available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Impact of aging on diurnal expression patterns of CLOCK and BMAL1 in the mouse brain

Cathy A. Wyse[a], *Andrew N. Coogan*[a,b,*]

[a]*Neuroscience and Molecular Psychiatry, Institute of Life Sciences, School of Medicine, University of Swansea, Singleton Park, Swansea, SA2 8PP, UK*

[b]*Department of Psychology, National University of Ireland, Maynooth, Co. Kildare, Republic of Ireland*

ARTICLE INFO

Article history:
Accepted 31 March 2010
Available online 9 April 2010

Keywords:
Aging
Brain
Circadian
Diurnal
BMAL1
CLOCK

ABSTRACT

Mammalian circadian rhythms are generated by a network of transcriptional and translational loops in the expression of a panel of clock genes in various brain and peripheral sites. Many of the output rhythms controlled by this system are significantly affected by ageing, although the mechanisms of age-related circadian dysfunction remain opaque. The aim of this study was to investigate the effect of aging on the daily oscillation of two clock gene proteins (CLOCK, BMAL1) in the mouse brain. Clock gene protein expression in the brain was measured by means of immunohistochemistry in groups of young (4 months) and older (16 months) mice sampled every 4 h over a 24-h cycle. CLOCK and BMAL1 were constitutively expressed in the suprachiasmatic nucleus (SCN; the master circadian pacemaker) in young adult animals. We report novel rhythmic expression of CLOCK and BMAL1 in a number of extra-SCN sites in the young mouse brain, including the hippocampus, amygdala and the paraventricular, arcuate and dorsomedial nuclei of the hypothalamus. Aging altered the amplitude and/or phase of expression in these regions. These results indicate hitherto unreported expression patterns of CLOCK and BMAL1 in non-SCN brain circadian oscillators, and suggest that alterations of these patterns may contribute to age-related circadian dysfunction.

1. Introduction

Circadian rhythms are daily oscillations in behaviour and physiology that are defined by a period close to 24 h and persistence in constant environmental conditions. Circadian rhythms are conserved throughout eukaryotic biology, indicating an ancient origin of these mechanisms, and the significance of their function to coordinate internal temporal order. Circadian timekeeping is mediated by a network of feedback and feedforward transcriptional and translational loops in the expression of a panel of "clock" genes (e.g. *clock, per1, 2, cry 1, 2, bmal1, rev-erbα*) and their respective protein products (Reppert and Weaver, 2002). The current view of the mammalian circadian system has moved from an outlook

* *Corresponding author*. Department of Psychology, National University of Ireland, Maynooth, Co. Kildare, Republic of Ireland. Fax: +353 1 7084767.
E-mail address: andrew.coogan@nuim.ie (A.N. Coogan).

Abbreviations: ARC, arcuate nucleus of the hypothalamus; BAOT, bed nucleus of the accessory olfactory tract; BLA, basolateral amygdale; BNST –OV, oval nucleus of the bed nucleus of the stria terminalis; CC, cingulate cortex; CeA, central nucleus of the amygdala; DG, dentate gyrus; DMH, dorsomedial hypothalamic nuclei; LHb, lateral habenula; MeA, medial amygdala; Pir, piriform cortex; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; SCN, suprachiasmatic nucleus

0006-8993/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2010.03.113

centred on the suprachiasmatic nucleus (SCN) to regarding the system as distributed and complex, with circadian clocks being described throughout the brain and periphery (Guilding and Piggins, 2007; Schibler, 2009). Most of these oscillators are reliant on entrainment from the master pacemaker located in the SCN, but some neural loci (e.g. retina, olfactory bulb, habenula) are capable of autonomous or semi-autonomous oscillation (Guilding and Piggins, 2007).

Aging has potent effects on output rhythms of the circadian clock. For example, aging in humans is characterised by phase advance of melatonin and body temperature rhythms, and older individuals typically show earlier habitual times of sleeping and waking as well as more disturbed sleep compared with young control subjects (e.g. Yoon et al., 2003; Duffy et al., 2002; Benloucif et al., 2006). Human aging is also accompanied by altered synchronisation of rhythms in the sleep-wake cycle, and other output parameters of the circadian clock, including melatonin secretion (Duffy et al., 2002) and body temperature (Yoon et al., 2003).

These findings in humans are corroborated by similar age-related changes in output rhythms in laboratory rodents, where aging is associated with striking changes in locomotor activity patterns in mice, including altered phase angles of entrainment, increased rhythm fragmentation, increased variability of activity onset and reduced capacity for re-entrainment after a photoperiod phase advance (Weinert, 2000).

Aging is associated with changes in SCN organisation and function, including alterations in the neurochemistry of the SCN (Hofman and Swaab, 2006) and electrophysiological output of SCN neurons (Watanabe et al., 1995; Aujard et al., 2001; Nygård et al., 2005). Further, aged rodents show increased photic thresholds for light-induced behavioural phase shifts, and for light-induced expression of *c-fos* and *per1* in the SCN (Benloucif et al., 2006; Kolker et al., 2003), altered re-setting of SCN electrophysiological rhythms (Biello, 2009) and reduced SCN responsiveness to melatonin (Von Gall and Weaver, 2008). There are also altered phase-relationships between the SCN and peripheral oscillators measured by *per1*-driven luciferase rhythms in aged rats (Yamazaki et al., 2002), suggesting that the strength of phase-coupling between the master SCN clock and peripheral oscillators may become weakened with age. Davidson et al. (2008) report that phase-shifts of the light:dark cycle lead to tissue-specific changes in re-entrainment rates in aged rats of *per-1* driven bioluminescence rhythms, again indicating a role for a circadian-system wide age-related dysfunction.

The emerging view of a physiologically and anatomically distributed system of circadian oscillators lead us to examine whether aging is associated with significant changes in clock gene expression patterns across the brain, as such widespread dysregulation would be consistent with a scenario of significant circadian dysfunction with advancing age. We have chosen to compare mice aged 16 months with young animals, as mice of this age display significant alterations in behavioural circadian parameters (Weinert, 2000). We have examined two core clock gene products, CLOCK and BMAL1 in several brain sites in young and older mice. The brain sites examined have previously been implicated in circadian timekeeping, as either semi-autonomous or "slave" oscillators linked to the SCN master clock (Guilding and Piggins, 2007). The exceptions to this are areas of the accessory olfactory system, whose potential involvement with the circadian clock is described here for the first time. We have chosen these clock genes to examine given the relative dearth of information on their expression outside the SCN, the fact that genetic deletion of these factors produces significant behavioural phenotypes (e.g. Roybal et al., 2007; McDearmon et al., 2006) and *bmal1* has been directly implicated in the ageing process (Kondratov et al., 2006). Therefore one may envisage that these factors play important roles both inside and outside of the SCN, and understanding their expression with increasing age may be of note in understanding age-related circadian and neuronal dysfunction.

2. Results

2.1. *Expression of BMAL1 in young and older mice*

BMAL1 was found to be expressed in all of the regions examined in this study. BMAL1 was not rhythmic in the SCN (Fig. 1), but diurnal patterns of expression in young mouse brain were detected by cosinor analysis in the CA1, CA3, CeA (Fig. 2), LHb and PVT (Fig. 3), with acrophases during the mid day (Fig. 5). All of the other areas examined showed constitutive BMAL1 expression in young tissue, including the SCN, with immunostain being nuclear in all of the regions examined.

Aging was found to affect BMAL1 expression across the mouse brain. ANOVA revealed a significant effect of age on mean BMAL1 expression ($F_{1,191}=81.3$, $p<0.001$), as well as significant interactions between time and age ($F_{5,\ 191}=5.72$, $p<0.001$) and age and region ($F_{15,\ 191}=2.018$, $p<0.05$). *Post-hoc* testing showed that the mean level of BMAl1 was lower in older animals than young animals in the ARC, BAOT, BNST, CA3, CC, DMH, LHb, MeA, PC and PVN. Of the five brain regions that showed rhythmic expression of BMAL1 in the young animals, only the CA3 and LHb retained rhythmic expression in the older age group, and then the acrophase was delayed towards the late day/dusk (Fig. 5). In older animals the BLA showed rhythmic expression of BMAL1 at a low amplitude, something that was not observed in younger animals.

2.2. *Expression of CLOCK in young and older mice*

CLOCK was found to be expressed in all of the regions examined in this study, and the nature of the stain was nuclear in all of the regions examined (Figs. 1–4). There were significant rhythmic patterns of expression across the diurnal cycle being found in a number of these regions as revealed by cosinor analysis. In the hypothalamus of young animals the ARC, DMH and PVN showed rhythmic CLOCK expression (Fig. 1), in the limbic forebrain the BLA, CeA, DG displayed rhythmic CLOCK in young animals (Fig. 2) whilst the LHb, PVT and CC (Fig. 3) and the BaOT and MeA, areas of the accessory olfactory system, also displayed rhythmic CLOCK expression in the young animals (Fig. 4). The peak of the CLOCK expression (acrophase) in these regions was found to be predominantly during the mid lights-on phase (Fig. 5).

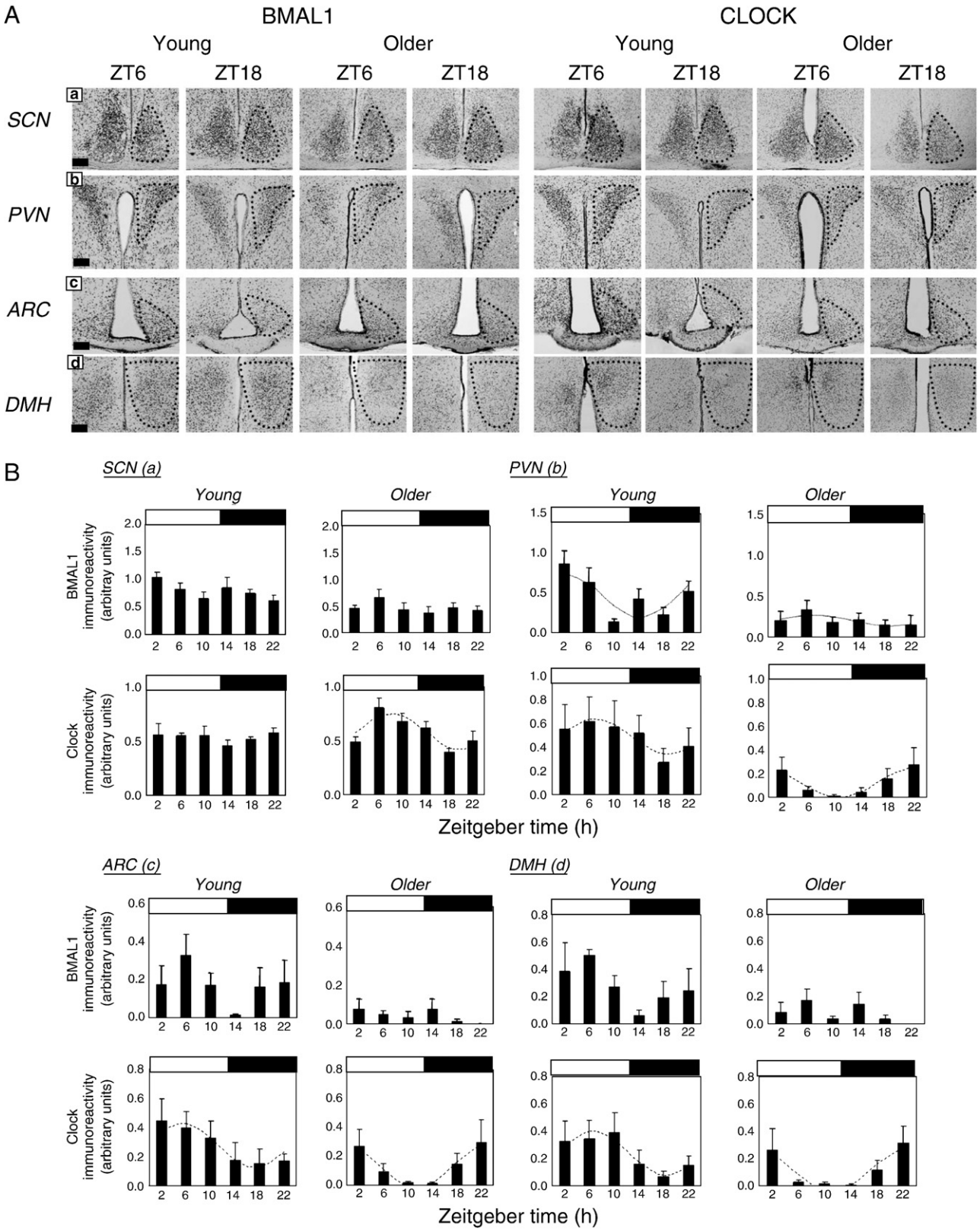


Fig. 1 – Expression of BMAL1 and CLOCK in the hypothalamus of young adult and older mice. (A) Photomicrographs of BMAL1 and CLOCK expression during the mid day (ZT6) and mid night (ZT18) phases in the (a) suprachiasmatic nucleus (SCN), (b) paraventricular nucleus of the hypothalamus (PVN), (c) arcuate nucleus of the hypothalamus (ARC) and (d) dorsomedial hypothalamic nucleus (DMH). Scale bars = 100 μm. (B) Diurnal expression patterns of BMAL1 and CLOCK immunoreactivity in the (a) SCN, (b) PVN, (c) ARC and (d) DMH. The presence of the dashed fitted line indicates a statistically significant cosinor fitted curve indicating rhythmic expression.

