Research Report

Daily methylphenidate and atomoxetine treatment impacts on clock gene protein expression in the mouse brain

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Abstract

Circadian rhythms are repeating patterns of physiological and other parameters that recur with periods of approximately 24 h, and are generated by an endogenous circadian timekeeping mechanism. Such circadian rhythms, and their underlying molecular mechanisms, are known to be altered by a number of central nervous system acting pharmacological compounds, as well as becoming perturbed in a number of common psychiatric and neurological conditions. The psychostimulant methylphenidate and the non-stimulant atomoxetine are used in the pharmacotherapy of attention deficit hyperactivity disorder, a common condition in which circadian rhythms have been reported to be altered. In the present study we have examined the effects of daily methylphenidate or atomoxetine treatment across 7 days on circadian clock gene product expression across numerous brain regions in the male mouse to test the potential impact of such compounds on circadian timing. We report drug, brain region and molecular specific effects of such treatments, including alterations in expression profiles in the suprachiasmatic nucleus, the master circadian pacemaker. These results indicate that drugs used in the clinical management of attention deficit hyperactivity disorder can alter molecular factors that are believed to underpin circadian timekeeping, and such effects may be of importance in both the therapeutic and side effect profiles of such drugs.

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Abbreviations: AcbC, core of the nucleus accumbens; AcbSh, shell of the nucleus accumbens; ADHD, attention deficit hyperactivity disorder; ATO, atomoxetine; BLA, basolateral amygdala; CC, cingulate cortex; CEA, central nucleus of the amygdala; CPu, caudate putamen; DAT, dopamine transporter; DG, dentate gyrus; DMH, dorsomedial nucleus of the hypothalamus; ILC, infralimbic cortex; MPD, methylphenidate; NET, noradrenaline transporter; PER, PERIOD; PLC, prelimbic cortex; PVN, paraventricular nucleus of the hypothalamus; SAL, saline; SCN, suprachiasmatic nucleus; ZT, zeitgeber time

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Introduction

Attention deficit-hyperactivity disorder (ADHD) is a common psychiatric condition of both childhood and adulthood, characterised by the core symptoms of impulsivity, inattention and hyperactivity (Coogan et al., 2012). The psychostimulant methylphenidate (MPD) and the non-stimulant atomoxetine (ATO) are used for the management of attention deficit hyperactivity disorder (ADHD) in both children and adults (Biederman and Faraone, 2005). Both drugs exert their therapeutic action through manipulation of the catecholaminergic systems, with MPD increasing the synaptic concentration of both dopamine and noradrenaline, though inhibition of the dopamine transporter (DAT) and the norepinephrine transporter (NET), whilst ATO is an inhibitor of the NET and increases synaptic noradrenaline levels (Madras et al., 2005).

Sleep deficits are commonly observed in ADHD (Sobanski et al., 2008), and both beneficial and adverse effects of MPD and ATO on various aspects of sleep have been documented in ADHD (Boonstra et al., 2007). Circadian disturbances in adult ADHD at the behavioural, molecular and endocrine levels have also been shown (Baird et al., 2011) as well as genetic associations between ADHD and clock gene polymorphisms (Kissling et al., 2008). The circadian clock is responsible for the generation of circadian rhythms, which are recurring patterns of behaviour and physiology on a near twenty-four period base and plays a key role in determining the sleep/wake cycle (Dibner et al., 2010; