima et al., 1997). These mice were found to have increased anxiety-like behaviour and increased pre-pulse inhibition, a measure of sensorimotor gating which is usually reduced in human schizophrenic patients (Amann et al., 2010).

Another study investigated the behaviour of mice which were heterozygous for SCRAPPER, a synapse-localized E3 ubiquitin ligase whose absence in homozygous knockout mice is lethal (Yao et al., 2011). Mice were found to have decreased anxiety-

like behaviour in one test, as well as increased social behaviour. Additionally, heterozygous mice displayed reduced freezing behaviour in contextual fear conditioning.

A third study looked at the role of the RING Finger 41 E3 ubiquitin ligase in behaviour using mice congenic for the chromosomal region where this enzyme is located (Kim et al., 2009). Like in the abovementioned studies, these mice were found to have significant differences in anxiety-like behaviour, displaying increased anxiety in two separate behavioural paradigms.

Interestingly, the only study looking at a DUB focused on USP46 which, as discussed above, associates with major depressive disorder in Japanese populations, and found that *Usp46* KO mice as well as mice with a lysine codon deletion in the *Usp46* gene displayed increased depression-like behaviour, supporting the human data (Imai et al., 2012; Tomida et al., 2009). In conjunction with the human studies presented above, these data suggest an important role of various components of the ubiquitin-proteasome system in the regulation of behaviour.

1.3 Ubiquitination in the circadian clock

Circadian rhythms are endogenous cycles of about 24 h generated by a master clock in the suprachiasmatic nucleus (SCN) and clocks located in other tissues. Circadian clocks exist in most organisms and allow physiological function to anticipate predictable daily occurrences, such as changes in light and temperature levels or food availability (Dibner et al., 2010). Through synchronization with the external environment, the organism can then adapt its behaviour in a way that ensures that internal homeostasis is maintained (Nakagawa and Okumura, 2010).

1.3.1 Circadian clocks and clock genes: At the molecular level, the circadian clock relies on self-sustained transcription-translation feedback loops. A set of clock genes and their protein products generate rhythmicity in the clock through negative feedback, a mechanism by which a protein inhibits its own transcription (Dardente and Cermakian, 2007; Reppert and Weaver, 2002). In the mammalian clock, the proteins CLOCK and BMAL1 dimerize and act as transcriptional activators of the *Period (Per) 1* and *2* and *Cryptochrome* (*Cry*) *1* and *2* genes (Gekakis et al., 1998; Ueda et al., 2005). The PER1,2 and CRY1,2 protein products then later enter the nucleus and inhibit the activity of CLOCK and BMAL1, thereby shutting down their own transcription (Kiyohara et al., 2006; Kume et al., 1999). Other feedback loops also exist, namely one which involves the induction of the transcription of the *Retinoic acid receptor-related orphan receptor (Ror)* and *Rev-erb* genes, with the REV-ERB protein subsequently inhibiting transcription of the *Bmal1* gene (Akashi and Takumi, 2005; Guillaumond et al., 2005; Preitner et al., 2002; Sato et al., 2004).

The timing of these feedback loops is dictated by post-translational modifications (PTM) of the various proteins involved (Gallego and Virshup, 2007; Vanselow and Kramer, 2007). Indeed, the core clock proteins CLOCK, BMAL1, PER1/2 and CRY1/2 are all subject to post-translational modifications such as acetylation, phosphorylation, SUMOylation and ubiquitination (Duguay and Cermakian, 2009). The importance of these modifications has been highlighted by many studies (Dardente et al., 2007; Etchegaray et al., 2003; Etchegaray et al., 2009; Gallego et al., 2006; Harada et al., 2005; Lowrey et al., 2000; Toh et al., 2001; Yin et al., 2006).

The light/dark cycle is the dominant synchronizing signal for the circadian system, and light detection by the clock is largely independent of the classic image forming vision pathway. Light is perceived by intrinsically photosensitive retinal ganglion cells (ipRGCs) which contain the photoreceptor melanopsin and project along the retinohypothalamic tract to the SCN and a dozen other brain regions (Berson et al., 2002; Hattar et al., 2002). In the SCN, light information activates the mitogen-activated protein kinase (MAPK) signaling cascade, which in turn leads to the activation of cAMP reponse element-binding protein (CREB), a transcription factor (Coogan and Piggins, 2003; Obrietan et al., 1998; Tischkau et al., 2003). Through the presence of CRE binding elements in their promoter regions, the key clock genes *Period 1* and *Period 2* are then rapidly induced, allowing the circadian clock to synchronize to environmental light cues. Recently, it has been found that the mammalian target of rapamycin (mTOR) protein, a target of the MAPK signaling cascade which is activated in the SCN in response to light, can directly modulate PERIOD 1 and 2 protein levels, indicating that light responses in the SCN occur at both the gene and protein level (Cao et al., 2010).

1.3.2 Ubiquitin ligases in the circadian clock: Of the various PTMs discussed in the previous section, ubiquitination is of particular interest due to the aforementioned diversity of signals that this modification can generate and due to its direct role in determining protein half-life via control of degradation by the proteasome (Duguay and Cermakian, 2009). Several ubiquitin ligases have been found to play important roles in the mammalian circadian clock.

FBXL3 is an F-box protein which is part of SCF E3 ubiquitin ligase complex (Siepka et al., 2007). FBXL3 was identified as the protein which was mutated in the *Overtime*

mouse, whose free-running period is 2h longer than that of WT littermates. It was found that loss of FBXL3 activity in the mutant lead to a stabilization of the CRY1,2 proteins due to decreased targeting of the proteins for proteasomal degradation by ubiquitination (Siepka et al., 2007) . Around the same time, another mouse mutant with a long free-running period (26.5 h) was identified. Dubbed the "after hours" mutants, these mice were also found to have a mutation in the FBXL3 gene. Work on these animals revealed that the "after hours" mutation also leads to increased stabilization of the CRY proteins, thereby prolonging inhibition of the BMAL1/CLOCK transcriptional activators. Peak levels of the *Per*, *Cry* and *Bmal1* clock genes and their protein products were therefore found to be reduced (Godinho et al., 2007). Further work on FBXL3 revealed that ubiquitination of CRY1,2 by this enzyme was necessary for the timely degradation of the CRY proteins and the reactivation of BMAL1/CLOCK (Busino et al., 2007). An FBXL3 homolog, FBXL21, was identified in sheep where it was also found to bind to CRY1, thereby affecting transcriptional activation by BMAL1 and CLOCK (Dardente et al., 2008). Recent work in mice has revealed that FBXL3 and FBXL21 have opposing roles, with FBXL21 stabilizing the CRY proteins rather than degrading them (Hirano et al., 2013). It has been proposed that the coordinated action of these two proteins in distinct subcellular compartments is important to maintain the robustness of circadian oscillations.

β-TRCP1 and β-TRCP2 are E3 ubiquitin ligase F-box proteins recruited to PER2 following phosphorylation of this protein by the kinases CK1ε and CK1δ, which leads to polyubiquitination and subsequent degradation of PER2 (Eide et al., 2005). Indeed, expression of a dominant negative form of β-TRCP was found to lead to the inhibition of

PER2 ubiquitination and degradation. β-TRCP1,2 were also shown to interact with PER1 in a CK1ε-depedent manner and a knockdown of both proteins was found to stabilize PER1, and reduce levels of transcriptional activation by CLOCK/BMAL1 (Shirogane et al., 2005). Interestingly, it was found that the circadian period of fibroblasts could be elongated by either inhibiting the kinases which phosphorylate the PER proteins, downregulating β-TRCP1 or expressing a dominant negative β-TRCP1 (Reischl et al., 2007). Accordingly, the introduction of a PER2 protein incapable of interacting with β-TRCP1,2 leads to disrupted circadian rhythms in these cells. *In vitro* experiments have shown that the stabilization of PER protein levels caused by the knockdown of β-TRCP1,2 leads to abnormal circadian rhythms (Maier et al., 2009; Ohsaki et al., 2008). Surprisingly, mice lacking β-TRCP1 were not found to have any disruptions in circadian period, phase shifting behaviour or PER2 levels, suggesting either that the SCN clock behaves differently from peripheral oscillators or that there is redundancy at the level of the ubiquitin ligases (Ohsaki et al., 2008).

Ubiquitination is also important for the regulation of the REV-ERBα protein. Indeed, REV-ERBα was found to interact with two E3 ubiquitin ligases, Arf-bp1 and Pam, and the depletion of either one of these proteins stabilized REV-ERBα levels (Yin et al., 2010). Accordingly, downregulation of Arf-bp1 and Pam also lead to decreased levels of *Bmal1* gene expression, and disrupted oscillations *in vitro*, suggesting that ubiquitination by these proteins plays an important role in the regulation of the circadian clock. In conjunction with the studies on FBXL3 and β-TRCP1,2, this work highlights the importance of ubiquitination in the circadian system.

1.3.3 Deubiquitination in the circadian clock: Given the importance of ubiquitination in circadian rhythms, it is reasonable to believe that deubiquitination would play a role in the regulation of clock proteins as well. It is therefore interesting that the mRNA levels of a DUB, ubiquitin-specific protease 2 (USP2), have been found to cycle in a circadian fashion in most tissues tested, a rare property shared by core clock components (Kita et al., 2002; Storch et al., 2002; Yan et al., 2008). In addition, the circadian rhythm of *Usp2* is dampened in *Clock* mutant mice, suggesting that the transcription of *Usp2* is at least partially controlled by CLOCK (Oishi et al., 2003). Together, these data make USP2 an interesting candidate to study the role of deubiquitination in the clock.

USP2 has two isoforms, USP2a and USP2b (Fig. 1), which result from the use of alternative promoters, leading to the use of distinct first exons that are spliced onto the core catalytic region (Bedard et al., 2011). While it is unclear whether these isoforms have different functions, it has been found that they differ in their temporal expression pattern, particularly in the SCN where USP2b appears to be the dominant circadian form (Scoma et al., 2011).

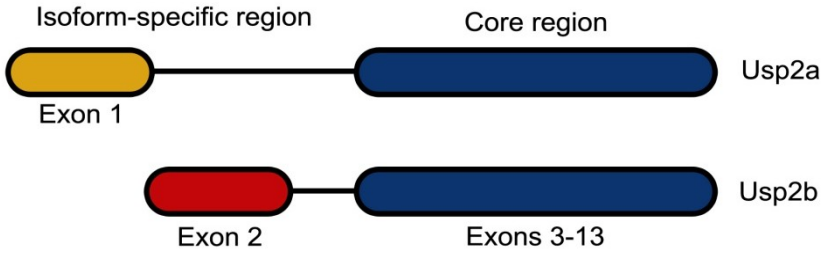

Figure 1: The isoforms of Usp2. Distinct promoter use leads to the use of distinct first exons.

It was further shown that USP2b can interact with several clock proteins in co-immunoprecipitation experiments, leading to a stabilization of BMAL1 protein *in vitro*.

Circadian characterization of *Usp2* knockout (KO) mice showed no overt abnormalities, except for a mild difference in responses to low irradiance light. USP2b was also identified as the dominant circadian isoform in the liver, appearing to be involved in the generation of a diurnal rhythm in glucose metabolism (Molusky et al., 2012). Finally, USP2a has been found to interact with the clock protein CRY1, resulting in deubiquitination and stabilization of CRY1 and decreased *Per2* levels. Interestingly, *Usp2a* expression is increased in response to inflammatory signals, suggesting that deubiquitination by USP2a is involved in circadian disruptions during inflammation (Tong et al., 2012). Together, these studies implicate USP2 in the functioning of both central and peripheral clocks.

In parallel to these studies, our lab has previously conducted both molecular and behavioural experiments to elucidate the role of USP2 in the circadian clock (Yang et al., 2012). Co-immunoprecipitations revealed an interaction of both isoforms of USP2 with PER1/2, CRY1/2 and BMAL1. Using *in vitro* translated proteins, a direct interaction was only confirmed for PER1, suggesting that the other proteins interact with USP2 through the formation of a complex. Ubiquitination assays further revealed that USP2 can deubiquitinate PER1 in transfected cells, suggesting that USP2 participates in the clock machinery via modulation of the PER1 protein. To see whether the involvement of USP2 in the circadian machinery could translate in differences in circadian behaviour, the circadian rhythms of *Usp2* KO mice were monitored in running wheels under different light regimens. In contrast to Scoma et al, our *Usp2* KO mice display clear alterations of their circadian behaviour: the free-running period in these animals was significantly longer than that of WT littermates, suggesting that the interaction between USP2 and

PER1 has an effect on locomotor activity rhythms, an output of the SCN clock. While levels of *Cry* mRNA were not affected, peak levels of *Bmal1* were significantly reduced in *Usp2* KO mouse embryonic fibroblasts. It is possible that repression of BMAL1/CLOCK by PER and CRY could be increased due to stabilized levels of PER1 in the *Usp2* KO, leading to the observed elongated period. Finally, the mice were subjected to phase shifts and the number of days it took them to adapt to the new phase was measured. *Usp2* knockout mice were faster to adapt to a 6 h phase delay while they were slower to adapt to a 6 h phase advance. Another experiment which consisted of administering phase shifting light pulses at either the advancing or the delaying portion of the circadian clock corroborated these findings. However, the exact molecular mechanism underlying the altered light responses and the long period have not yet been elucidated.

CHAPTER 2: HYPOTHESES AND AIMS

While it is well established that ubiquitination plays a role in circadian rhythms and behaviour, the role of deubiquitination remains poorly studied. The purpose of this project is to investigate the role of the deubiquitinating enzyme USP2 on circadian rhythms, at the level of behavioural locomotor rhythms, light responses by the clock and the molecular clock, and in the brain, with a focus on expression and behaviour. Studying circadian rhythms and broader brain function is particularly important given the recent interest in the role of clock proteins in various psychiatric disorders. Indeed, it has been suggested that the discrepancy between psychiatric patients' endogenous rhythms and their sleep cycles contribute to their pathology (Lamont et al., 2007).

Hypotheses: We hypothesize that *Usp2* knockout mice will show altered circadian rhythms both at the molecular and behavioural levels. Moreover, we predict that general behaviour in these mice will also be altered. In order to explore these hypotheses, we will more specifically:

Aim 1: Study circadian locomotor activity rhythms of *Usp2* KO mice under different circadian protocols. By subjecting mice to different lighting conditions and analyzing several activity and entrainment parameters, we can determine whether differences exist in the SCN clock.

Aim 2: Explore the effects of the absence of USP2 on the molecular clockwork of the suprachiasmatic nucleus clock. We focus on responses of the molecular clock to the administration of light pulses and on protein rhythms in isolated SCN slices.

Aim 3: Determine the importance of USP2 in the brain. We focus on the expression of the two isoforms of *Usp2* in the brain and, at a functional level, on behaviour in *Usp2* KO mice.

Together, the results of these experiments should provide insights into the contribution of deubiqutination to both circadian rhythms, and general behaviour and brain function.

CHAPTER 3: AIM 1: THE CIRCADIAN BEHAVIOUR OF *USP2* KO MICE UNDER DIFFERENT CIRCADIAN PROTOCOLS

Studies focusing on the role of ubiquitination in circadian rhythms (Busino et al., 2007; Godinho et al., 2007; Maier et al., 2009; Ohsaki et al., 2008; Reischl et al., 2007; Shirogane et al., 2005; Siepka et al., 2007) have highlighted the importance of this post-translational modification both at the level of the molecular clockwork and on locomotor activity rhythms, which are a direct output of the SCN clock (LeSauter and Silver, 1998). Since previous work in our lab has shown differences in the endogenous period and phase resetting of *Usp2* KO mice (Yang et al., 2012), we were interested in continuing the characterization of the circadian behaviour of these mice under different protocols so as to have a complete picture of the way in which SCN outputs are affected by the loss of deubiquitination by USP2.