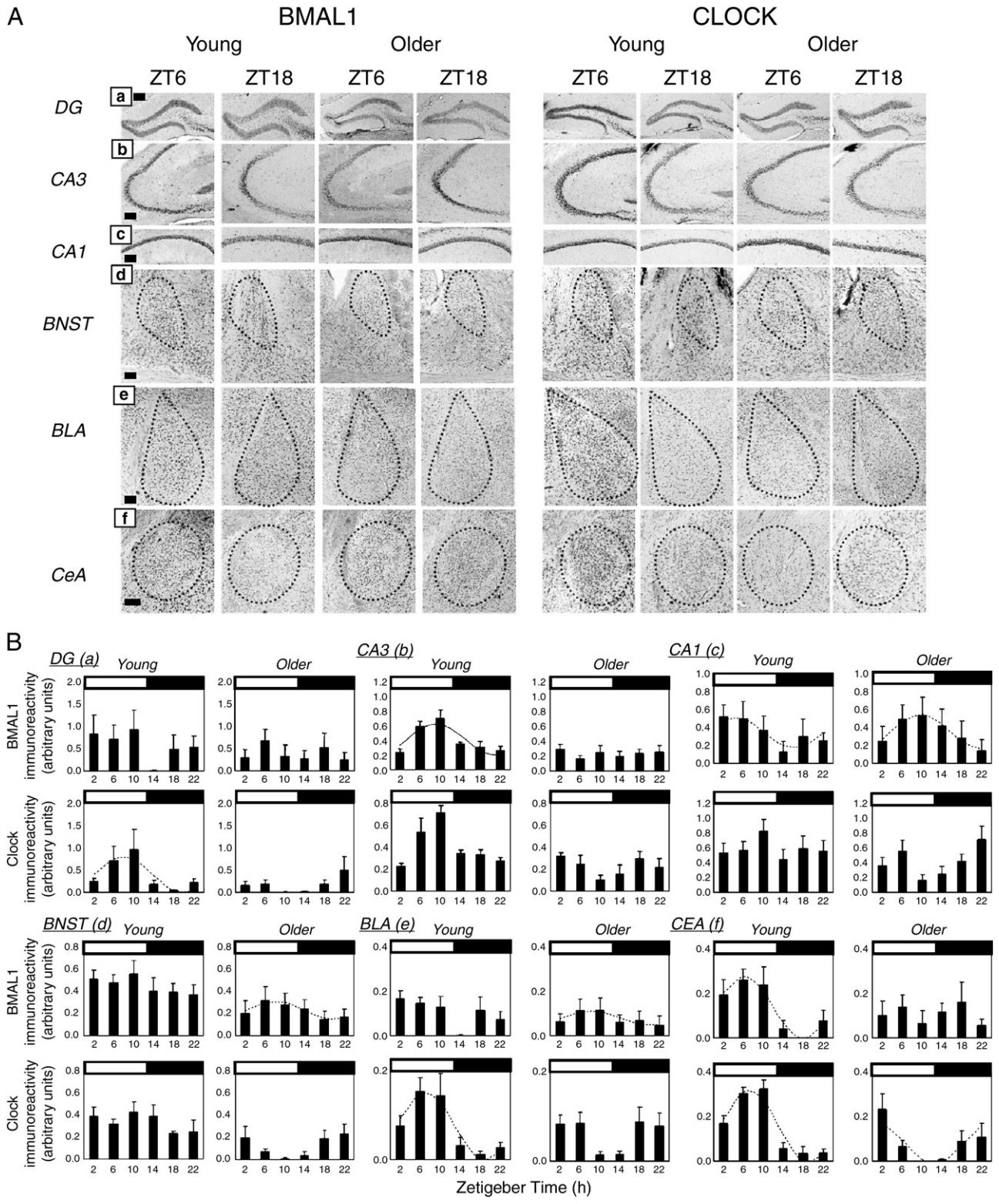


Fig. 2 – Expression of BMAL1 and CLOCK in the limbic forebrain of young adult and older mice. (A) Photomicrographs of BMAL1 and CLOCK expression during the mid day (ZT6) and mid night (ZT18) phases in the (a) dentate gyrus (DG), (b) CA3, (c) CA1, (d) oval nucleus of the bed nucleus of the stria terminalis (BNST), (e) basolateral amygdala (BLA) and (f) central nucleus of the amygdale (CeA). Scale bars = 100 μm. (B) Diurnal expression patterns of BMAL1 and CLOCK immunoreactivity in the (a) DG, (b) CA3, (c) CA1, (d) BNST, (e) BLA and (f) CeA. The presence of the dashed fitted line indicates a statistically significant cosinor fitted curve indicating rhythmic expression.

When CLOCK expression was examined in the older animals, the general trend was towards lower expression and a loss of rhythmicity. ANOVA revealed a significant main effect of ageing for mean CLOCK ($F_{1,173}=72$, $p<0.001$), a significant interaction between time and age for CLOCK ($F_{5,173}=13.76$, $p<0.001$) as well as significant interactions between age and region for CLOCK ($F_{15,173}=3.13$, $p<0.001$). Further *post-hoc* analysis revealed significant effects of age on mean CLOCK expression in the ARC, BNST, BAOT, CA1, CA3, CeA, DMH, LHb, MeA PVT and PVN . Diurnal patterns in CLOCK expression was retained in the older animals in the ARC, DMH, PVN (Fig. 1), CeA (Fig. 2), LHb, CC (Fig. 3) and BAOT (Fig. 4) with all of these regions displaying acrophase in the late night (Fig. 5). In older animals the SCN displayed rhythmic CLOCK expression, whereas in young animals it is constitutively expressed (Fig. 1). Aside from changes in the acrophase, aging was also associated with diminished amplitudes of the 24 rhythm in some of the areas examined that remained rhythmic in the older animals (CeA, LHb, MeA, Supplementary Fig. 1).

3. Discussion

The results of this study reveal profound changes in the amplitude and pattern of expression of CLOCK and BMAL1 in the brains of older mice, as well as demonstrating hitherto undescribed diurnal patterns of CLOCK and BMAL1 protein expression in extra-SCN brain clocks. These results indicate that CLOCK and BMAL1 expression may be regulated in a different manner in extra-SCN sites than in the master SCN circadian clock. Further, these findings suggest that age-related circadian dysfunction may involve changes across the brain's circadian timekeeping system. Indeed, signs of circadian dysfunction were reported across a range of physiological parameters in mice of the same age as those in the current study, including decreased amplitude of circadian rhythms of activity, body temperature and corticosterone secretion, changes in the length and stability of the free-running period and altered response to photic stimulation as well as to non-photic stimuli (Weinert, 2000).

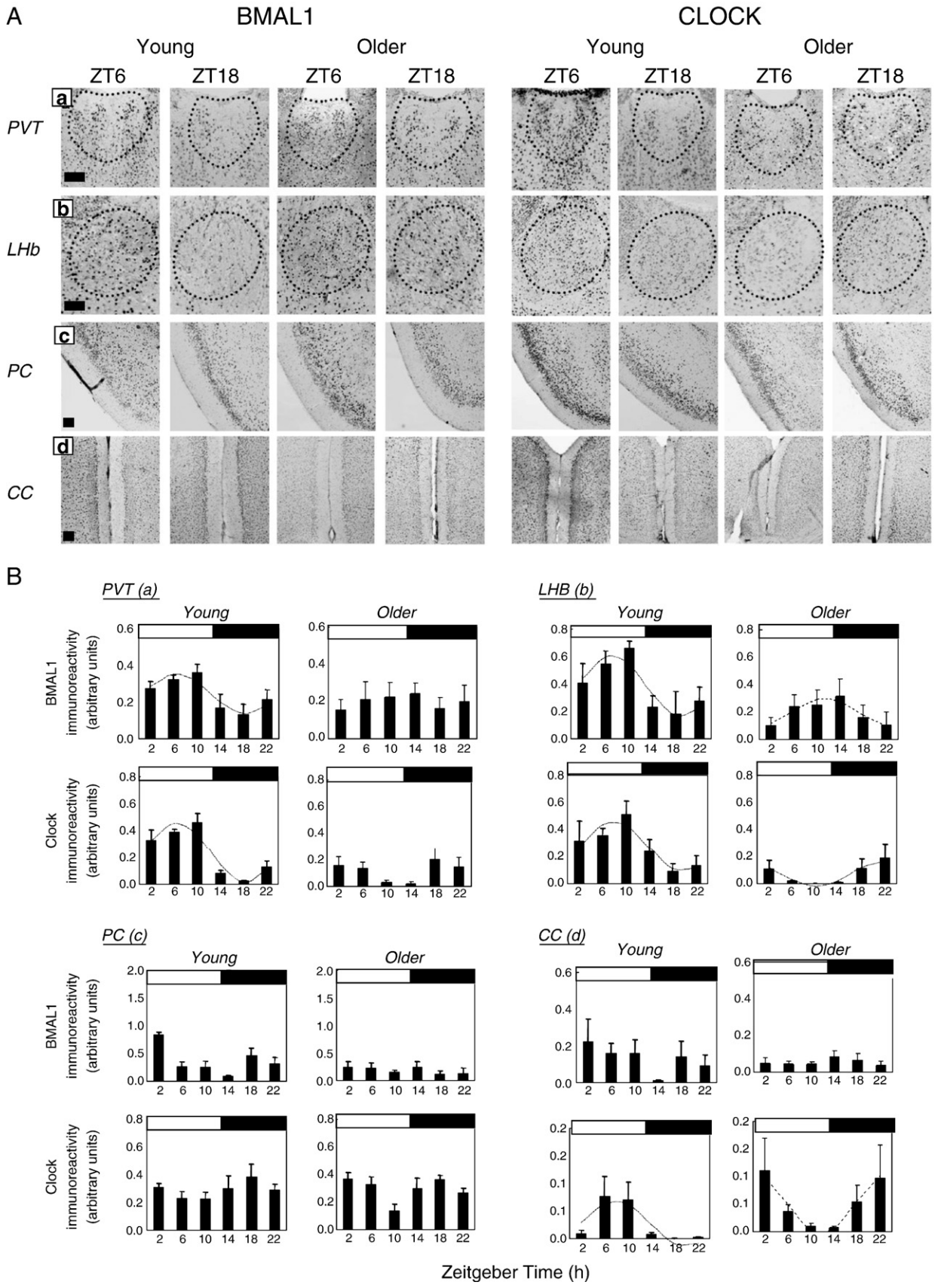
3.1. *Age-related alterations in circadian function*