3.1 Experimental methods

3.1.1 Animals: The *Usp2* KO mice were originally generated in the lab of Dr. Simon Wing (McGill University) using the Cre-Lox recombination system to excise exons 4-6, which are part of the core catalytic domain (Bedard et al., 2011). The mice *Usp2* KO and WT mice used for experiments were generated in house by breeding heterozygous mice whose genetic backgrounds were 99% C57BL/6 (9 backcrosses with C57BL/6 starting from 50:50 C57BL/6:129Sv mice). We put 11 *Usp2* KO and 8 *Usp2* WT male mice between 2 and 4 months of age in individual running wheel cages. Mice were moved from the breeding room to the experimental room and allowed to habituate to a 12 h light

(~200 lux), 12 h dark (12:12 LD) cycle in the light-proof ventilated cabinets for two weeks before starting the experiment. All animal procedures described in chapters 3, 4 and 5 were conducted in accordance with the guidelines of the Canadian Council for Animal Care, and approved by the Douglas Institute Facility Animal Care Committee.

3.1.2 Experimental setup: Experiments aimed at studying circadian locomotor activity rhythms were conducted in isolated ventilated cabinets. Light was controlled via an external timer and the cabinets were light-proof, thereby ensuring precise control over the light schedule and the intensity of light perceived by the animals (see Jud et al., 2005 for a review of the standard methods). Mice were placed in individual cages equipped with running wheels (Actimetrics, Wilmette, IL, USA), some bedding and no other enrichments. Wheel rotations were recorded by a computer and analyzed using the ClockLab program (Actimetrics, Wilmette, IL, USA). During the experiment, food and water were given *ad libitum*. Cages were changed every 7-10 days so as to avoid health problems. When changes in the light/dark cycle were planned, cage changes were scheduled to occur at least 2 days before to avoid any potential effects of cage changing on measured behaviour (Jud et al., 2005). Light was provided by white light bulbs placed in the middle of each compartment of the cabinet. Cages were arranged to achieve a light intensity of ~200 lux for each cage.

3.1.3 Measurement of locomotor activity under entrained conditions: This experiment aimed to characterize the circadian rhythms of *Usp2* KO mice under different photoperiods *i.e.* when exposed to light for varying lengths of time during a 24 h day. Following an initial habituation to a 12:12 LD cycle, cages were changed and mice were left undisturbed under the same photoperiod for 10 days. Following this initial

entrainment, the mice were put on a short day with 8 h of light, 16 h dark (8:16 LD) for a period of two weeks. Mice were then re-entrained to a 12:12 LD cycle for two weeks before being transferred to a long day with 16 h of light, 8 h dark (16:8 LD) for another two weeks. The last 10 days of each photoperiod were used to calculate the phase angle of activity onset and offset (*i.e.* when the mice start and stop running relative to lights on and off), overall activity, the percentage of activity in the light phase, the duration of activity (alpha) and period length (through Chi^2 periodogram analysis).

3.1.4 Measurement of locomotor activity under a skeleton photoperiod: Prior to starting this experiment, mice were re-entrained to a 12:12 LD for two weeks. Mice were then subjected to a skeleton photoperiod which consisted of two 30 min light pulses at the beginning and the end of what would be the light phase of a regular 12:12 LD cycle (i.e. 30 min light pulse followed by 11 h of darkness and another 30 min light pulse before the lights go off for 12 h). This protocol was used to minimize the masking effect of light. Indeed, light suppresses locomotor activity in mice, making it difficult to know whether the observed behavioural rhythm is reflective of an entrained SCN rhythm or simply due to the presence of light (Aschoff, 1960). While the two light pulses given in the skeleton photoperiod are sufficient to entrain the animal, they minimize the amount of light thereby eliminating the masking effect (Jud et al., 2005). Again, the last 10 days were used to calculate the parameter listed in section 3.1.3.

3.1.5 Measurement of locomotor activity under constant light: After another two week re-entrainment to 12:12 LD, the mice were subjected to a constant light protocol where they were exposed to two weeks of dim constant light (20-50 lux) and two weeks of normal constant light (200 lux). This allowed us to test the responses of these mice to different

light intensities as well as whether they followed "Aschoff's rule" whereby the endogenous period increases with increasing light intensity (Aschoff, 1952).

3.1.6 Statistical analysis: Data are presented as means ± SEM and statistical analysis was done using the STATISTICA software (StatSoft, Tulsa, OK, USA). Since we were interested in the differences between the two genotypes within each light condition (and not the effect of genotype across all light conditions), we conducted Student's unpaired 2-tailed t-tests for each parameter for each light condition. Differences were considered to be significant at $p < 0.05$.

3.2 Results

We found no differences between genotypes in period length, total activity levels, percentage of activity in the rest phase and phase angle of activity onset in any of the photoperiods tested. The phase angle of activity offset was significantly different in 12:12 LD ($p=0.03$ and $p=0.01$ in two different 12:12 LD photoperiods recorded, unpaired 2-tailed t-test), with the *Usp2* KO mice running until lights off unlike the WT mice which stop running on average 1 h 40 before lights off (Fig. 2A). A similar trend was seen in the skeleton photoperiod but the difference did not reach significance ($p=0.06$). These differences in the phase angle lead to significant differences in alpha (activity duration) in both 12:12 LD photoperiods and the skeleton photoperiod ($p=0.038, 0.009$ and 0.045, respectively), with the *Usp2* KO mice running on average 2 h longer than their WT counterparts in all three conditions (Fig. 2B).

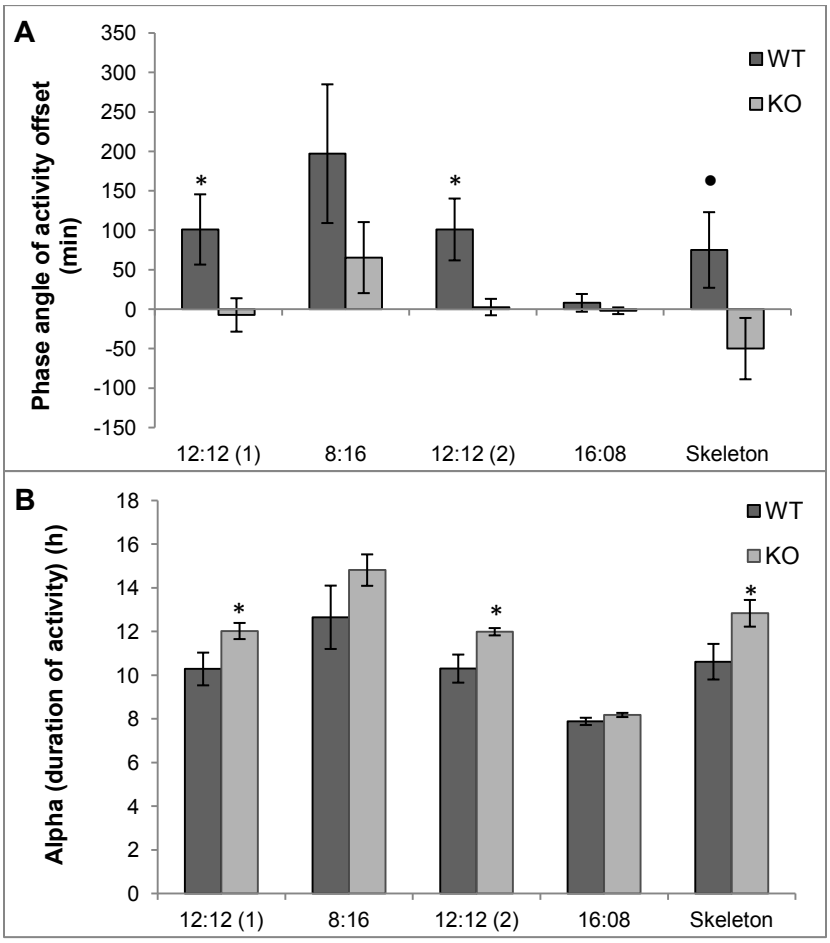

Figure 2: Circadian locomotor activity. Phase angle of activity offset (A) and alpha (B) for each of the photoperiods tested. (*) is a significant difference between Usp2 KO and WT ($p<0.05$) .(•)is a trend towards a significant difference between Usp2 KO and WT ($p=0.06$).

No differences in any of the parameters were observed in 8:16 LD, 16:8 LD or constant light. However, we suspect that the way in which the constant light protocol was carried out (12:12 LD to 200 lux constant light then 20 lux constant light) may have prevented us from obtaining good data, since further research into the subject revealed that constant light should follow a period of DD and light intensity should be increasing throughout the protocol (Jud et al., 2005).

CHAPTER 4: AIM 2: THE EFFECTS OF THE ABSENCE OF USP2 ON THE MOLECULAR CLOCK OF THE SUPRACHIASMATIC NUCLEUS

The administration of light pulses at different circadian times was found to produce different phase shifts in the *Usp2* KO compared to the WT animals, with the KO animals showing larger phase shifts in the delaying portion of the night (ZT14, with ZT12 referring to the time of lights off) and smaller phase shifts in the advancing portion of the night (ZT20), suggesting that USP2 is important for light responses by the molecular clockwork of the SCN (Yang et al., 2012).

To study the molecular basis of this, we sought to determine whether the absence of USP2 would affect light responses in the SCN at the molecular level. Furthermore, we examined the role of USP2 in SCN tissue to see how impaired deubiquitination affects both rhythms and stimulus responses.

4.1 Experimental methods

4.1.1 Animals: Only male mice 6-16 weeks of age were used for all experiments. Refer to section 3.1.1 for information on the breeding of *Usp2*KO mice. For the light pulse experiments, we first conducted a pilot experiment at ZT14 using 4 *Usp2* KO and 4 WT animals, then re-did this time point with 4 *Usp2* KO and 5 WT animals which received a light pulse and 2 *Usp2* KO and 2 WT control animals that did not receive a light pulse. For the ZT20 time point, we used 4 *Usp2* KO and 4 WT mice, and only 1 WT control mouse.

To address the role of USP2 in the molecular clockwork and light responses by the SCN, *mPer2*^{luciferase} knock-in mice were used (Jackson Laboratory, B6.129S6-*Per2*^{tm1Jt}/J). These mice express an mPER2::LUCIFERASE (PER2::LUC) fusion protein. This fusion protein allows for the visualization of the dynamics of PER2 expression and, by extension, of circadian rhythms in live cultured tissues (Yoo et al., 2004). Bioluminescence (produced by the LUCIFERASE protein) can be monitored with a Lumicycle (Actimetrics) via photomultiplier tube detector assemblies. A *mPer2*^{luciferase} homozygous male was bred with *Usp2* homozygous knockout females to generate mice which all have one copy of PER2::LUC and *Usp2*. The resulting mice were bred amongst themselves to create mice containing at least one copy of PER2::LUC and being either homozygous WT or KO for *Usp2*. So far, 3 *Usp2* KO and 1 WT mice between 6-10 weeks of age have been tested.

Mice were moved from the breeding room to the experimental room and allowed to habituate to a 12 h light (~200 lux), 12 h dark (12:12 LD) cycle in light-proof ventilated cabinets for two weeks before any experimental procedures were carried out.

4.1.2 Experimental setup: Refer to section 3.1.2.

4.1.3 Administration of light pulses: In order to be able to compare our data to the phase response curve made for behavioural phase shifts (Yang et al., 2012), we have chosen the time points that showed the largest differences in phase shifts between *Usp2* KO and WT animals. We tested mice at ZT14, the delaying portion of the circadian cycle where larger phase shifts were observed in KO animals, and ZT20, the advancing portion of the circadian cycle where KO animals showed reduced phase shifts.

Mice were entrained to a 12:12 LD cycle for at least two weeks prior to testing. At ZT14 or ZT20, the mice were given a 30 min light pulse (~200 lux). Since we were looking at the induction of the protein product of the immediate early gene *c-fos* which is known to be induced by light and to reach maximal levels about 1 h after the beginning of the light pulse (Colwell and Foster, 1992), the mice were put back in the dark for another 30 min before tissue collection. Control mice did not receive a light pulse but were sacrificed at the same time as experimental animals.

4.1.4 Tissue collection: Mice were anesthetized with 0.1 mL/10 g body weight of mouse cocktail (ketamine 100 mg/kg, xylazine 10 mg/kg, acepromazine 3 mg/kg). Once anesthesia was complete, mice were perfused with 0.1 M phosphate buffered saline (PBS) followed by 4 % paraformaldehyde (PFA). The brains were then collected, stored in 4% PFA overnight and transferred to 30 % sucrose. The perfused brains were then cut on the cryostat into floating 20 μm coronal sections that include the SCN and sections were stored in Watson's cryoprotectant (a solution containing 30% sucrose and 30% ethylene glycol in 0.1 M PBS).

4.1.5 Immunohistochemistry: DAB staining was done on brain sections to detect cFOS protein in the SCN. The floating sections were washed in PBS and then quenched in a 1% H_2O_2 solution to remove peroxidases and scavenge remaining oxygen. Slices were then blocked in a solution containing 0.3%Triton X-100 and 1.5% Normal Goat Serum (Vector laboratories, Burlingame, CA, USA) in 10 mL of 0.1 M PBS before incubation with the primary antibody (Ab5 rabbit anti c-Fos, 1:20000 in blocking solution; Oncogene, Cat. # PC38). The primary incubation was carried out for 48 h at 4°C. Incubation with the secondary antibody, biotinylated goat anti-rabbit IgG (1:500, Vector

Laboratories), was done for 1 h, followed by a 1 h incubation with the avidin-biotin-horseradish peroxidase complex (VECTASTAIN Elite ABC kit, Vector laboratories). Finally, DAB staining was done with a solution of 1 mL of 5 mg/mL DAB in 15 mL PBS supplemented with 1 mL of 1% nickel chloride which was allowed to react with hydrogen peroxide (10 μlLof 3% H_2O_2 in 10 mL of 0.1 M PBS) until staining was visible (10-15 min). The reaction was stopped with PBS washes. Stained slices were mounted on slides and pictures containing both SCNs were taken using a bright field microscope.

4.1.6 Quantification of cFOS staining: The number of cFOS positive cells was estimated using the "count mask" function in ImageJ, using the same criteria (number of pixels and circularity) for all the sections. The number of cells was counted for both SCNs on each section and the sections were added to give a total number of cFOS positive cells for a given animal.

4.1.7 PER2::LUC SCN sections: In order to minimize the time it took to get the tissue in culture, thereby minimizing tissue death, mice were taken from the animal facility into the Lumicycle room where they were sacrificed by cervical dislocation. Brains were rapidly extracted and sliced at 250 μm with a vibratome. Slices containing medial SCN were selected, the two SCNs were separated and each put in a separate culture dish. They were then placed in the Lumicycle where bioluminescence was recorded every 5 min. Slices which survived and remained rhythmic for at least 3 circadian cycles were analyzed using the LumiCycle analysis software (Actimetrics) to determine period length.

We administered glutamate to SCN slices to generate a molecular correlate of the light pulse phase response curve. Glutamate, the main neurotransmitter in the retinohypothalamic tract, which is responsible for photic input to the circadian system, was shown to mimic the phase shifting effects of light on SCN slices (Colwell et al., 1991; Colwell and Menaker, 1992) and, since we see behavioural differences in light response in the *Usp2* KO mice, looking at how SCN rhythms respond to glutamate receptor activation can help determine whether the phenotype is caused by differences in the detection of light in the retina or differences in downstream transduction of light information. For this experiment, culture media was removed and replaced with media containing 20 μM glutamate for 30 min at CT14 (circadian time estimated from bioluminescence traces from previous days, with CT12 defined as peak PER2::LUC bioluminescence). The glutamate solution was then removed, replaced by regular culture medium and bioluminescence was recorded over the next 72 h. By recording the phase of the PER2 rhythm before and after the application of glutamate, we can determine whether phase shifts have been induced and compare the magnitude of these shifts between *Usp2* KO and WT mice.

4.1.8 Statistical analysis: Following the quantification of cFOS, we calculated the percent of mean (i.e. number of stained cells for animal x/average number of stained cells for all animals) to account for differences in staining intensity between experiments. Data are represented as a scatter plot with the mean. Student's unpaired 2-tailed t-test was done to identify differences between genotypes. For the PER2::LUC experiments, individual data points are shown. Since we only have 1 WT animal to date for this experiment, statistical analysis could not be done.

4.2. Results

4.2.1 cFOS induction in response to light pulse administration: The pilot study was conducted with 4 *Usp2* KO and 4 WT mice which all received a light pulse (no control animals in this experiment). However one of the KO mice had to be excluded because the SCN sections were lost during staining. A 2-tailed unpaired t-test revealed a significant difference between *Usp2* KO and WT mice ($p=0.02$), with KO mice showing a larger cFOS induction after a light pulse administered at ZT14 (Fig. 3A). Given these results, we decided to continue with these experiments. The ZT14 light pulse experiment was done again using 4 *Usp2* KO and 5 WT mice in the light pulse group and 2 *Usp2* KO and 2 WT in the control group (no light pulse). No cFOS positive cells were seen in the mice that did not receive the light pulse. Variability in the cFOS response was higher in this group of mice and the 2-tailed unpaired t-test did not find a significant difference between the two groups ($p=0.46$), although the response again seemed to be larger in the *Usp2* KO mice than in the WT (Fig. 3B). Since the variability prevented a significant difference, we wanted to see whether increasing group size would remedy this issue. We therefore pooled the data from the two ZT14 experiments and a 2-tailed unpaired t-test found a significant difference between *Usp2* KO and WT mice ($p=0.03$, Fig. 3C), suggesting that increasing our group sizes will allow to get a significant difference between genotypes.

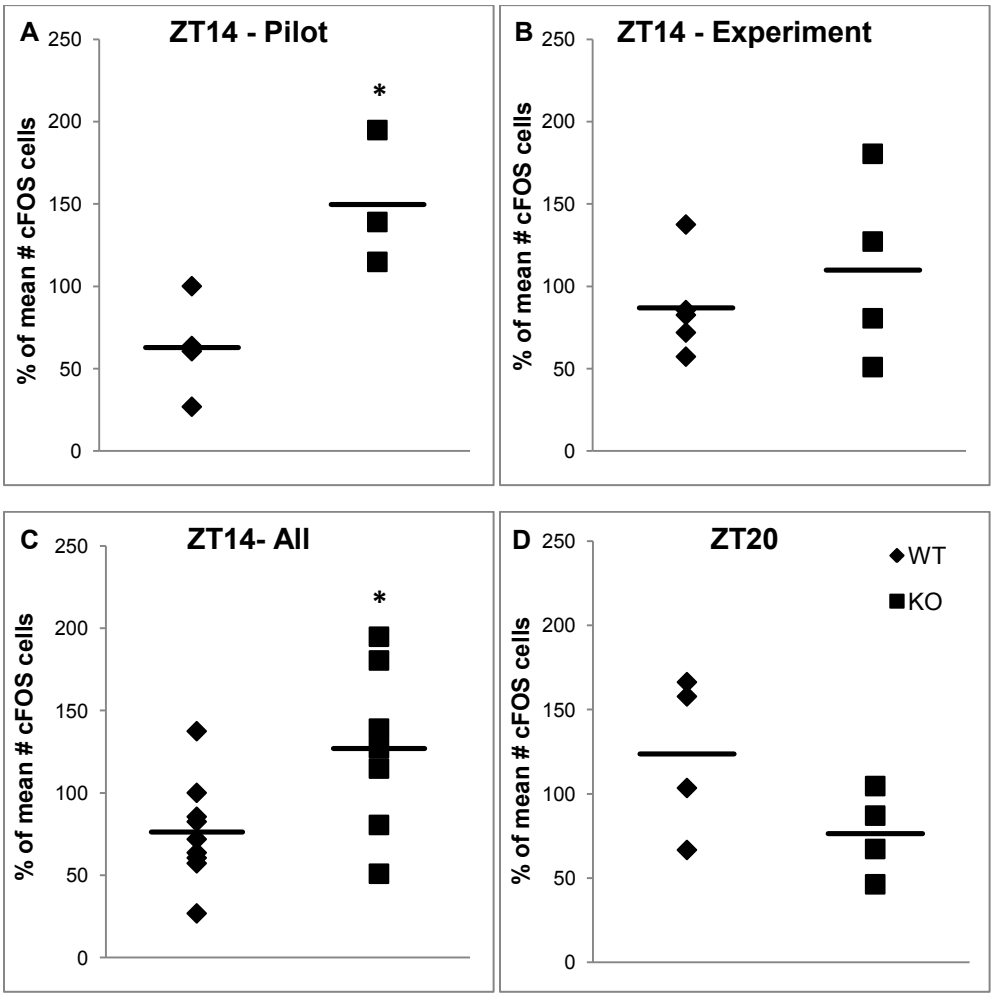

Figure 3: Induction of cFOS in the SCN after a light pulse. Data is shown for the pilot study (A), the experiment (B) and the pool of both experiments(C) for the ZT14 time point and for the ZT20 experiment (D). (*) is a significant difference between Usp2 KO and WT ($p<0.05$).

Next, we did a light pulse at ZT20 with 4 *Usp2* KO and 4 WT mice in the light pulse group and one WT mouse in the control group (no light pulse). Again, no staining was seen in the control mouse. A 2-tailed unpaired t-test did not find a significant difference between the two genotypes ($p=0.12$), although *Usp2* KO mice appeared to have lower cFOS induction than their WT counterparts (Fig. 3D). This experiment will be repeated on more animals to see if increasing group size will allow seeing a significant difference. Interestingly, the cFOS response seems to follow the behavioural phase shift data at both

time points, with *Usp2* KO mice responding more than WT at ZT14, and less at ZT20 (Yang et al., 2012).

4.2.2 PER2::LUC bioluminescence: We were able to get rhythmic bioluminescence traces for 3 *Usp2* KO and 1 WT brain (Fig. 4A). In some cases, there was variability between the two sides of the SCN, a phenomenon which we do not know how to explain. The period was calculated using 3 circadian cycles for each side of the SCN, and then averaged for each mouse. Although we were unable to do statistics at this time due to our small group sizes, preliminary results suggest that the period is longer in the *Usp2* KO mice than in WT mice (Fig. 4B), similarly to what was previously seen at the behavioural level (Yang et al., 2012). The molecular phase response curve experiments are in progress.

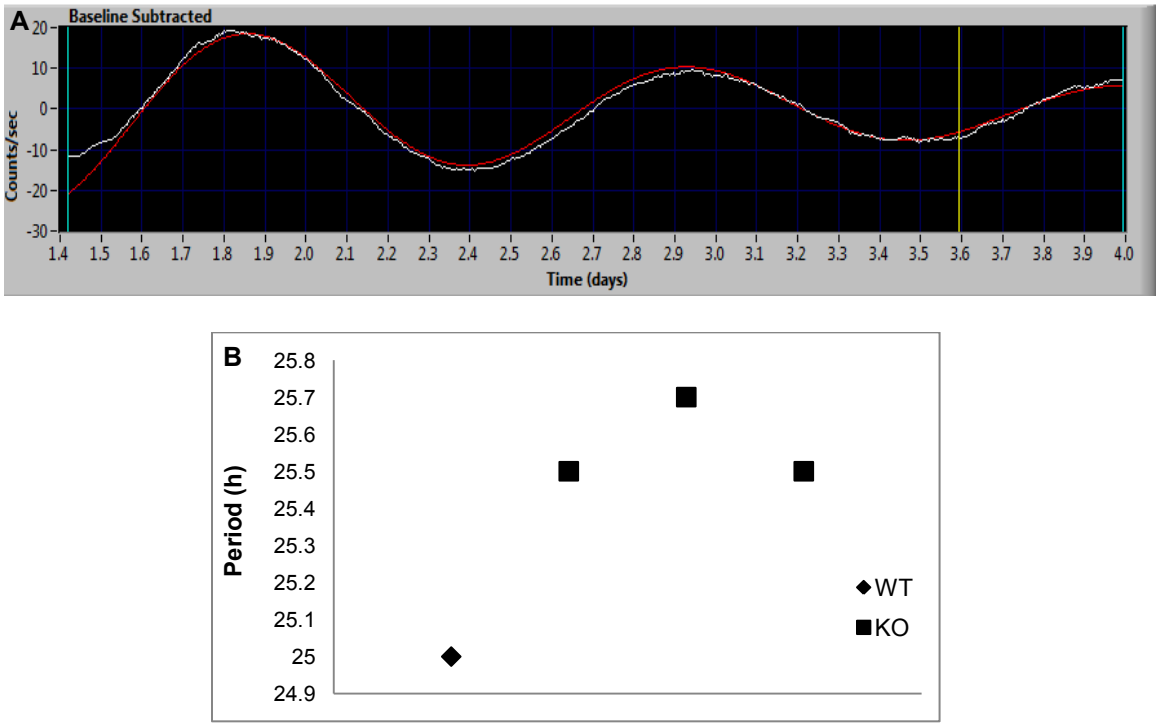

Figure 4: Period of PER2::LUCIFERASE bioluminescent rhythms in Usp2 KO and WT SCN slices. An example trace is shown in (A) and the period of the SCN slices tested so far is shown in (B).

CHAPTER 5: AIM 3: THE IMPORTANCE OF USP2 IN THE BRAIN

It is known from both human and rodent studies (discussed above) that ubiquitination is important in a variety of neuropsychiatric and neurodegenerative diseases. It is therefore reasonable to believe that deubiquitination may be at least as important, although it has received less attention in so far. While it is known that the two isoforms of *Usp2* are expressed differentially in certain tissues (Gousseva and Baker, 2003), it is unknown whether a difference exists at the level of brain expression. We are therefore interested in determining where *Usp2a* and *Usp2b* are expressed in the mouse brain.

Additionally, since *Usp2* is expressed in the brain, we expect that KO mice might have a defect in certain behaviours (Gousseva and Baker, 2003). Since the *Usp2* knockout mouse strain is relatively new and has yet to be tested behaviourally, we were interested in performing a battery of neurophenotyping tests to determine whether USP2 plays a role in behaviour.

5.1 Experimental methods

5.1.1 Animals: All mice used in these experiments were male. We used a total of 6 *Usp2* WT, and 6 *Usp2* KO mice as controls for non-specific labeling, to examine the expression of the two *Usp2* isoforms in the brains.

Unless otherwise noted, all behavioural tests were done on mice between 8-16 weeks of age. The first cohort consisted of 11 *Usp2* KO and 6 WT mice and was subjected to all tests except the beam walking assay. The mice which were tested in the running wheels

(see section 3.1.1) were also tested on the rotarod, elevated plus maze and Thatcher-Britton test. Finally, another group of 9 *Usp2* KO and 5 WT mice was tested on the rotarod, the beam walking assay, the elevated plus maze and the Thatcher-Britton test. All behavioural tests were done in the morning, under a light intensity of around 200 lux in the appropriate rooms of the Douglas neurophenotyping center.

5.1.2 Tissue collection: Mice were entrained to a 12:12 LD cycle for 2 weeks prior to the experiment and the brains were collected at ZT11, since this is when *Usp2* mRNA levels were found to peak in whole brain tissue in a time series microarray study (Yan et al., 2008). Mice were euthanized via cervical dislocation and fresh brains were collected and frozen in cold isopentane. Sagittal sections (20 μm thick) were cut on a cryostat, mounted on slides and stored at -80°C.

5.1.3 In situ hybridization: In order to perform *in situ* hybridization against the two isoforms of *Usp2*, we have generated probes targeting the isoform-specific region of *Usp2* mRNA (Fig. 5). Using the available sequence data, we have designed probes which cover a portion of the coding sequence of exon 1 (308 base pairs) for *Usp2a* and of exon 2 (144 base pairs) for *Usp2b*. The probes were produced via molecular cloning and were labeled with digoxigenin. Briefly, probes were incubated at 37°C for 2 h with T7 RNA Polymerase, RNAse inhibitor, transcription buffer (Promega), DTT and Dig-RNA labeling mix (Roche), then purified using NucAway Spin Columns (Applied Biosystems).

The slides were thawed in PBS, fixed in 4% paraformaldehyde and treated with an acetylation solution (4.64 g Triethanolamine, 560 μL 10 N NaOH and 625 μL acetic

anhydride in 250 mL H_2O) to acetylate any positive charges remaining on the brain slices and reduce background staining (Hayashi et al., 1978). The slides are then pre-hybridized in hybridization solution (25 mL formamide 100%, 12.5 mL 20X SSC, 12.5 mg E.coli tRNA (Sigma), 5 mL DEPC water, 5 mL Denhardt's solution (Sigma) and 2.5 mL salmon sperm DNA (Sigma)) for 2 h at room temperature. The probe is added to the hybridization solution (final concentration of 200-400 ng/mL) and denatured at 80°C for 5 min, then quenched on ice prior to being applied to the slide and left to hybridize overnight at 62°C. The next day, slides are washed in decreasing concentrations of SSC and then in B1 buffer (10% 1 M Tris pH 7.5 and 3% 5 M NaCl in 1 L H_2O) before adding the blocking solution (10 % fetal bovine serum in B1 solution) for 1 h at room temperature. DIG-labeling is then detected after a 2 h incubation at room temperature using anti-DIG-AP (Roche, Cat. # 11 093 274 910) at a final concentration of 1:7500 in blocking solution. Slides are washed in B1 solution and incubated with B3 solution (10% 1 M Tris pH 9.5, 2% 5 M NaCl and 5% $MgCl_2$ in 1 L H_2O) for 5 min before exposure with B4 solution (11.25 µL NBT (Roche, 100 mg/mL), 17.5 µL BCIP (Roche, 50 mg/mL) and 1.2 mg Levamisole in 5 mL B3 solution). Slides have to be monitored at this last step as the duration of exposure necessary for clear staining can range from 30 min to overnight (4-8 h with our probes). Slides are rinsed in TE before being cover slipped.

Unlike radioactive *in situ* hybridization, this technique does not allow us to quantify the amount of expressed mRNA but it does allow us to visualize cells which express *Usp2*. We performed *in situ* hybridization on sagittal sections to create an atlas of *Usp2* expression. Pictures were taken using a microscope to identify the regions where each of the two *Usp2* isoforms are expressed. Due to issues discussed below, we also generated a

probe which specifically targets exons 4, 5 and 6 of the *Usp2* gene, which are excised out in the *Usp2* KO mice (Fig. 5, (Bedard et al., 2011).