A number of studies have addressed age-related changes in the SCN that may contribute to age-related circadian dysfunction. These studies have demonstrated, amongst other things, changes in electrophysiological properties of SCN neurons (Watanabe et al., 1995; Nygård et al., 2005; Aujard et al., 2001; Biello, 2009), changes in the clock gene *mper2* expression (Weinert et al., 2001), changes in neuropeptide content of the SCN (Hofman and Swaab, 2006; Kalló et al., 2004; Beynon et al., 2009), changes in the GABAergic network of the SCN (Palomba et al., 2008), changes in SCN responsiveness to melatonin (von Gall and Weaver, 2008) and changes in serotoninergic input to the SCN (Jagota and Kalyani, 2009). All of these lines of evidence suggest that SCN organisation and function is altered with aging, although it is not clear as to what gives rise to these changes (for example increased neuroinflammation in the aging brain may impact on SCN neurophysiology, Nygård et al., 2009).

A couple of previous studies have suggested that we need to look wider than the SCN in order to understand age-related circadian dysfunction. Yamazaki et al. (2002) report that in *per1*::luc rats aging is associated with modest changes in *per1* expression in the SCN, but is also associated with either loss of rhythmicity (lung and retrochiasmatic area) or phase-advanced rhythms (pineal, kidney). These results demonstrate some age-related changes in the intrinsic properties of these peripheral pacemakers. A further study by Davidson et al. (2008), again using *per1*::luc rats, showed that following a 6-h phase-shift of the light/dark cycle in either the advance or delay direction *per1* rhythms in the SCN re-entrained in 2-year-old rats similar to young animals, although re-entrainment of rhythms in the liver, arcuate nucleus and the pineal were altered with age. These results indicate the possibility that the communication from the SCN to peripheral oscillators is altered with age, or that the ability of extra-SCN clock to entrain to SCN-derived signals is altered in ageing. Further startling evidence of the global impact of circadian dysfunction in senescence was provided by the observation in this study, and a follow up study in mice (Davidson et al., 2006) that phase-advances of the light/dark cycle induced significant mortality in aged animals.

3.2. *Rhythmic clock gene expression in the brain*

We have examined the expression of two core clock gene protein products in brain areas that have been previously been associated with the circadian system (e.g. cortical and sub-cortical limbic areas, hypothalamic areas; Guilding and Piggins, 2007; Amir and Stewart, 2009; Saper et al., 2005) and areas that have not previously been described to be part of the circadian timekeeping systems (areas of the accessory olfactory system). A number of studies have demonstrated either rhythmic clock gene mRNA expression or rhythmic immediate early gene expression in a number of brain sites (reviewed in Guilding and Piggins, 2007), with a number of these studies using clock gene reporter systems to monitor *per1* or *per2* expression (e.g. Abe et al., 2002). However, no previous studies had comprehensively assessed rhythmic expression of the core clock gene products CLOCK and BMAL1 in brain areas outside of the SCN. We chose to examine BMAL1, as *bmal1* appears to a non-redundant requirement for circadian functioning (the *bmal1* knockout mouse is arrhythmic; Bunger et al., 2000) and *bmal1* knockouts also show accelerated aging, shortened lifespan and increased free-radical production (Kondratov et al., 2006). In most clock systems examined, CLOCK dimerises with BMAL1, and as such is a central component of the clock gene cycle, although its loss can be compensated for by NPAS2 in the SCN (DeBruyne et al., 2007). Previous studies of *clock* mRNA expression outside the SCN have shown rhythmic expression in the rat striatum (Cai et al., 2009), or no significant diurnal expression of *clock* mRNA in the nucleus accumbens, the hippocampus, the amygdala and ventral tegmental area, although *clock* expression in these regions was regulated by morphine withdrawal (Li et al., 2009). Sellix et al. (2006) describe no rhythmic expression of CLOCK in neuroendocrine dopaminergic neurons. Interestingly in the context of the current study, Karolczak et al. (2004) describe rhythmic expression of CLOCK, but not BMAL1, in the pineal glands of C57/Bl6 mice. This rhythm in CLOCK was found in


A
BMAL1
CLOCK
Young
Older
Young
Older
ZT6
ZT18
ZT6
ZT18
ZT6
ZT18
ZT6
ZT18
a
PVT
b
LHb
c
PC
d
CC
B
PVT (a)
LHB (b)
PC (c)
CC (d)
Young
Older
BMAL1 immunoreactivity (arbitrary units)
Clock immunoreactivity (arbitrary units)
Zeitgeber Time (h)

the absence of a rhythmic pattern of *clock* mRNA, whilst BMAL1 protein was constitutively expressed although *bmal1* mRNA oscillated. There is also a recent report of rhythmic expression of CLOCK and BMAL1 in the hippocampus of C3H mice (Jilg et al., 2010). Other paradigms in which regulation of CLOCK and/or BMAL1 (in the SCN) have been shown are caloric restriction (Mendoza et al., 2007) and interferon-γ treatment (Koyanagi and Ohdo, 2002).