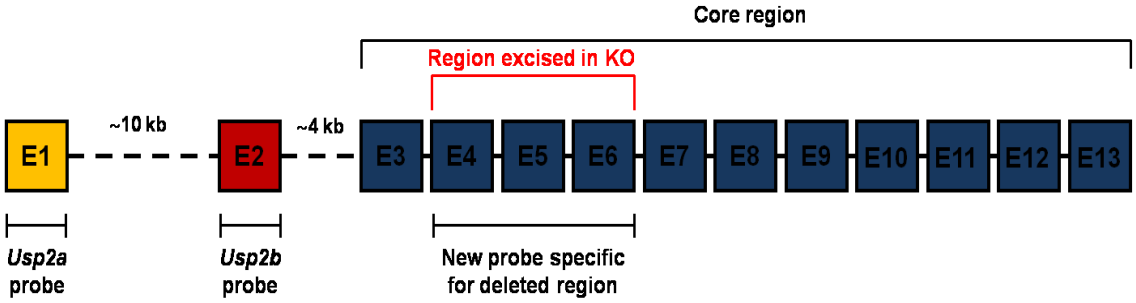

Figure 5: In situ hybridization probes. The isoform-specific probes are shown as well as the new probe which specifically targets the exons which are excised out in the Usp2 KO mice.

5.1.4 Actimetry: The actimetry test assesses spontaneous locomotor activity by placing individual mice in plexiglass chambers equipped with laser beams for a 30 min habituation period and a 60 min recording period, during which the number of beam breaks per 10 min is recorded (Karl et al., 2003). Total horizontal activity/h was measured.

5.1.5 Accelerating rotarod: The accelerating rotarod test assesses motor coordination and equilibrium (Jones and Roberts, 1968). Mice are individually placed on rotating cylinders where they are habituated to increased acceleration and to increasing speed (from 4 to 24 rpm) over 4 training trials. The cylinders are elevated 20 cm over a press-plate which detects when the mice fall off. Mice are then tested over four trials on the next day, at 25 rpm, and the time spent on the cylinder is recorded for each trial. At least 30 min are given between each trial for each animal on both days. The four test trials (day 2) are averaged for each mouse.

5.1.6 Beam walking: The beam walking assay also assesses motor coordination and balance in mice (Carter et al., 2001). It is of interest to perform both tests as some studies suggest that there are differences in the sensitivity of the beam walk and the accelerating rotarod tests in determining motor coordination deficits (Hamm et al., 1994; Stanley et al., 2005). Mice are tested on 1 meter beams which are placed 50 cm above a table, on two poles. A black box is placed at one end of the beam and nesting material from the home cage of the animal is placed inside the box to motivate it to cross the beam. A lamp illuminates the start site, creating an aversive stimulus which also motivates the mouse to cross the beam. The time to cross the beam is measured and each trial is recorded by video camera to allow subsequent scoring of the number of foot slips. Each test day consists of 3 trials and mice are tested on 3 beam widths (1 inch, ½ inch and ¼ inch) over 3 days.

5.1.7 Forced swimming test: The forced swimming test is used to measure depression-like behaviour in mice (Karl et al., 2003). Mice are individually placed in a cylinder filled with 25°C water for 6 min, during which time they are observed by the experimenter. While mice swim to attempt to escape, they eventually resign themselves to making minimal movements to keep themselves afloat. This immobilization behaviour is measured by the experimenter during the last 4 min of the session.

5.1.8 Elevated plus maze: The elevated plus maze is used to test anxiety-like behaviour (Lister, 1987). Individual mice are placed in a plus-shaped maze, consisting of two closed and two open arms, which is elevated 50 cm from the ground. During a 5 min session, the number of arm entries, the latency to enter an open arm and the time spent in the arms is measured. Since the test creates a conflict between the anxiogenic effect of open spaces

and the natural tendency to explore, mice which more readily enter or spend more time in the open arms are considered less anxious.

5.1.9 Thatcher-Britton: The Thatcher-Britton test also evaluates anxiety-like behaviour (Rochford et al., 1997). Individual mice are food deprived for 24 h and placed in an open field apparatus with food pellets in the middle, creating a conflict between the fear of open spaces and the desire to get the food. Mice are tested for 10 min and the latency to eat the food, carefully excluding instances where food was only sniffed or manipulated, is recorded.

5.1.10 Morris water maze: The Morris water maze assesses spatial memory and consists of a large circular swimming pool filled with opaque water which hides a platform which mice must swim to in order to be able to rest (Morris et al., 1986). The platform measures 14 cm in diameter and is submerged about 1 cm below the water, which is adjusted to 25°C. Spatial cues are placed on the pool walls to allow the mice to learn the location of the platform. The learning stage lasts 4 days, with each day consisting of 4 trials starting at 4 different locations in the pool. The time to reach the platform is recorded at every trial so as to ensure that learning proceeds normally in all animals. On the 5^{th} day, the platform is removed and the time spent in each quadrant of the pool is recorded during a 60 sec trial to test how well the location was learned. A cue trial, where the platform is placed in a new location along with a visual cue, is also done to verify that the visual system is intact in the animals. The latency to reach the platform and thigmotaxis time (time spent circling the edge of the pool) were analyzed.

5.1.11 Food intake: Since some of the above tests rely on food deprivation, we wanted to exclude any confounding effects of food consumption or motivation to eat food after a deprivation. We measured food consumption daily for 9 days and then every 3 days for the next 6 days. We also weighed the mice every other day during 9 days. We then food deprived mice for 24 h and measured the latency to eat the food and the amount consumed 10 min, 1 h and 24 h after food was returned.

5.1.12 Statistical analysis: Data are shown as mean ± SEM. Statistical analysis was performed using the STATISTICA software (StatSoft, Tulsa, OK, USA). Student's unpaired 2-tailed t-test was conducted on the data from all neurophenotyping tests except for the beam walking assay, which was analyzed with a repeated measures ANOVA, and learning in the Morris water maze, which was analyzed with a 2-way ANOVA (factors: genotype and trial). Subsequent post-hoc comparisons were done using Tukey's HSD test. Food intake and body weight data were analyzed with a 2-way ANOVA (factors: genotype and day) and food deprivation data were analyzed with Student's unpaired 2-tailed t-test. Differences were considered significant at $p<0.05$.

5.2 Results

5.2.1 Expression of Usp2 in the brain: The isoform-specific probes were generated and tested on several sections. Staining was detected in a number of specific brain regions. In the hippocampus, *Usp2* expression was detected in the granule cell layer of the dentate gyrus and the pyramidal cell layer of Ammon's horn (Fig. 6, 7A). In the cerebellum, stained cells were seen in the Purkinje and granule cell layers (Fig. 7B). Additionally, staining was found in the olfactory bulb and certain areas of the cortex, although

insufficient data precludes the identification of specific sub-regions (data not shown). A similar pattern of expression was found for the two isoforms (Fig. 6A, B). Surprisingly, we could see the same staining on *Usp2* WT and KO brain sections, making it difficult to assess the specificity of the labeling (compare Fig. 6A, B and Fig. 6C, D).

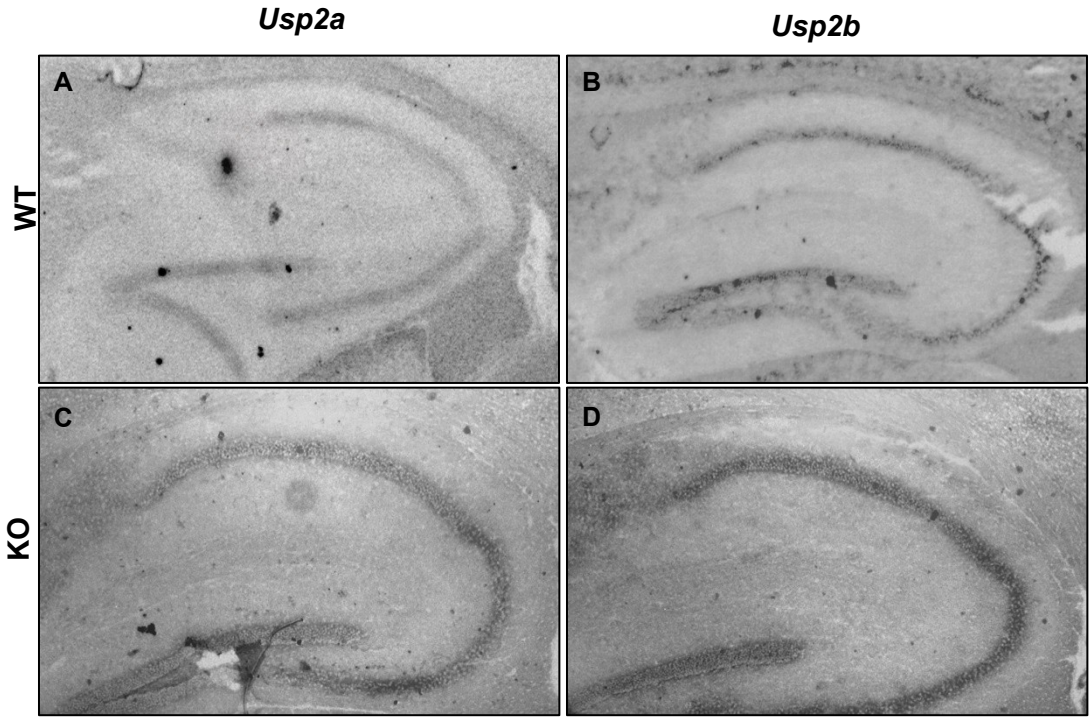

Figure 6: In situ hybridization with isoform-specific Usp2 probes. No differences were seen between staining for Usp2a (A) and Usp2b (B). However, identical staining was seen in WT (A, B) and KO (C, D) brain sections, raising questions about the specificity of the probe.

Looking at the way in which the *Usp2* KO mice were generated, we saw that the excision which renders *Usp2* non-functional occurs downstream of the isoform-specific region and that the first exons continue to be expressed in the KO mice (Fig. 5, (Bedard et al., 2011)). While this explains why there is staining in the control *Usp2* KO brains, it means that we cannot generate an isoform-specific probe for which the KO control can be used. We therefore decided to use the sense probe as a control for these *in situ* hybridization experiments and to generate another probe that can be used with the appropriate KO

controls. An *in situ* hybridization using both the sense and antisense probes revealed no detectable staining in the brain with the sense probe, and confirmed previous results for the antisense probe (Fig. 7). The new probe targeting exons 4-6 was generated and is being tested. By combining the different probes, a comprehensive atlas of the brain expression of the two *Usp2* isoforms will be built.

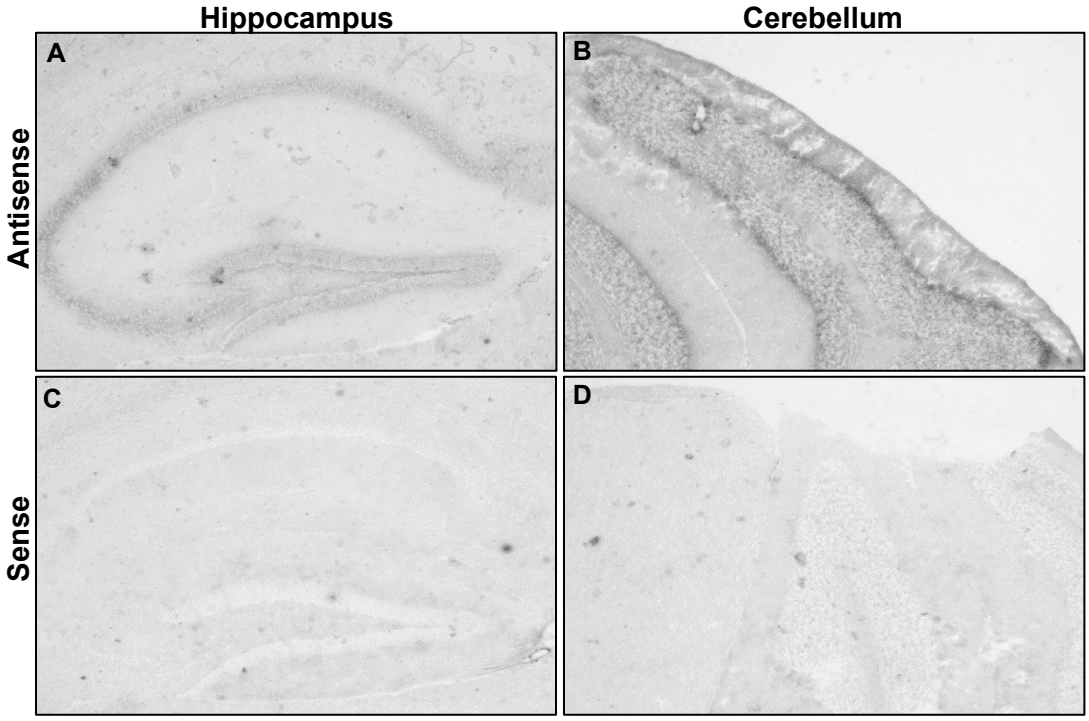

Figure 7: In situ hybridization for Usp2a using the sense probe as a control. DIG-labeled cells were found in the hippocampus and the cerebellum using the antisense Usp2a probe (A and B, respectively). No labeling was seen in these regions with the sense Usp2a probe (C and D, respectively). Similar results were obtained with the sense and antisense Usp2b probes. The stronger staining seen in the CA2 region of Ammon's horn (A) was not seen in other brain sections and should therefore not be considered significant.

5.2.2 Neurophenotyping: We found no differences in motor coordination, locomotor activity and depression-like behaviour in the first group of 11 *Usp2* KO and 6 WT mice (Fig. 8). We also found no differences in learning, or in performance in the probe or the cue trials of the Morris Water Maze (Fig. 9). We found that thigmotaxis time was

significantly shorter in *Usp2* KO mice (*p=0.046*) in the second cue trial but not in any of the other trials, making it difficult to draw conclusions about this result. However, we found that the *Usp2* KO mice have decreased anxiety-like behaviour as seen by decreased latency to eat in the Thatcher-Britton (see Fig. 10A) and decreased latency to enter the open arms of the elevated plus maze (see Fig. 10B; *p=0.004* and *p=0.048* on unpaired 2-tailed t-tests, respectively).

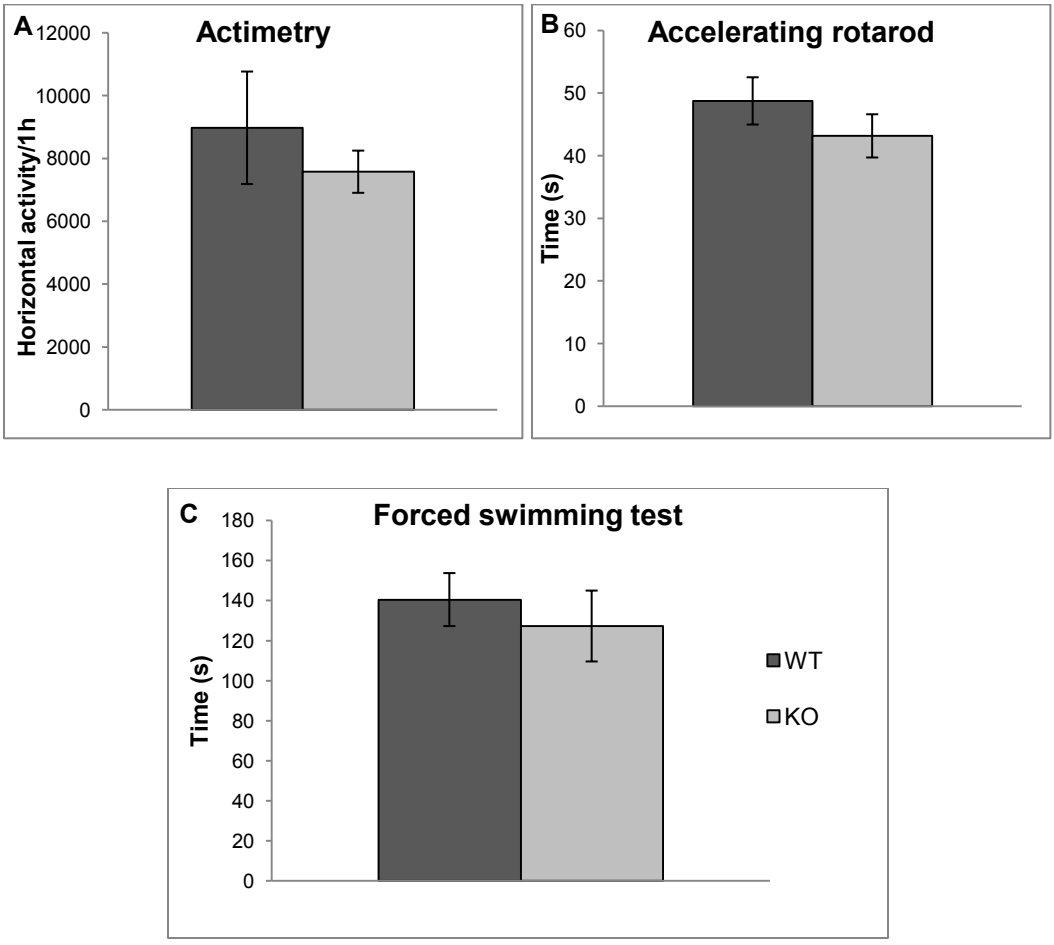

Figure 8: Neurophenotyping of Usp2 KO mice. Mice have been tested for locomotor activity on the actimetry test (A), for motor coordination on the accelerating rotarod (B) and for depression-like behaviour on the forced swim test (C).

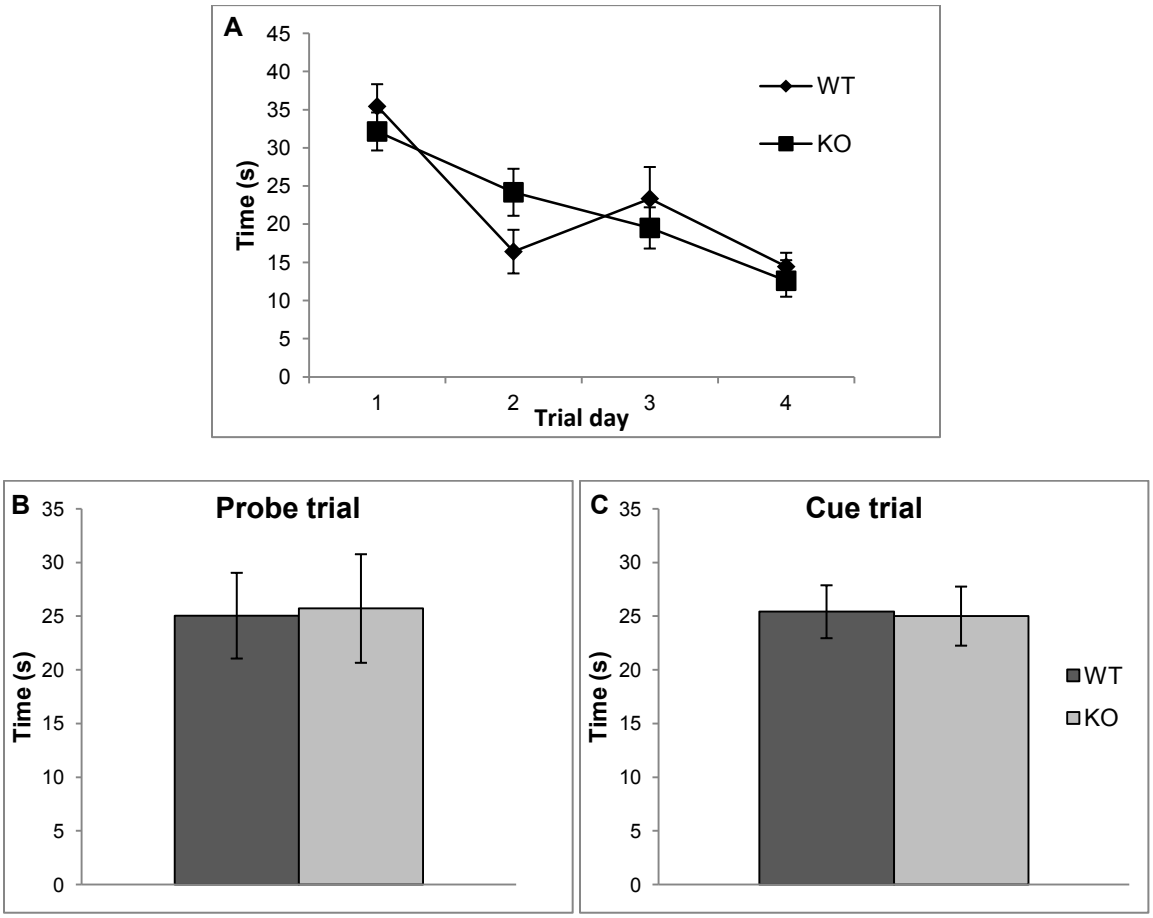

Figure 9: Morris Water maze. No differences were seen in learning over the training days (A), performance on the probe trial (B) or performance on the cue trials (C).

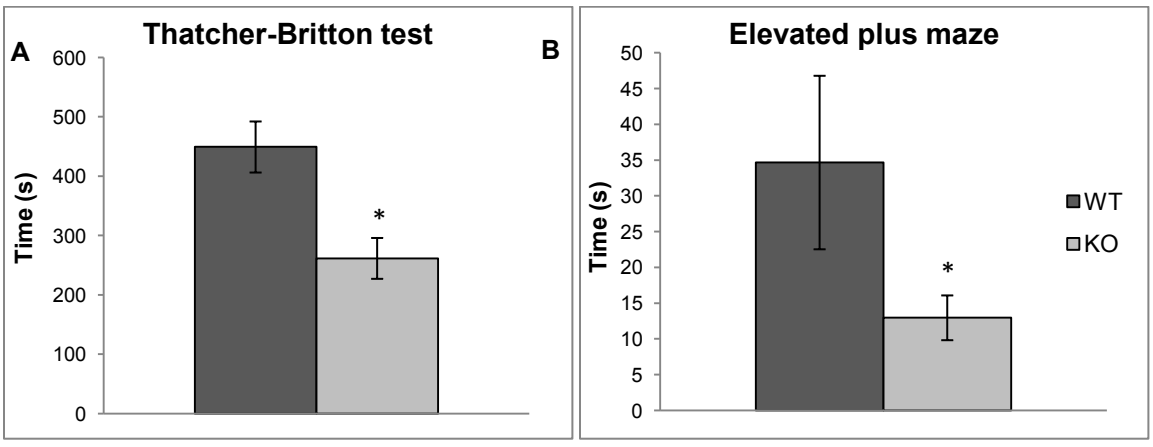

Figure 10: Thatcher-Britton and elevated plus maze. Latency to eat is reduced in the Thatcher-Britton (A) as is the latency to enter the open arms in the elevated plus maze (B). (*) is a significant difference between Usp2 KO and WT ($p<0.05$).

In order to exclude that the decreased latency to eat observed in *Usp2* KO mice was caused by differences in food consumption, daily food consumption and body weight were assessed over the course of two weeks (Fig. 11). A 2-way ANOVA revealed no significant differences between genotypes for either measurement. Additionally, we performed a 24 h food deprivation and subsequently measured both latency to eat and the amount of food consumed over 24 h (Fig. 12). No differences were found between genotypes, suggesting that the observed phenotype relates specifically to anxiety.

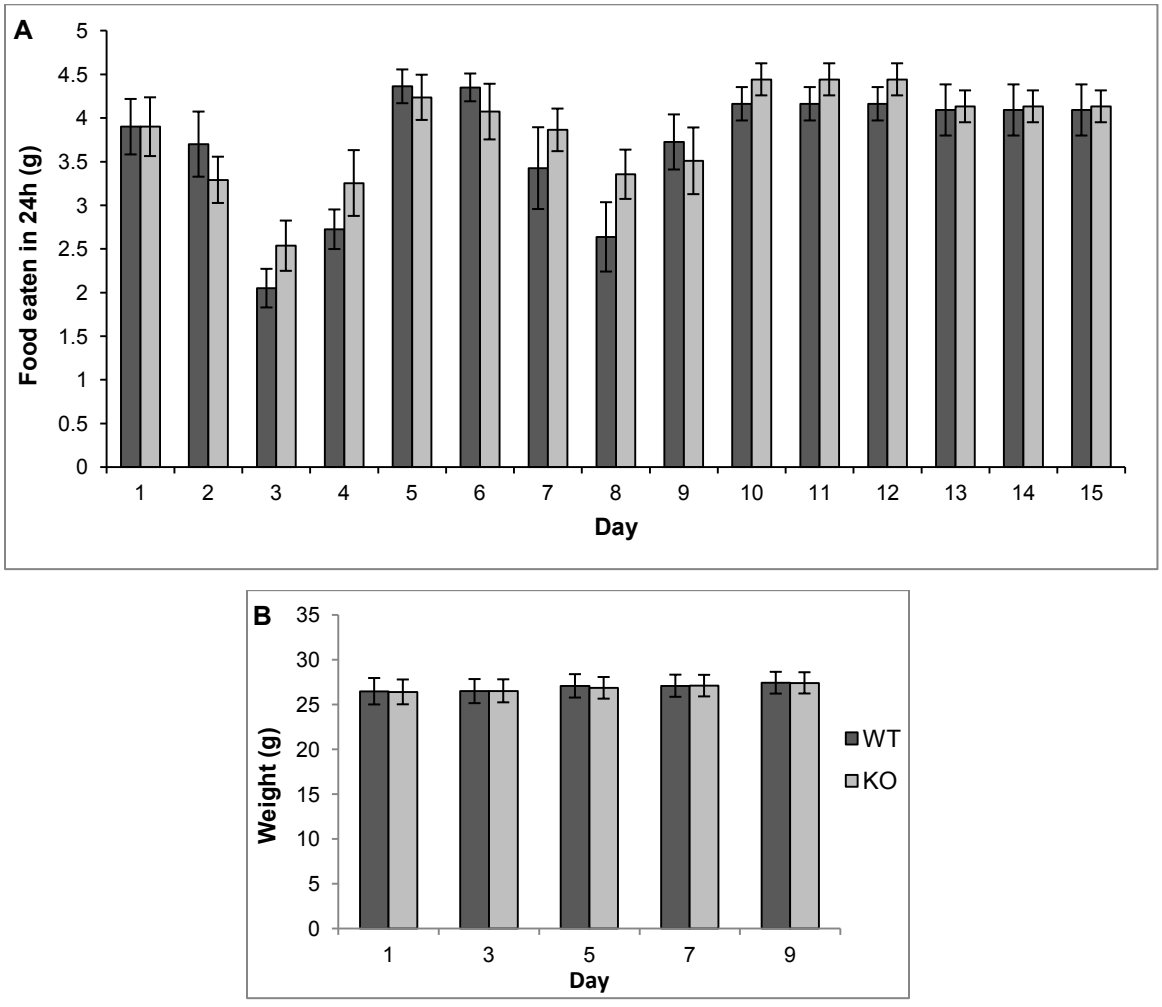

Figure 11: Food consumption and body weight. Food consumption (A) was measured every day for 9 days and then every 3 days so the quantities plotted for days 10-12 and 13-15 represent averages. Body weight (B) was measured every other day for 9 days. No significant differences were seen between genotypes.

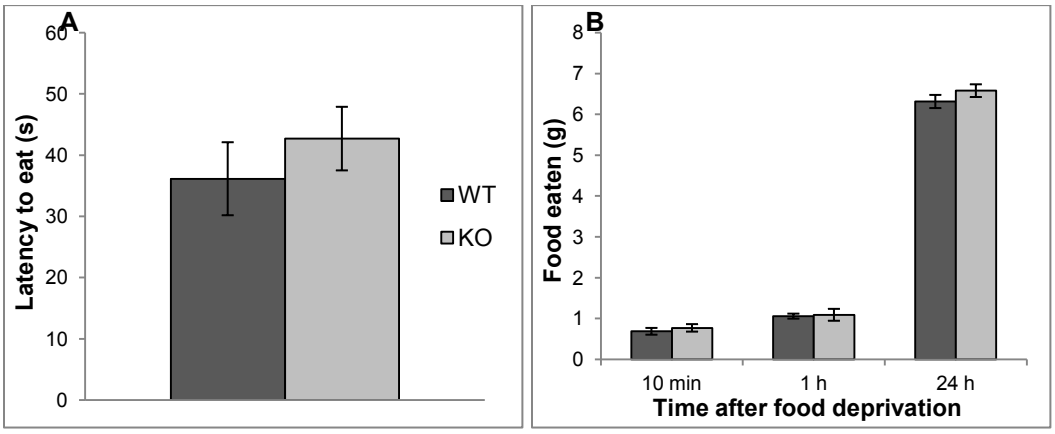

Figure 12: 24 h food deprivation. Latency to eat (A) and amount of food eaten (B) are shown. No differences were found between genotypes.

In the second group, mice were between 18 and 22 months old. No differences were found in the Thatcher-Britton. In the elevated plus maze, only a trend towards significantly reduced time spent by *Usp2* KO mice in the closed arms and a trend towards an increased percentage of time spent in the open arms relative to the total time spent in any arm was found ($p=0.059$ and $p=0.072$ on unpaired 2-tailed t-tests, respectively). While this does not corroborate our previous findings, it is possible that the differences are due to the age of the mice (the mice were over a year old when tested compared to 8-16 weeks old in the other group) or to their experience in the running wheels. Interestingly, while the young mice showed no difference in motor coordination as tested on the rotarod (Fig. 13A), these older *Usp2* KO mice were able to spend significantly less time on the rotating cylinder than their WT counterparts (Fig. 13B; $p=0.007$ on an unpaired two-tailed t-test). Since these mice showed no differences in overall daily activity levels in the running wheels, we believe that this difference is specifically due to an age-related coordination impairment, which may indicate a neurodegeneration-like phenotype associated with the loss of *Usp2* function.