With regards to the SCN, most studies have reported that *clock* mRNA and protein are constitutively expressed, whilst other core components of the clock gene cycle show significant circadian patterns of their expression in both mRNA and protein levels (Reppert and Weaver, 2002). *Bmal1,* which encodes the heterodimer partner of CLOCK protein, has been shown to express a circadian pattern of expression in its mRNA in the SCN (Maywood et al., 2003), but BMAL1 was found not to oscillate in the SCN (Von Gall et al., 2003). Thus, our findings of constitutive expression of CLOCK and BMAL1 in the SCN of young mice are in agreement with previous findings. Aging was not associated with marked changes in BMAL1 expression in the SCN, although we have shown that the older SCN exhibits a significant diurnal rhythm in CLOCK expression. This finding is of interest, as Kolker et al. (2003) reported an age-related decrease in *clock* expression in the hamster SCN. Given that previous studies have shown a disconnection between mRNA and protein levels (Reddy et al., 2006), it will be of interest in the future to probe alterations in the relationship between transcriptional and translational/post-translational regulation of core clock gene cycle components in the aged SCN. As the current study examined diurnal patterns of expression, the possibility that CLOCK and BMAL1 expression might reflect a masking effect of light must be considered, with future studies of free-running animals required to delineate these effects more clearly. Another important distinction to make in comparing the present data to previous studies using clock-gene driven reporter systems or electrophysiology monitored *in vitro* is the idea that some brain oscillators may be "slaves" to the SCN, or at best semi-autonomous, and so may display rhythms when assayed in the intact brain, but may not be rhythmic when examined *in vitro*. Conversely, one might also imagine a situation where some oscillatory sites may be under phasic/tonic inhibition *in vivo*, which is then relieved with *in vitro* preparations allowing for the expression of rhythmicity.

A striking finding of our study is the diurnal patterns of expression of CLOCK and BMAL1 in a number of regions outside the SCN, with a consistent acrophase in the mid light-phase in young adult animals. Of interest here is the report by Jilg et al. (2010) that demonstrated synchronous rhythmic expression of CLOCK and BMAL1 in the hippocampus. The amplitude of expression of CLOCK was reduced in the present study in older mice, but diurnal patterns of expression were retained in many regions, with the acrophase shifted to the late night phase. Likewise, regions that expressed diurnal patterns of BMAL1 expression in the young adult brain (CA1, CA3, CeA, PVT, LHb) either displayed altered phases or loss of rhythmicity in the older brain. Interestingly, the only 2 areas in young animals to show rhythmic expression of both BMAL1 and CLOCK, the LHb and the PVT, are areas that have previously been strongly implicated in the circadian system and have significant neuroanatomical connections with the SCN (Tavakoli-Nezhad and Schwartz, 2006; Moga and Moore, 1997; Feillet et al., 2008).

3.3. *Functions of rhythmic clock gene expression across the brain and significance of their alteration with age*

The functional significance of clock gene oscillations in regions outside the central clock is unknown, although there are some indications that clock genes play important region-specific roles. For example, knockout of *per2* results in attenuated hippocampal long-term potentiation (Wang et al., 2009) and impaired performance on memory tasks (Jilg et al., 2010), suggesting a role for circadian processes in memory formation and learning. Hypothalamic clock gene expression outside of the SCN may be of particular importance for energy balance (Kalsbeek et al., 2006), with the DMH being implicated in the food-entrainable oscillator (an area of some current controversy, Fuller et al., 2008; Mistlberger et al., 2008). A novel finding of the present data is the rhythmic expression of CLOCK in areas of the accessory olfactory system (BAOT and MeA). The accessory olfactory, or vomeronasal system, is a chemosensory system that evolved specifically for detection of pheromones, and mediation of the innate behavioural and neuroendocrine responses evoked by these olfactory signals. Future examination of interactions between the accessory olfactory and circadian systems should prove enlightening. Given the likelihood that various brain regions express components of the circadian clock for good physiological reasons, the perturbations of some of these circadian components demonstrated in the present study have the potential to contribute to age-related neuronal dysfunction in these areas and impaired overall brain function and behaviour in senescence.

4. Experimental procedures

4.1. *Animals*

Young adult male C57/Bl6 mice (Charles River UK; $n=36$; 4 months old) and older mice ($n=36$; 16 months old) were group housed, with *ad libitum* access to food and water, under a 12:12 light–dark cycle with lights on at 05:00 (defined as

Fig. 3 – Expression of BMAL1 and CLOCK in cortical and sub-cortical regions of the young adult and older mouse brain. (A) Photomicrographs of BMAL1 and CLOCK expression during the mid day (ZT6) and mid night (ZT18) phases in the (a) paraventricular nucleus of the thalamus (PVT), (b) the lateral habenula (LHb), (c) the piriform cortex (PC) and (d) the cingulate cortex (CC). Scale bars = 100 μm. (B) Diurnal expression patterns of BMAL1 and CLOCK immunoreactivity in the (a) PVT, (b) LHb, (c) PC and (d) CC. The presence of the dashed fitted line indicates a statistically significant cosinor fitted curve indicating rhythmic expression.