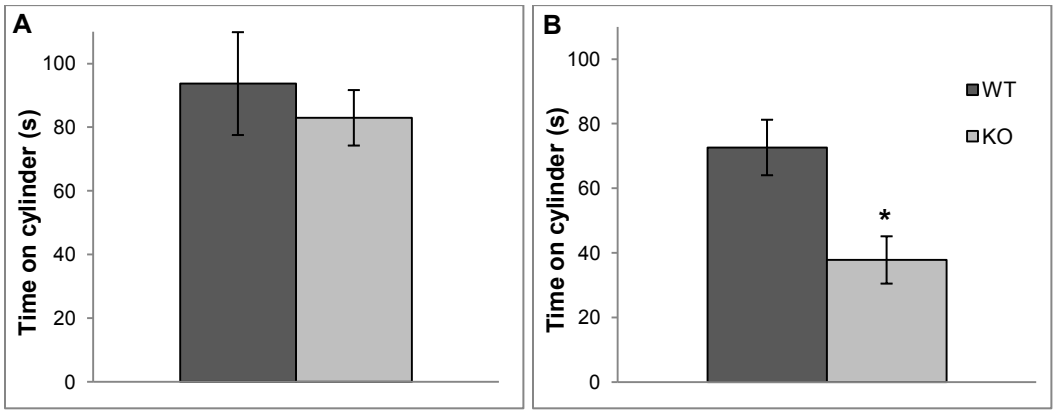

Figure 13: Rotarod in young and old mice. Motor coordination is not impaired in young Usp2 KO mice (A, same data as Fig. 8B) but is impaired in older mice (B). (*) is a significant difference between Usp2 KO and WT ($p<0.05$).

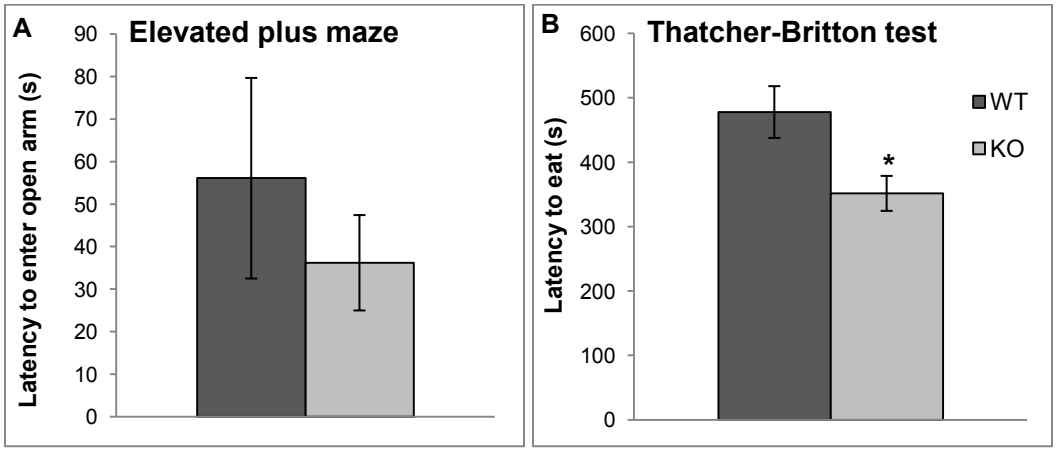

Figure 14: Elevated plus maze and Thatcher-Britton in the last group of mice tested. The latency to enter the open arm in the elevated plus maze (A) and latency to eat in the Thatcher-Britton (B) are shown. (*) is a significant difference between Usp2 KO and WT ($p<0.05$).

Finally, a group of 9 *Usp2* KO and 5 WT mice was tested on the elevated plus maze, the Thatcher-Britton test and the beam walking assay. In the elevated plus maze, there were no significant differences for either latency to enter the open arms (Fig. 14A), time spent in the open arms or the number of open arm entries (*$p=0.4$, 0.95 and 0.28* on an 2-tailed unpaired t-test, respectively), although the large inter-subject variability in the WT group and the apparent difference between genotypes suggest that a significant difference could have been found with larger groups. However, a significant difference was found in the latency to eat in the Thatcher-Britton test, with the *Usp2* KO mice consuming the food

faster than their WT counterparts, as was seen in the first group (Fig. 14B, *p=0.02* on a 2-tailed unpaired t-test).

While no differences were found in the latency to cross the beam during the beam walking assay, we were surprised to find a main effect of genotype for the number of foot slips (*p=0.94 and 0.0004* on Tukey's HSD). Indeed, while no differences in rotarod performance were seen in mice of the same age, *Usp2* KO mice had significantly more foot slips, indicating that their fine motor coordination was already disrupted in some ways at 8-16 weeks (Fig. 15). Both these mice and a group of older mice will soon be tested on the rotarod to further investigate these results.

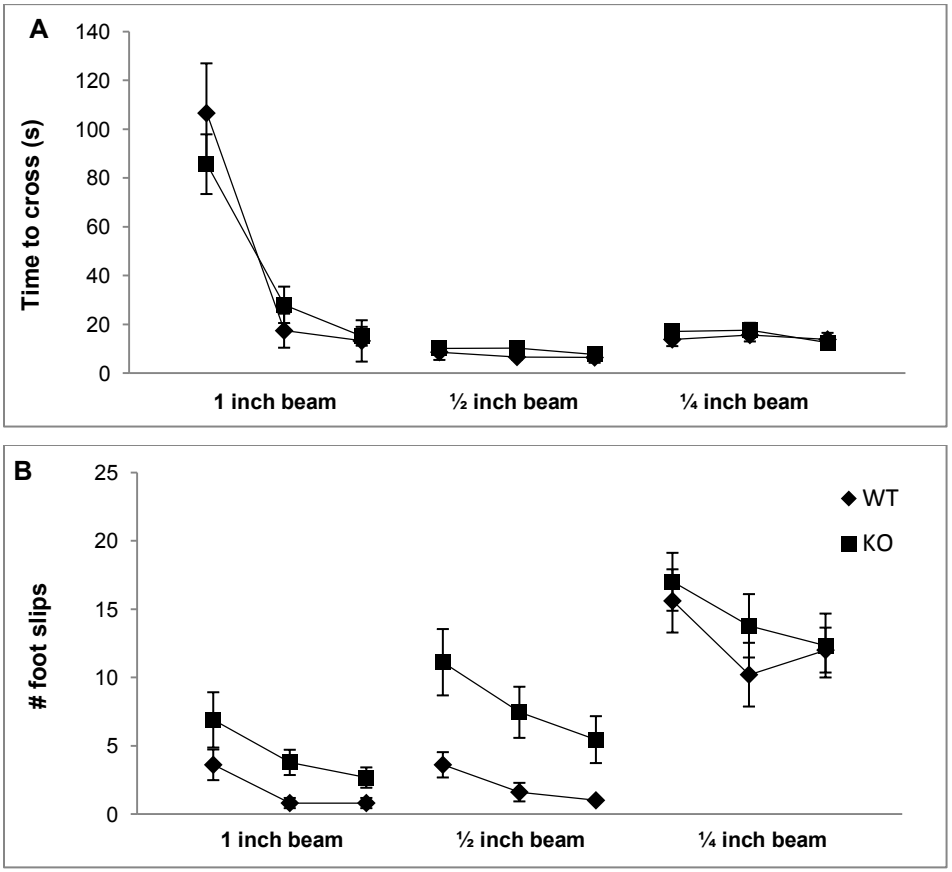

Figure 15:Beam walking assay. The time to cross the beam (A) and the number of footslips recorded (B) during the beam walking assay are shown. No significant differences were seen for the time to cross but a repeated measures ANOVA revealed a main effect of genotype for the number of foot slips ($p<0.05$).

CHAPTER 6: DISCUSSION

The present studies aimed at characterizing the implication of the deubiquitinating enzyme USP2 in the circadian clock and in the brain, through the study of the *Usp2* KO mouse model. As it was previously shown that USP2 affected circadian locomotor behaviour and light responses in addition to interacting with the clock protein PER1 (Yang et al., 2012), mice lacking the *Usp2* gene were tested to detect any differences in their ability to entrain to different photoperiods, in their responses to light or in clock protein rhythms in their SCN. Further, the expression of the *Usp2* gene and its isoforms was tested in the brain and *Usp2* KO mice were subjected to neurophenotyping tests in order to study possible differences in their behaviour.

6.1 Role of USP2 in the circadian clock

6.1.1 USP2 and circadian locomotor behaviour: Previous work in the laboratory has revealed that the free-running period is longer in *Usp2* KO mice than in their WT littermates (Yang et al., 2012). Additionally, *Usp2* KO mice respond differently to 30 min light pulses and show differences in entrainment following a phase shift. Based on these data, we believed that *Usp2* KO mice could display differences in their ability to entrain to photoperiods with different lengths of light exposure. While *Usp2* KO mice were capable of entraining to all photoperiods, we saw significant differences in the phase angle of activity offset under two 12:12 LD cycles, with a trend towards significance under a skeleton photoperiod and, consequently, differences in the duration

of activity (alpha, see Fig. 2). Even though the biological significance of the phase angle of activity offset is unclear, these data suggest that, even though *Usp2* KO mice can entrain to a light/dark cycle, USP2 is important for the precise timing of activity.

The role of USP2 in entrainment to either short (8:16 LD) or long (16:8 LD) days is of particular interest as it has been proposed that coding for day length at the level of the SCN depends on the range of the phases of individual oscillating neurons, with a broader range coding for a longer day (VanderLeest et al., 2007). This suggests that either network connectivity is altered or that clocks in individual cells behave differently, raising the possibility that the regulation of protein degradation could be involved in this process. However, we found no differences between genotypes in entrainment or other activity parameters under 8:16 and 16:8 LD cycles (section 3.2, Fig. 2), suggesting that USP2 is not involved in the encoding of day length despite its role in light responses by the clock. This apparent discrepancy might be reconciled by the fact that two distinct populations of light responding cells exist in the SCN, with one population being sensitive to acute light exposure while the other is sensitive to tonic light exposure (Yan and Silver, 2008).

6.1.2 USP2 and light responses by the clock: Induction of cFOS, the protein product of an immediate early gene known to respond to light in the SCN, was found to be significantly larger in *Usp2* KO mice in the delaying portion of the circadian cycle and may be (although small numbers of animals and large variability prevent us from reaching statistical significance) smaller in the advancing portion of the cycle. If this is confirmed in new larger groups of mice, this cFOS response would correlate with the behavioural phase shifting data previously obtained in our lab (Yang et al., 2012),

suggesting that the differences in phase shifting in *Usp2* KO mice originate at the level of the SCN. The opposite effects observed on both behavioural phase shifting and cFOS induction in the delaying and advancing portions of the cycle further lend credence to this belief, as it is known that the clock in the SCN responds differently to light at these two times. Indeed, it has been shown that *Per1* and *Per2* mRNA and protein increase in response to light perceived in the delaying portion of the phase response curve, while only *Per1* mRNA and protein increase in the advancing portion (Yan and Silver, 2004). Accordingly, it was found that *Per1* mutant mice could not phase advance and *Per2* mutant mice could not phase delay in response to light pulse administered at specific times (Albrecht et al., 2001). Phase response curves involving the administration of light pulses throughout the circadian cycle have revealed that, while *Per* KO mice retain their ability to phase shift, the timing and amplitude of phase shifts is different between *Per1* and *Per2* KO mice (Pendergast et al., 2010). Together, these data reinforce the differential roles of *Per1* and *Per2* mRNA and protein in light responses by the clock. Without testing the pupillary light reflex in *Usp2* KO mice, however, we cannot exclude that differences at the level of light perception in the retina may be involved.

6.1.3 USP2 and PER2 protein rhythms: To further investigate the origin of the differences in light responses by the clock of *Usp2* KO mice, a molecular correlate of the behavioural phase response curve is being produced via the addition of glutamate to SCN slices *in vitro*. If the magnitude of phase shifts induced by glutamate resembles the behavioural phase response curve (Yang et al., 2012), it would further reinforce the idea that the differences in phase shifting in *Usp2* KO mice originate in the SCN. The experiments pertaining to PER2 protein rhythms in the SCN are still in the preliminary

phase and are therefore difficult to interpret. From the few data points obtained so far, it appears that PER2 rhythms may be longer in the SCN of *Usp2* KO mice, corroborating previous data obtained in our lab regarding a longer free-running period in KO animals (Yang et al., 2012).

6.2 Role of USP2 in the brain

6.2.1 USP2 and brain expression: We were able to find *Usp2* mRNA expression in the hippocampus, cerebellum, olfactory bulb and cortex. So far, we have no evidence for differential expression between the two isoforms of *Usp2*, suggesting that, although they are known to have certain tissue-specific functions, *Usp2a* and *Usp2b* do not have different roles in the brain. It is important to note, however, that we cannot conclusively say that the staining is specific until the *in situ* hybridization is repeated with the new probe designed to specifically target the region which is excised out in *Usp2* KO mice (see section 5.2.1 and Fig. 5).

6.2.2 USP2 and behaviour: To examine the role of USP2 in behaviour, several groups of mice were tested on a battery of neurophenotyping tests. While no differences were observed in the forced swimming test, the Morris water maze and actimetry, we found differences in anxiety-like behaviour and, in certain cases, motor coordination.

The first group of *Usp2* KO mice showed a reduced latency to enter the open arms of the elevated plus maze (see section 5.2.2) and a reduced latency to eat in the Thatcher-Britton test but data from a second group of mice of similar age failed to replicate the elevated plus maze results. These differences in the elevated plus maze can be attributed to the

large variability seen in the second group of mice (particularly the WT) which may have prevented us from getting statistically significant results, despite the fact that the mean latency appeared to be reduced in *Usp2* KO mice. Further, the lack of differences observed in these two tests in 18-22 month old mice can be attributed to their age and to their previous experience in the running wheels. The latter point is of particular interest given that anxiety tests, especially the elevated plus maze, are known to be very sensitive to handling and previous experience (Crawley, 2008; Holmes and Rodgers, 2003; Karl et al., 2003). On the other hand, replication of the Thatcher-Britton data in two cohorts of mice, in addition to the absence of any differences in food consumption, body weight and latency to eat after a food deprivation, suggests that *Usp2* KO mice present decreased anxiety-like behaviour compared to their WT counterparts.

We found that motor coordination deficits in *Usp2* KO mice were test- and age-specific (see Figs. 12 and 14). While young mice displayed no differences in the accelerating rotarod test, older *Usp2* KO mice spent significantly less time on the cylinder than WT counterparts. Given these data, we were surprised to see that young *Usp2* KO mice had significantly more footslips in the beam walking assay. While both tests should be repeated to confirm the results, these differences can be attributed to the sensitivity of each test to specific causes of motor impairment (Hamm et al., 1994; Stanley et al., 2005). Interestingly, the accelerating rotarod data suggests that the aging process may affect *Usp2* KO animals differently than WT.

6.3 Possible crosstalk between the circadian system and anxiety behaviour

As it is well known that psychiatric disorders and sleep and circadian rhythm disruptions are often comorbid in humans (Jagannath et al., 2013), it is reasonable to believe that there is crosstalk between the circadian system and neuropsychiatric disorders. Indeed, patients suffering from anxiety disorders are known to experience sleep disturbances and reduced REM sleep (Lamont et al., 2007). This begs the question: is USP2 affecting anxiety-like behaviour through its involvement in the circadian clock or are these two distinct roles of the enzyme? While it is hard to draw conclusions based on the data presented here in the absence of experiments directly aimed at answering this question, there are some interesting hints in the literature.

First, other mice with altered circadian rhythms have also been found to have differences in anxiety-like behaviour. Indeed, both the *ClockΔ19* and the $Fbxl3^{Afh}$ mutants have been found to be less anxious than their WT counterparts (Keers et al., 2012; Roybal et al., 2007). While it is not impossible that both CLOCK and FBXL3 have roles outside of the circadian system that lead to changes in anxiety-like behaviour, these data suggest that disruptions of the circadian clock in general play an important role.

Second, it was found that knocking down both *Per1* and *Per2* in the nucleus accumbens using RNA interference leads to increased anxiety-like behaviour in WT mice, suggesting that the *Period* genes are involved in the regulation of anxiety (Spencer et al., 2013). Interestingly, one study also showed that *Per1* levels were reduced in the mouse cerebellum following the administration of antianxiety medications (Akiyama et al., 1999). Again, these data suggest that key genes of the circadian clock also play a role in

the modulation of anxiety behaviour. Furthermore, the involvement of *Per1* in the nucleus accumbens raises the possibility that USP2 could affect anxiety through its effects on the PER1 protein.

Third, it has been shown that exposing mice to abnormal light cycles can induce changes in behaviour. WT mice subjected to constant darkness for 4 weeks develop depression-like behaviour, as evidenced by increased immobilization in the forced swimming test, suggesting that the absence of light can cause behavioural abnormalities (Monje et al., 2011). Alternatively, depression-like behaviour can also be induced by exposure to very short days (T7, *i.e.* 3.5 h light, 3.5 h dark), which also cause impairments in hippocampal learning (LeGates et al., 2012). Similarly, exposure to a T20 photoperiod (*i.e.* 10 h light, 10 h dark) leads to changes in cognition and altered responses to novel environments (Karatsoreos et al., 2011). As both the T7 and T20 photoperiods are outside the range of entrainment (*i.e.* the endogenous circadian clock cannot entrain to these environmental conditions and free-runs instead), these results suggest that it is the exposure to light at aberrant times throughout the circadian cycles that results in these behavioural deficits.

Finally, the presence of *Usp2* in the hippocampus, detected in our *in situ* hybridization experiment, also raises the possibility that this enzyme exerts its effects via the ventral hippocampus, a region known to be involved in anxiety (Bannerman et al., 2004). In that case, it is hard to say whether USP2 would modulate the clock in the cells of the ventral hippocampus or would be involved in some other way.

6.4 Limitations of the study

6.4.1 Duration of wheel running experiments: It should be noted that the characterization of circadian locomotor activity in the running wheels was done over quite a long period of time. Mice were 14 months old by the time all the experiments were finished. Therefore, if *Usp2* KO mice do indeed age differently from WT mice (as suggested above), the outcome of the tests might be affected. Importantly, overall daily wheel running activity was not different between *Usp2* KO and WT mice at any time during the experiments, suggesting that at least wheel running itself was not differentially affected by age.

6.4.2 Other roles of USP2: In addition to its role in the circadian clock, USP2 has been involved in skeletal muscle atrophy, inflammation, cancer and metabolism, amongst other things (Kim et al., 2012; Molusky et al., 2012; Tong et al., 2012; Wing, 2013). While it is unclear whether any of these other effects of USP2 are mediated through its involvement in the circadian system, it is possible that some of the behavioural deficits observed in our study could be in part due to its other roles.

6.4.3 Compensation mechanisms: As mentioned in the introduction, it is possible that there is redundancy at the level of the ubiquitin pathway in the circadian clock. This raises the possibility that other deubiquitinating enzymes could assume some of the roles of USP2 in the KO mice, thereby reducing the magnitude of the effects observed in our study. However, DUBs are believed to be highly regulated in order to achieve target specificity, which may reduce the likelihood of such compensation occurring (Komander et al., 2009).

6.5 Future directions

6.5.1 Circadian characterization: It would be interesting to continue characterizing the circadian behaviour of *Usp2* KO mice. Using a similar experimental group in the same setup outlined above, mice could be subjected to a 23 h day (11.5:11.5 LD) and a 25 h day (12.5:12.5 LD). By imposing T cycles outside of the natural 24 h day, one would be testing the ability of the circadian pacemaker to consistently phase-shift to entrain to a cycle that is different from its endogenous period (Pittendrigh and Daan, 1976). This is of particular interest because previous work in the laboratory and results discussed here have identified alterations in the phase shifting ability of *Usp2* KO mice. Additionally, it would be interesting to repeat the constant light experiment using the right protocol (DD followed by LL with increasing light intensity), as this would allow us to study network connectivity in *Usp2* KO mice since constant light induces desynchrony between the neurons in the SCN (Ohta et al., 2005).

6.5.2 Light responses by the clock: For previously discussed reasons, we will repeat both the ZT14 and ZT20 light pulse experiments. Since *Per1* and its protein product increase in response to light (Albrecht et al., 1997), it may also be of interest to look at their induction following the same experimental procedure used in the cFOS immunohistochemistry experiment. This is of particular interest given the known interaction between PER1 and USP2 (Yang et al., 2012), as it raises the possibility that the absence of USP2 deubiquitinating activity in *Usp2* KO mice could lead to differences in the stability and function of the PER proteins, thereby directly affecting light responses in the SCN. We are also continuing to record bioluminescence traces, in addition to

giving glutamate pulses at CT14 and CT20 using more mice in order to be able to run statistical analyses on these data.

6.5.3 Behaviour: The *in situ* hybridization experiments need to be completed. Additionally, since we have seen a difference in rotarod performance between older and younger mice, it would be of interest to repeat the rotarod test in these two groups (experiment in progress) and to observe performance on the balance beam assay in older mice (data already acquired). In order to address the question of crosstalk between the circadian clock and anxiety behaviour, it would be of interest to test behaviour in *Usp2* KO mice at different phases of the light/dark cycle and under different photoperiods. For example, constant light has been found to increase depressive-like behaviour and decrease anxiety-like behaviour in one strain of mice (Fonken et al., 2009) and to increase both behaviours in rats (Tapia-Osorio et al., 2013), suggesting that it could be interesting to investigate the responses of *Usp2* KO mice compared to their WT counterparts under this light condition. Further, the data discussed in section 6.3 suggest that it may also be of interest to study behaviour of *Usp2* KO mice under different T cycles.

CONCLUSION

The involvement of deubiquitination in circadian rhythms and, more broadly, in the brain has been relatively poorly studied to date. The objective of this study was to further characterize the role of the deubiquitinating enzyme USP2, an enzyme known to be involved in the circadian clock (Yang et al., 2012), in circadian rhythms and to investigate, for the first time, its role in behaviour and brain function. We have found that deubiquitination by the USP2 protein is important for the regulation of entrainment to light/dark cycles and light responses by the clock, as well as in the control of anxiety-like behaviour and, in certain cases, motor coordination. Given the coexistence of circadian disruptions and neuropsychiatric disorders in humans (Lamont et al., 2007), the data obtained so far could implicate that USP2 is at the interface of light responses, circadian rhythms and affective disorders.

REFERENCES

Akashi, M., and Takumi, T. (2005). The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. Nat Struct Mol Biol *12*, 441-448.

Akiyama, M., Kirihara, T., Takahashi, S., Minami, Y., Yoshinobu, Y., Moriya, T., and Shibata, S. (1999). Modulation of mPer1 gene expression by anxiolytic drugs in mouse cerebellum. Br J Pharmacol *128*, 1616-1622.

Albrecht, U., Sun, Z.S., Eichele, G., and Lee, C.C. (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell *91*, 1055-1064.

Albrecht, U., Zheng, B., Larkin, D., Sun, Z.S., and Lee, C.C. (2001). MPer1 and mper2 are essential for normal resetting of the circadian clock. J Biol Rhythms *16*, 100-104.

Altar, C.A., Jurata, L.W., Charles, V., Lemire, A., Liu, P., Bukhman, Y., Young, T.A., Bullard, J., Yokoe, H., Webster, M.J.*, et al.* (2005). Deficient hippocampal neuron expression of proteasome, ubiquitin, and mitochondrial genes in multiple schizophrenia cohorts. Biol Psychiatry *58*, 85-96.

Amann, L.C., Gandal, M.J., Halene, T.B., Ehrlichman, R.S., White, S.L., McCarren, H.S., and Siegel, S.J. (2010). Mouse behavioral endophenotypes for schizophrenia. Brain Res Bull *83*, 147-161.

Amerik, A.Y., and Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta *1695*, 189-207.

Aschoff, J. (1952). [Changes of frequency of periods of activity of mice in constant light and lasting darkness]. Pflugers Arch *255*, 197-203.

Aschoff, J. (1960). Exogenous and endogenous components in circadian rhythms. Cold Spring Harb Symp Quant Biol *25*, 11-28.

Bannerman, D.M., Rawlins, J.N., McHugh, S.B., Deacon, R.M., Yee, B.K., Bast, T., Zhang, W.N., Pothuizen, H.H., and Feldon, J. (2004). Regional dissociations within the hippocampus--memory and anxiety. Neurosci Biobehav Rev *28*, 273-283.

Bedard, N., Yang, Y., Gregory, M., Cyr, D.G., Suzuki, J., Yu, X., Chian, R.C., Hermo, L., O'Flaherty, C., Smith, C.E.*, et al.* (2011). Mice lacking the USP2 deubiquitinating enzyme have severe male subfertility associated with defects in fertilization and sperm motility. Biol Reprod *85*, 594-604.

Berson, D.M., Dunn, F.A., and Takao, M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. Science *295*, 1070-1073.

Bousman, C.A., Chana, G., Glatt, S.J., Chandler, S.D., Lucero, G.R., Tatro, E., May, T., Lohr, J.B., Kremen, W.S., Tsuang, M.T., and Everall, I.P. (2010). Preliminary evidence of ubiquitin proteasome system dysregulation in schizophrenia and bipolar disorder: convergent pathway analysis findings from two independent samples. Am J Med Genet B Neuropsychiatr Genet *153B*, 494-502.

Busino, L., Bassermann, F., Maiolica, A., Lee, C., Nolan, P.M., Godinho, S.I., Draetta, G.F., and Pagano, M. (2007). SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. Science *316*, 900-904.

Cao, R., Li, A., Cho, H.Y., Lee, B., and Obrietan, K. (2010). Mammalian target of rapamycin signaling modulates photic entrainment of the suprachiasmatic circadian clock. J Neurosci *30*, 6302-6314.

Carter, R.J., Morton, J., and Dunnett, S.B. (2001). Motor coordination and balance in rodents. Curr Protoc Neurosci *Chapter 8*, Unit 8 12.

Chen, X., Wang, X., Sun, C., Chen, Q., O'Neill, F.A., Walsh, D., Fanous, A., and Kendler, K.S. (2008). FBXL21 association with schizophrenia in Irish family and case-control samples. Am J Med Genet B Neuropsychiatr Genet *147B*, 1231-1237.

Colwell, C.S., and Foster, R.G. (1992). Photic regulation of Fos-like immunoreactivity in the suprachiasmatic nucleus of the mouse. J Comp Neurol *324*, 135-142.

Colwell, C.S., Foster, R.G., and Menaker, M. (1991). NMDA receptor antagonists block the effects of light on circadian behavior in the mouse. Brain Res *554*, 105-110.

Colwell, C.S., and Menaker, M. (1992). NMDA as well as non-NMDA receptor antagonists can prevent the phase-shifting effects of light on the circadian system of the golden hamster. J Biol Rhythms *7*, 125-136.

Coogan, A.N., and Piggins, H.D. (2003). Circadian and photic regulation of phosphorylation of ERK1/2 and Elk-1 in the suprachiasmatic nuclei of the Syrian hamster. J Neurosci *23*, 3085-3093.

Crawley, J.N. (2008). Behavioral phenotyping strategies for mutant mice. Neuron *57*, 809-818.

Dardente, H., and Cermakian, N. (2007). Molecular circadian rhythms in central and peripheral clocks in mammals. Chronobiol Int *24*, 195-213.

Dardente, H., Fortier, E.E., Martineau, V., and Cermakian, N. (2007). Cryptochromes impair phosphorylation of transcriptional activators in the clock: a general mechanism for circadian repression. Biochem J *402*, 525-536.

Dardente, H., Mendoza, J., Fustin, J.M., Challet, E., and Hazlerigg, D.G. (2008). Implication of the F-Box Protein FBXL21 in circadian pacemaker function in mammals. PLoS One *3*, e3530.

Dawson, T.M., and Dawson, V.L. (2010). The role of parkin in familial and sporadic Parkinson's disease. Mov Disord *25 Suppl 1*, S32-39.

Dibner, C., Schibler, U., and Albrecht, U. (2010). The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol *72*, 517-549.

Duguay, D., and Cermakian, N. (2009). The crosstalk between physiology and circadian clock proteins. Chronobiol Int *26*, 1479-1513.

Eide, E.J., Woolf, M.F., Kang, H., Woolf, P., Hurst, W., Camacho, F., Vielhaber, E.L., Giovanni, A., and Virshup, D.M. (2005). Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. Mol Cell Biol *25*, 2795-2807.

Etchegaray, J.P., Lee, C., Wade, P.A., and Reppert, S.M. (2003). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature *421*, 177-182.

Etchegaray, J.P., Machida, K.K., Noton, E., Constance, C.M., Dallmann, R., Di Napoli, M.N., DeBruyne, J.P., Lambert, C.M., Yu, E.A., Reppert, S.M., and Weaver, D.R. (2009). Casein kinase 1 delta regulates the pace of the mammalian circadian clock. Mol Cell Biol *29*, 3853-3866.

Fonken, L.K., Finy, M.S., Walton, J.C., Weil, Z.M., Workman, J.L., Ross, J., and Nelson, R.J. (2009). Influence of light at night on murine anxiety- and depressive-like responses. Behav Brain Res *205*, 349-354.

Fukuo, Y., Kishi, T., Kushima, I., Yoshimura, R., Okochi, T., Kitajima, T., Matsunaga, S., Kawashima, K., Umene-Nakano, W., Naitoh, H.*, et al.* (2011). Possible association between ubiquitin-specific peptidase 46 gene and major depressive disorders in the Japanese population. J Affect Disord *133*, 150-157.

Gallego, M., Kang, H., and Virshup, D.M. (2006). Protein phosphatase 1 regulates the stability of the circadian protein PER2. Biochem J *399*, 169-175.

Gallego, M., and Virshup, D.M. (2007). Post-translational modifications regulate the ticking of the circadian clock. Nat Rev Mol Cell Biol *8*, 139-148.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564-1569.

Godinho, S.I., Maywood, E.S., Shaw, L., Tucci, V., Barnard, A.R., Busino, L., Pagano, M., Kendall, R., Quwailid, M.M., Romero, M.R.*, et al.* (2007). The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. Science *316*, 897-900.

Gousseva, N., and Baker, R.T. (2003). Gene structure, alternate splicing, tissue distribution, cellular localization, and developmental expression pattern of mouse deubiquitinating enzyme isoforms Usp2-45 and Usp2-69. Gene Expr *11*, 163-179.

Guillaumond, F., Dardente, H., Giguere, V., and Cermakian, N. (2005). Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J Biol Rhythms *20*, 391-403.

Hamm, R.J., Pike, B.R., O'Dell, D.M., Lyeth, B.G., and Jenkins, L.W. (1994). The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. J Neurotrauma *11*, 187-196.

Harada, Y., Sakai, M., Kurabayashi, N., Hirota, T., and Fukada, Y. (2005). Ser-557-phosphorylated mCRY2 is degraded upon synergistic phosphorylation by glycogen synthase kinase-3 beta. J Biol Chem *280*, 31714-31721.

Hattar, S., Liao, H.W., Takao, M., Berson, D.M., and Yau, K.W. (2002). Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science *295*, 1065-1070.