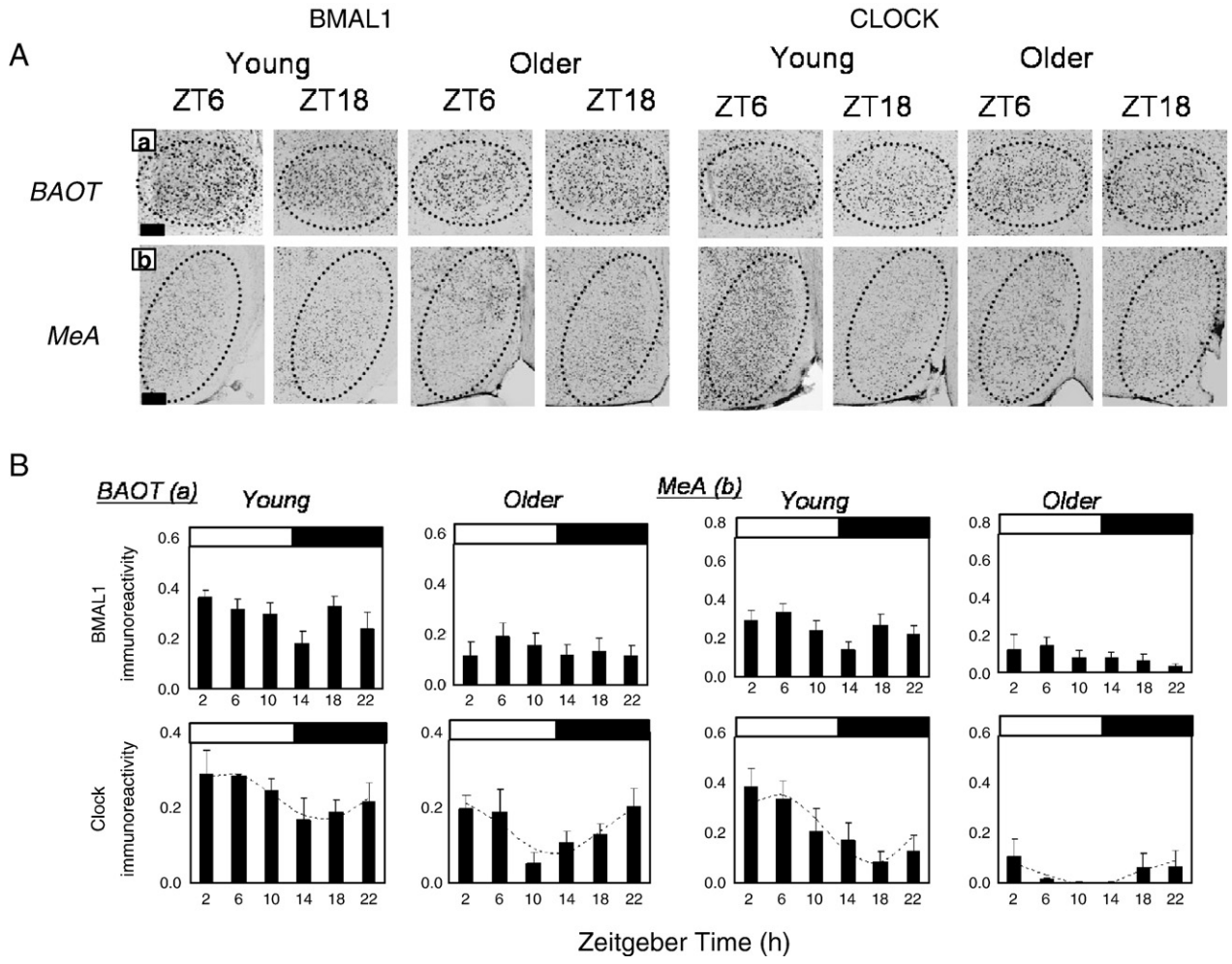


Fig. 4 – Expression of BMAL1 and CLOCK in regions of the accessory olfactory systems in young adult and older mouse brain. (A) Photomicrographs of BMAL1 and CLOCK expression during the mid day (ZT6) and mid night (ZT18) phases in the (a) bed nucleus of the accessory tract (BAOT) and (b) the medial amygdala (MeA). Scale bars = 100 μm. (B) Diurnal expression patterns of BMAL1 and CLOCK immunoreactivity in the (a)BAOT and (b) MeA. The presence of the dashed fitted line indicates a statistically significant cosinor fitted curve indicating rhythmic expression.

Zeitgeber Time (ZT) 0). The light source was standard fluorescent light bulbs, with illuminance levels of ~100 lux at cage level. These studies were carried out in accordance with the Animals (Scientific Procedures) Act, 1986, the European Communities Council Directive 86/609 and with the guidelines of the Research Ethics Committee, School of Medicine, Swansea University. All efforts were made to minimise the numbers of animals used and any suffering experienced.

4.2. *Immunohistochemistry*

Groups of animals ($n = 6$ for each age group per time point) were killed by CO_2 inhalation at ZT 2, 6, 10, 14, 18 and 22 and their brains fixed in 4% paraformaldehyde. Those animals sampled during the lights off phase were done so with the use of an infra-red viewer. Following fixation, antigen retrieval was performed by overnight incubation in 0.1 M citrate buffer (pH 4.5) followed by 2.5 min heating in a microwave oven at 700 W. All of the brains gathered were subsequently examined for immunohistochemical staining for CLOCK and BMAL1 and quantitatively analysed. The brains were transferred into cryoprotectant sucrose (30%) in 0.1 M phosphate buffer (pH 7.4) for a further 24 h, and then sectioned at 30 μm on a freezing microtome (Leica) with 4 serial sets of sections collected from each brain. Therefore sections were collected at a section interval of 120 μm for each serial set. Free floating sections were processed for BMAL1 and CLOCK immunohistochemistry, using a rabbit polyclonal antibody against BMAL1, raised against epitopes in the human sequences, (sc-48790); or for detection of CLOCK proteins, a goat polyclonal antibody (sc-6928) raised against an epitope of mouse origin (both Santa Cruz, USA). The use, and specificity, of these antibodies has previously been described in mouse and other rodents (e.g. anti CLOCK: Mendoza et al., 2007; Sellix et al., 2006; McClung et al., 2005; Koyanagi and Ohdo, 2002; anti-BMAL1: Herwig et al., 2007; Wood et al., 2006; Koyanagi and Ohdo, 2002). Primary antibodies were diluted at 1:500. Immunostaining was abolished by omission of the primary

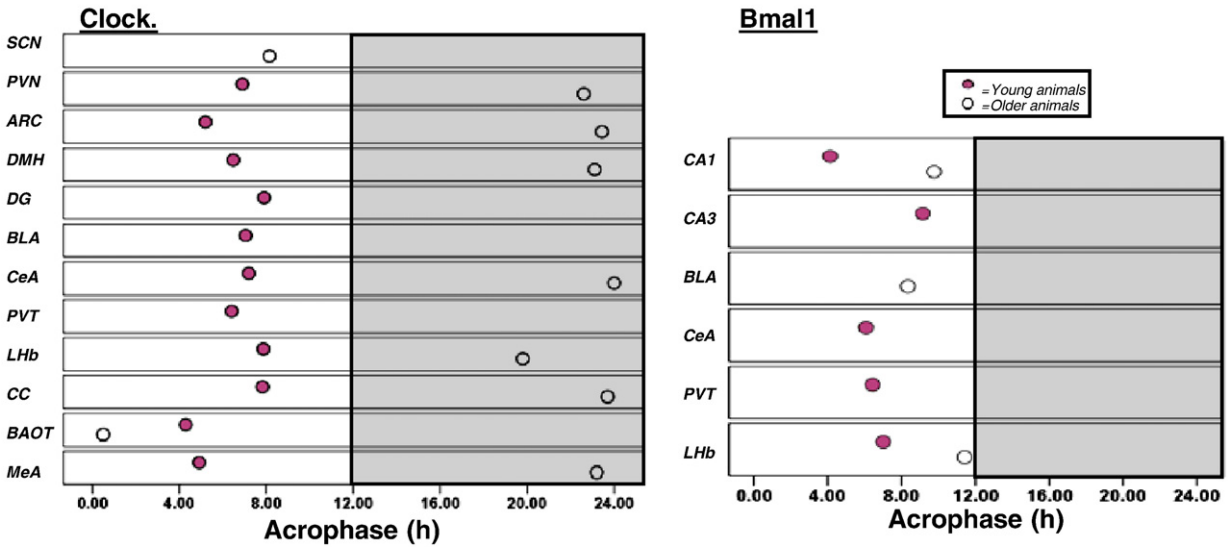


Fig. 5 – Scatterplot showing the distribution of acrophases of CLOCK and BMAL1 expression rhythms in young and old animals. Filled circles represent young animals, empty circles represent older animals. Acrophases are only indicated for expression patterns deemed to de significant via cosinor analysis, thus where in older animals expression becomes arrhythmic no point is indicated for that region. The shaded portion represents the night phase of the diurnal cycle.

antibodies, or by pre-incubation of the CLOCK primary antibody with its immunising peptide (this was not available for the BMAL1 antibody).

Immunoreactivity was visualised using avidin–biotin/peroxidase and nickel-enhanced diaminobenzidine chromogen (Vector Labs, UK) according to a standard protocol (Coogan and Piggins, 2005). All sections were treated in exactly the same manner at all stages of tissue processing to minimise any inter-assay variability. Sections were mounted on gelatin-coated slides, dehydrated and cover-slipped using mounting media (Entellan, Merck, UK). Control sections were treated in the same way but with the primary antibody omitted.