Hayashi, S., Gillam, I.C., Delaney, A.D., and Tener, G.M. (1978). Acetylation of chromosome squashes of Drosophila melanogaster decreases the background in autoradiographs from hybridization with [125I]-labeled RNA. J Histochem Cytochem *26*, 677-679.

Hirano, A., Yumimoto, K., Tsunematsu, R., Matsumoto, M., Oyama, M., Kozuka-Hata, H., Nakagawa, T., Lanjakornsiripan, D., Nakayama, K.I., and Fukada, Y. (2013). FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. Cell *152*, 1106-1118.

Holmes, A., and Rodgers, R.J. (2003). Prior exposure to the elevated plus-maze sensitizes mice to the acute behavioral effects of fluoxetine and phenelzine. Eur J Pharmacol *459*, 221-230.

Husnjak, K., and Dikic, I. (2012). Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. Annu Rev Biochem *81*, 291-322.

Imai, S., Mamiya, T., Tsukada, A., Sakai, Y., Mouri, A., Nabeshima, T., and Ebihara, S. (2012). Ubiquitin-specific peptidase 46 (Usp46) regulates mouse immobile behavior in the tail suspension test through the GABAergic system. PLoS One *7*, e39084.

Jagannath, A., Peirson, S.N., and Foster, R.G. (2013). Sleep and circadian rhythm disruption in neuropsychiatric illness. Curr Opin Neurobiol *23*, 1-7.

Jones, B.J., and Roberts, D.J. (1968). A rotarod suitable for quantitative measurements of motor incoordination in naive mice. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol *259*, 211.

Jud, C., Schmutz, I., Hampp, G., Oster, H., and Albrecht, U. (2005). A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. Biol Proced Online *7*, 101-116.

Karatsoreos, I.N., Bhagat, S., Bloss, E.B., Morrison, J.H., and McEwen, B.S. (2011). Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. Proc Natl Acad Sci U S A *108*, 1657-1662.

Karl, T., Pabst, R., and von Horsten, S. (2003). Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol *55*, 69-83.

Keers, R., Pedroso, I., Breen, G., Aitchison, K.J., Nolan, P.M., Cichon, S., Nothen, M.M., Rietschel, M., Schalkwyk, L.C., and Fernandes, C. (2012). Reduced anxiety and depression-like behaviours in the circadian period mutant mouse afterhours. PLoS One *7*, e38263.

Kim, J., Kim, W.J., Liu, Z., Loda, M., and Freeman, M.R. (2012). The ubiquitin-specific protease USP2a enhances tumor progression by targeting cyclin A1 in bladder cancer. Cell Cycle *11*, 1123-1130.

Kim, S., Zhang, S., Choi, K.H., Reister, R., Do, C., Baykiz, A.F., and Gershenfeld, H.K. (2009). An E3 ubiquitin ligase, Really Interesting New Gene (RING) Finger 41, is a candidate gene for anxiety-like behavior and beta-carboline-induced seizures. Biol Psychiatry *65*, 425-431.

Kita, Y., Shiozawa, M., Jin, W., Majewski, R.R., Besharse, J.C., Greene, A.S., and Jacob, H.J. (2002). Implications of circadian gene expression in kidney, liver and the effects of fasting on pharmacogenomic studies. Pharmacogenetics *12*, 55-65.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature *392*, 605-608.

Kiyohara, Y.B., Tagao, S., Tamanini, F., Morita, A., Sugisawa, Y., Yasuda, M., Yamanaka, I., Ueda, H.R., van der Horst, G.T., Kondo, T., and Yagita, K. (2006). The BMAL1 C terminus regulates the circadian transcription feedback loop. Proc Natl Acad Sci U S A *103*, 10074-10079.

Komander, D., Clague, M.J., and Urbe, S. (2009). Breaking the chains: structure and function of the deubiquitinases. Nat Rev Mol Cell Biol *10*, 550-563.

Kume, K., Zylka, M.J., Sriram, S., Shearman, L.P., Weaver, D.R., Jin, X., Maywood, E.S., Hastings, M.H., and Reppert, S.M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell *98*, 193-205.

Kushima, I., Aleksic, B., Ito, Y., Nakamura, Y., Nakamura, K., Mori, N., Kikuchi, M., Inada, T., Kunugi, H., Nanko, S.*, et al.* (2010). Association study of ubiquitin-specific peptidase 46 (USP46) with bipolar disorder and schizophrenia in a Japanese population. J Hum Genet *55*, 133-136.

Lamont, E.W., Legault-Coutu, D., Cermakian, N., and Boivin, D.B. (2007). The role of circadian clock genes in mental disorders. Dialogues Clin Neurosci *9*, 333-342.

LeGates, T.A., Altimus, C.M., Wang, H., Lee, H.K., Yang, S., Zhao, H., Kirkwood, A., Weber, E.T., and Hattar, S. (2012). Aberrant light directly impairs mood and learning through melanopsin-expressing neurons. Nature *491*, 594-598.

LeSauter, J., and Silver, R. (1998). Output signals of the SCN. Chronobiol Int *15*, 535-550.

Lister, R.G. (1987). The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) *92*, 180-185.

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., and Takahashi, J.S. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science *288*, 483-492.

Maier, B., Wendt, S., Vanselow, J.T., Wallach, T., Reischl, S., Oehmke, S., Schlosser, A., and Kramer, A. (2009). A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. Genes Dev *23*, 708-718.

Molusky, M.M., Li, S., Ma, D., Yu, L., and Lin, J.D. (2012). Ubiquitin-specific protease 2 regulates hepatic gluconeogenesis and diurnal glucose metabolism through 11beta-hydroxysteroid dehydrogenase 1. Diabetes *61*, 1025-1035.

Monje, F.J., Cabatic, M., Divisch, I., Kim, E.J., Herkner, K.R., Binder, B.R., and Pollak, D.D. (2011). Constant darkness induces IL-6-dependent depression-like behavior through the NF-kappaB signaling pathway. J Neurosci *31*, 9075-9083.

Morris, R.G., Anderson, E., Lynch, G.S., and Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature *319*, 774-776.

Nakagawa, H., and Okumura, N. (2010). Coordinated regulation of circadian rhythms and homeostasis by the suprachiasmatic nucleus. Proc Jpn Acad Ser B Phys Biol Sci *86*, 391-409.

Obrietan, K., Impey, S., and Storm, D.R. (1998). Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. Nat Neurosci *1*, 693-700.

Ohsaki, K., Oishi, K., Kozono, Y., Nakayama, K., Nakayama, K.I., and Ishida, N. (2008). The role of {beta}-TrCP1 and {beta}-TrCP2 in circadian rhythm generation by mediating degradation of clock protein PER2. J Biochem *144*, 609-618.

Ohta, H., Yamazaki, S., and McMahon, D.G. (2005). Constant light desynchronizes mammalian clock neurons. Nat Neurosci *8*, 267-269.

Oishi, K., Miyazaki, K., Kadota, K., Kikuno, R., Nagase, T., Atsumi, G., Ohkura, N., Azama, T., Mesaki, M., Yukimasa, S.*, et al.* (2003). Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. J Biol Chem *278*, 41519-41527.

Pendergast, J.S., Friday, R.C., and Yamazaki, S. (2010). Photic entrainment of period mutant mice is predicted from their phase response curves. J Neurosci *30*, 12179-12184.

Pittendrigh, C.S., and Daan, S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: Pacemaker as clock. J Comp Physiol A *106*, 291-331.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell *110*, 251-260.

Reischl, S., Vanselow, K., Westermark, P.O., Thierfelder, N., Maier, B., Herzel, H., and Kramer, A. (2007). Beta-TrCP1-mediated degradation of PERIOD2 is essential for circadian dynamics. J Biol Rhythms *22*, 375-386.

Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian timing in mammals. Nature *418*, 935-941.

Reyes-Turcu, F.E., Ventii, K.H., and Wilkinson, K.D. (2009). Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem *78*, 363-397.

Riederer, B.M., Leuba, G., Vernay, A., and Riederer, I.M. (2011). The role of the ubiquitin proteasome system in Alzheimer's disease. Exp Biol Med (Maywood) *236*, 268-276.

Rochford, J., Beaulieu, S., Rousse, I., Glowa, J.R., and Barden, N. (1997). Behavioral reactivity to aversive stimuli in a transgenic mouse model of impaired glucocorticoid (type II) receptor function: effects of diazepam and FG-7142. Psychopharmacology (Berl) *132*, 145-152.

Roybal, K., Theobold, D., Graham, A., DiNieri, J.A., Russo, S.J., Krishnan, V., Chakravarty, S., Peevey, J., Oehrlein, N., Birnbaum, S.*, et al.* (2007). Mania-like behavior induced by disruption of CLOCK. Proc Natl Acad Sci U S A *104*, 6406-6411.

Ryan, M.M., Lockstone, H.E., Huffaker, S.J., Wayland, M.T., Webster, M.J., and Bahn, S. (2006). Gene expression analysis of bipolar disorder reveals downregulation of the ubiquitin cycle and alterations in synaptic genes. Mol Psychiatry *11*, 965-978.

Sato, T.K., Panda, S., Miraglia, L.J., Reyes, T.M., Rudic, R.D., McNamara, P., Naik, K.A., FitzGerald, G.A., Kay, S.A., and Hogenesch, J.B. (2004). A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron *43*, 527-537.

Scoma, H.D., Humby, M., Yadav, G., Zhang, Q., Fogerty, J., and Besharse, J.C. (2011). The de-ubiquitinylating enzyme, USP2, is associated with the circadian clockwork and regulates its sensitivity to light. PLoS One *6*, e25382.

Shirogane, T., Jin, J., Ang, X.L., and Harper, J.W. (2005). SCFbeta-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. J Biol Chem *280*, 26863-26872.

Siepka, S.M., Yoo, S.H., Park, J., Song, W., Kumar, V., Hu, Y., Lee, C., and Takahashi, J.S. (2007). Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. Cell *129*, 1011-1023.

Spencer, S., Falcon, E., Kumar, J., Krishnan, V., Mukherjee, S., Birnbaum, S.G., and McClung, C.A. (2013). Circadian genes Period 1 and Period 2 in the nucleus accumbens regulate anxiety-related behavior. Eur J Neurosci *37*, 242-250.

Stanley, J.L., Lincoln, R.J., Brown, T.A., McDonald, L.M., Dawson, G.R., and Reynolds, D.S. (2005). The mouse beam walking assay offers improved sensitivity over the mouse rotarod in determining motor coordination deficits induced by benzodiazepines. J Psychopharmacol *19*, 221-227.

Storch, K.F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F.C., Wong, W.H., and Weitz, C.J. (2002). Extensive and divergent circadian gene expression in liver and heart. Nature *417*, 78-83.

Strieter, E.R., and Korasick, D.A. (2012). Unraveling the complexity of ubiquitin signaling. ACS Chem Biol *7*, 52-63.

Tapia-Osorio, A., Salgado-Delgado, R., Angeles-Castellanos, M., and Escobar, C. (2013). Disruption of circadian rhythms due to chronic constant light leads to depressive and anxiety-like behaviors in the rat. Behav Brain Res *252C*, 1-9.

Tischkau, S.A., Mitchell, J.W., Tyan, S.H., Buchanan, G.F., and Gillette, M.U. (2003). Ca2+/cAMP response element-binding protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the suprachiasmatic nucleus circadian clock. J Biol Chem *278*, 718-723.

Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J., and Fu, Y.H. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science *291*, 1040-1043.

Tomida, S., Mamiya, T., Sakamaki, H., Miura, M., Aosaki, T., Masuda, M., Niwa, M., Kameyama, T., Kobayashi, J., Iwaki, Y.*, et al.* (2009). Usp46 is a quantitative trait gene regulating mouse immobile behavior in the tail suspension and forced swimming tests. Nat Genet *41*, 688-695.

Tong, X., Buelow, K., Guha, A., Rausch, R., and Yin, L. (2012). USP2a protein deubiquitinates and stabilizes the circadian protein CRY1 in response to inflammatory signals. J Biol Chem *287*, 25280-25291.

Tsujimura, A., Matsuki, M., Takao, K., Yamanishi, K., Miyakawa, T., and Hashimoto-Gotoh, T. (2008). Mice lacking the kf-1 gene exhibit increased anxiety- but not despair-like behavior. Front Behav Neurosci *2*, 4.

Ueda, H.R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., and Hashimoto, S. (2005). System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat Genet *37*, 187-192.

VanderLeest, H.T., Houben, T., Michel, S., Deboer, T., Albus, H., Vansteensel, M.J., Block, G.D., and Meijer, J.H. (2007). Seasonal encoding by the circadian pacemaker of the SCN. Curr Biol *17*, 468-473.

Vanselow, K., and Kramer, A. (2007). Role of phosphorylation in the mammalian circadian clock. Cold Spring Harb Symp Quant Biol *72*, 167-176.

Varshavsky, A. (2012). The ubiquitin system, an immense realm. Annu Rev Biochem *81*, 167-176.

Wing, S.S. (2003). Deubiquitinating enzymes--the importance of driving in reverse along the ubiquitin-proteasome pathway. Int J Biochem Cell Biol *35*, 590-605.

Wing, S.S. (2013). Deubiquitinases in skeletal muscle atrophy. Int J Biochem Cell Biol, http://dx.doi.org/10.1016/j.biocel.2013.05.002.

Yan, J., Wang, H., Liu, Y., and Shao, C. (2008). Analysis of gene regulatory networks in the mammalian circadian rhythm. PLoS Comput Biol *4*, e1000193.

Yan, L., and Silver, R. (2004). Resetting the brain clock: time course and localization of mPER1 and mPER2 protein expression in suprachiasmatic nuclei during phase shifts. Eur J Neurosci *19*, 1105-1109.

Yan, L., and Silver, R. (2008). Day-length encoding through tonic photic effects in the retinorecipient SCN region. Eur J Neurosci *28*, 2108-2115.

Yang, Y., Duguay, D., Bédard, N., Rachalski, A., Baquiran, G., Na, C.H., Fahrenkrug, J., Storch, K.F., Peng, J., Wing, S.S., and Cermakian, N. (2012). Regulation of behavioral circadian rhythms and clock protein PER1 by the deubiquitinating enzyme USP2. Biology Open *1*, 789-801.

Yao, I., Takao, K., Miyakawa, T., Ito, S., and Setou, M. (2011). Synaptic E3 ligase SCRAPPER in contextual fear conditioning: extensive behavioral phenotyping of Scrapper heterozygote and overexpressing mutant mice. PLoS One *6*, e17317.

Yasojima, K., Tsujimura, A., Mizuno, T., Shigeyoshi, Y., Inazawa, J., Kikuno, R., Kuma, K., Ohkubo, K., Hosokawa, Y., Ibata, Y.*, et al.* (1997). Cloning of human and mouse cDNAs encoding novel zinc finger proteins expressed in cerebellum and hippocampus. Biochem Biophys Res Commun *231*, 481-487.

Yin, L., Joshi, S., Wu, N., Tong, X., and Lazar, M.A. (2010). E3 ligases Arf-bp1 and Pam mediate lithium-stimulated degradation of the circadian heme receptor Rev-erb alpha. Proc Natl Acad Sci U S A *107*, 11614-11619.

Yin, L., Wang, J., Klein, P.S., and Lazar, M.A. (2006). Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. Science *311*, 1002-1005.

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J.*, et al.* (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A *101*, 5339-5346.