4.3. *Image analysis*

Slides were examined using an Olympus BX-51 light microscope attached to a CCD camera, and a PC. Between 3 and 6 images (depending on the rostro-caudal extent of the area being examined) were evaluated for each individual animal, region and timepoint. Background staining was subtracted from each image using image analysis software (Scion Image, Scion Corporation, USA), before thresholding to a predetermined optimal value that was specific for each antigen. Immunoreactive cells in each region of interest were quantified using image analysis software (Scion Image, Scion Corporation, USA) to count the number of particles stained above the threshold level, and normalising this value to the area of the region, in order to control for the variety of anatomical areas we examined (a modification of methods previously described (Coogan and Piggins, 2005)). Anatomical regions were defined by reference to the atlas of Paxinos and Franklin (2004). The entirety of each structure was analysed with the borders delineated according to the demarcations in Paxinos and Franklin (2004), referenced to the appropriate rostrocaudal level of the brain. Care was taken to ensure consistency between cases in the exact anatomical regions examined. The suprachiasmatic nuclei (SCN) were measured at the mid rostro-caudal level, including the core and shell regions of these structures. The oval nucleus of the bed nucleus of the stria terminalis (BNST-OV) was located in the dorsal aspect of the bed nucleus of the stria terminalis as defined in the Allen Brain Atlas (www.alleninstitute.org). Immunoreactivity was also measured in the bed nucleus of the accessory olfactory tract (BAOT), the hippocampus (CA1, CA3, dentate gyrus, DG), the cerebral cortex (cingulate, CC; piriform, PC), the lateral habenula (LHb), the paraventricular nucleus of the thalamus (PVT), the paraventricular nucleus of the hypothalamus (PVN), the dorsal medial nucleus of the hypothalamus (DMH), the arcuate nucleus (ARC), the basolateral (BLA) and the central (CeA) and medial (MeA) nuclei of the amygdala (Supplementary Fig. 2).

4.4. *Data analysis*

Mean immunoreactivity was calculated for each animal and at each region and timepoint. Data were found to conform to the parameters of the normal distribution and so 3-way analysis of variance (ANOVA) was conducted with the mean CLOCK/BMAL1 immunoreactivity per animal as the dependent variable, and Zeitgeber time, age and brain region as independent variables. Tukey HSD *post-hoc* testing was then applied to delineate the specific effects within any significant main effects (for example in which brain regions were mean levels of immunoreactivity altered with age).

Cosinor analysis was used to determine if significant diurnal patterns in protein expression were present. Non-linear regression analysis was used to predict a single cosinor

curve for each antigen at each region of the brain according to the equation:

$$Y_i = M + \beta Cos(2\pi t_i / 24) + \gamma Sin(2\pi t_i / 24) \quad (1)$$

Where Y is immunoreactivity (arbitrary units), t is Zeitgeber time, and M, β and γ were predicted by non linear regression, above. Diurnal patterns of expression were accepted when a variance test indicated that the amplitude of the fitted cosinor curve was not significantly different from zero. Where significant circadian periodicity was suggested, the acrophase (ϕ) and amplitude (A) was predicted using Eqs. (2) and (3), with β and γ predicted from Eq. (1) above.

$$\phi = tan^{-1}(-\gamma / \beta) \quad (2)$$

$$A = (\beta^2 + \gamma^2)^{1/2} \quad (3)$$

Data were expressed as the mean ± standard error of the mean, statistical significance was accepted at $p < 0.05$, and all analyses were performed using Microsoft Excel software.

Acknowledgments

We acknowledge the help and assistance of Professor David Carter of Cardiff University, UK. We also acknowledge financial support from Research into Ageing.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2010.03.113.

REFERENCES

Abe, M., Herzog, E.D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., Block, G.D., 2002. Circadian rhythms in isolated brain regions. J. Neurosci. 22, 350–356.

Amir, S., Stewart, J., 2009. Behavioral and hormonal regulation of expression of the clock protein, PER2, in the central extended amygdala. Prog. Neuropsychopharmacol. Biol. Psychiatry (Apr 17 Electronic publication ahead of print).

Aujard, F., Herzog, E.D., Block, G.D., 2001. Circadian rhythms in firing rate of individual suprachiasmatic nucleus neurons from adult and middle-aged mice. Neuroscience 106, 255–261.

Benloucif, S., Green, K., L'Hermite-Balériaux, M., Weintraub, S., Wolfe, L.F., Zee, P.C., 2006. Responsiveness of the aging circadian clock to light. Neurobiol. Aging 27, 1870–1879.

Beynon, A.L., Thome, J., Coogan, A.N., 2009. Age and time of day influences on transforming growth factor-beta and phosphorylated SMAD3 in the mouse suprachiasmatic and paraventricular nuclei. Neuroimmunomodulation 19, 392–399.

Biello, S.M., 2009. Circadian clock resetting in the mouse changes with age. Age (Dordr) (Jun 26. Electronic publication ahead of print).

Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S., Bradfield, C.A., 2000. Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103, 1009–1017.

Cai, Y., Liu, S., Li, N., Xu, S., Zhang, Y., Chan, P., 2009. Postnatal ontogenesis of molecular clock in mouse striatum. Brain Res. 1264, 33–38.

Coogan, A.N., Piggins, H.D., 2005. Dark pulse suppression of P-ERK and c-Fos in the hamster suprachiasmatic nuclei. Eur. J. NeuroSci. 22, 158–168.

Davidson, A.J., Sellix, M.T., Daniel, J., Yamazaki, S., Block, G.D., 2006. Chronic jetlag increases mortality in aged mice. Curr. Biol. 16, R914–R916.

Davidson, A.J., Yamazaki, S., Arble, D.M., Menaker, M., Block, G.D., 2008. Resetting of central and peripheral circadian oscillators in aged rats. Neurobiol. Aging 29, 471–477.

DeBruyne, J.P., Weaver, D.R., Reppert, S.M., 2007. CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. Nat. Neurosci. 10, 543–545.

Duffy, J.F., Zeitzer, J.M., Rimmer, D.W., Klerman, E.B., Dijk, D.J., Czeisler, C.A., 2002. Peak of circadian melatonin rhythm occurs later within the sleep of older subjects. Am. J. Physiol. Endocrinol. Metab. 282, E297–E303.

Feillet, C.A., Mendoza, J., Albrecht, U., Pévet, P., Challet, E., 2008. Forebrain oscillators ticking with different clock hands. Mol. Cell. Neurosci. 37, 209–221.

Fuller, P.M., Lu, J., Saper, C.B., 2008. Differential rescue of light- and food-entrainable circadian rhythms. Science 320, 1074–1077.

Guilding, C., Piggins, H., 2007. Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain. Eur. J. NeuroSci. 25, 3195–3216.

Herwig, A., Saboureau, M., Pevet, P., Steinlechner, S., 2007. Daily torpor affects the molecular machinery of the circadian clock in Djungarian hamsters (*Phodopus sungorus*). Eur. J. NeuroSci. 2007 (26), 2739–2746.

Hofman, M.A., Swaab, D.F., 2006. Living by the clock: the circadian pacemaker in older people. Ageing Res. Rev. 5, 33–51.

Jagota, A., Kalyani, D., 2009. Effect of melatonin on age induced changes in daily serotonin rhythms in suprachiasmatic nucleus of male Wistar rat. Biogerontology (Sep 23. Electronic publication ahead of print).

Jilg, A., Lesny, S., Peruzki, N., Schwegler, H., Selbach, O., Dehghani, F., Stehle, J.H., 2010. Temporal dynamics of mouse hippocampal clock gene expression support memory processing. Hippocampus 20, 377–388.

Kalló, I., Kalamatianos, T., Piggins, H.D., Coen, C.W., 2004. Ageing and the diurnal expression of mRNAs for vasoactive intestinal peptide and for the VPAC2 and PAC1 receptors in the suprachiasmatic nucleus of male rats. J. Neuroendocrinol. 16, 758–766.

Kalsbeek, A., Palm, I.F., La Fleur, S.E., Scheer, F.A., Perreau-Lenz, S., Ruiter, M., Kreier, F., Cailotto, C., Buijs, R.M., 2006. SCN outputs and the hypothalamic balance of life. J. Biol. Rhythms 21, 458–469.

Karolczak, M., Burbach, G.J., Sties, G., Korf, H.W., Stehle, J.H., 2004. Clock gene mRNA and protein rhythms in the pineal gland of mice. Eur. J. NeuroSci. 19, 3382–3388.

Kolker, D.E., Fukuyama, H., Huang, D.S., Takahashi, J.S., Horton, T. H., Turek, F.W., 2003. Aging alters circadian and light-induced expression of clock genes in golden hamsters. J. Biol. Rhythms 18, 159–169.

Kondratov, R.V., Kondratova, A.A., Gorbacheva, V.Y., Vykhovanets, O.V., Antoch, M., 2006. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. Genes Dev. 20, 1868–1873.

Koyanagi, S., Ohdo, S., 2002. Alteration of intrinsic biological rhythms during interferon treatment and its possible mechanism. Mol. Pharmacol. 62, 1393–1399.

Li, S.X., Liu, L.J., Jiang, W.G., Lu, L., 2009. Morphine withdrawal produces circadian rhythm alterations of Clock genes in mesolimbic brain areas and peripheral blood monouclear cells in rats. J. Neurochem. (Apr 4. Electronic publication ahead of print).

Maywood, E.S., O'Brien, J.A., Hastings, M.H., 2003. Expression of mCLOCK and other circadian clock-relevant proteins in the

mouse suprachiasmatic nuclei. J. Neuroendocrinol. 15, 329–334.
McClung, C.A., Sidiropoulou, K., Vitaterna, M., Takahashi, J.S., White, F.J., Cooper, D.C., Nestler, E.J., 2005. Regulation of dopaminergic transmission and cocaine reward by the clock gene. Proc. Natl. Acad. Sci. U. S. A. 102, 9377–9381.
McDearmon, E.L., Patel, K.N., Ko, C.H., Walisser, J.A., Schook, A.C., Chong, J.L., Wilsbacher, L.D., Song, E.J., Hong, H.K., Bradfield, C. A., Takahashi, J.S., 2006. Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice. Science 314, 1304–1308.
Mendoza, J., Pevet, P., Challet, E., 2007. Circadian and photic regulation of clock and clock-controlled proteins in the suprachiasmatic nuclei of calorie-restricted mice. Eur. J. NeuroSci. 25, 3691–3701.
Mistlberger, R.E., Yamazaki, S., Pendergast, J.S., Landry, G.J., Takumi, T., Nakamura, W., 2008. Comment on "Differential rescue of light- and food-entrainable circadian rhythms". Science 322, 675.
Moga, M.M., Moore, R.Y., 1997. Organization of neural inputs to the suprachiasmatic nucleus in the rat. J. Comp. Neurol. 389, 508–534.
Nygård, M., Hill, R.H., Wikström, M.A., Kristensson, K., 2005. Age-related changes in electrophysiological properties of the mouse suprachiasmatic nucleus in vitro. Brain Res. Bull. 65, 149–154.
Nygård, M., Lundkvist, G.B., Hill, R.H., Kristensson, K., 2009. Rapid nitric oxide-dependent effects of tumor necrosis factor-alpha on suprachiasmatic nuclei neuronal activity. NeuroReport 20, 213–217.
Palomba, M., Nygård, M., Florenzano, F., Bertini, G., Kristensson, K., Bentivoglio, M., 2008. Decline of the presynaptic network, including GABAergic terminals, in the aging suprachiasmatic nucleus of the mouse. J. Biol. Rhythms 23, 220–231.
Paxinos, G., Franklin, K.J., 2004. The mouse brain in stereotaxic coordinates. Elsevier Academic Press, USA.
Reddy, A.B., Karp, N.A., Maywood, E.S., Sage, E.A., Deery, M., O'Neill, J.S., Wong, G.K., Chesham, J., Odell, M., Lilley, K.S., Kyriacou, C.P., Hastings, M.H., 2006. Circadian orchestration of the hepatic proteome. Curr. Biol. 16, 1107–1115.
Reppert, S.M., Weaver, D., 2002. Co-ordination of circadian timekeeping in mammals. Nature 418, 935–941.
Roybal, K., Theobold, D., Graham, A., DiNieri, J.A., Russo, S.J., Krishnan, V., Chakravarty, S., Peevey, J., Oehrlein, N., Birnbaum, S., Vitaterna, M.H., Orsulak, P., Takahashi, J.S., Nestler, E.J., Carlezon, W.A. Jr, McClung, C.A., 2007. Mania-like behavior induced by disruption of CLOCK. Proc. Natl. Acad. Sci. 104, 6406–6411.
Saper, C.B., Lu, J., Chou, T.C., Gooley, J., 2005. The hypothalamic integrator for circadian rhythms. Trends Neurosci. 28, 152–157.
Schibler, U., 2009. The 2008 Pittendrigh/Aschoff lecture: peripheral phase coordination in the mammalian circadian timing system. J. Biol. Rhythms 24, 3–15.
Sellix, M.T., Egli, M., Poletini, M.O., McKee, D.T., Bosworth, M.D., Fitch, C.A., Freeman, M.E., 2006. Anatomical and functional characterization of clock gene expression in neuroendocrine dopaminergic neurons. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290, R1309–R1323.
Tavakoli-Nezhad, M., Schwartz, W.J., 2006. Hamsters running on time: is the lateral habenula a part of the clock? Chronobiol. Int. 23, 217–222.
von Gall, C., Weaver, D.R., 2008. Loss of responsiveness to melatonin in the aging mouse suprachiasmatic nucleus. Neurobiol. Aging 29, 464–470.
von Gall, C., Noton, E., Lee, C., Weaver, D.R., 2003. Light does not degrade the constitutively expressed BMAL1 protein in the mouse suprachiasmatic nucleus. Eur. J. NeuroSci. 18, 125–133.
Wang, L.M., Dragich, J.M., Kudo, T., Odom, I.H., Welsh, D.K., O'Dell, T.J., Colwell, C.S., 2009. Expression of the circadian clock gene Period2 in the hippocampus: possible implications for synaptic plasticity and learned behaviour. ASN Neuro. 1.
Watanabe, A., Shibata, S., Watanabe, S., 1995. Circadian rhythm of spontaneous neuronal activity in the suprachiasmatic nucleus of old hamster in vitro. Brain Res. 695, 237–239.
Weinert, D., 2000. Age-dependent changes of the circadian system. Chronobiol. Int. 17 (3), 261–283.
Weinert, H., Weinert, D., Schurov, I., Maywood, E.S., Hastings, M.H., 2001. Impaired expression of the mPer2 circadian clock gene in the suprachiasmatic nuclei of aging mice. Chronobiol. Int. 18, 559–565.
Wood, P.A., Du-Quiton, J., You, S., Hrushesky, W.J., 2006. Circadian clock coordinates cancer cell cycle progression, thymidylate synthase, and 5-fluorouracil therapeutic index. Mol. Cancer Ther. 5, 2023–2033.
Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., Block, G. D., 2002. Effects of aging on central and peripheral mammalian clocks. Proc. Natl. Acad. Sci. U. S. A. 99, 10801–10806.
Yoon, I.Y., Kripke, D.F., Elliott, J.A., Youngstedt, S.D., Rex, K.M., Hauger, R.L., 2003. Age-related changes of circadian rhythms and sleep–wake cycles. J. Am. Geriatr. Soc. 51, 1085–1091.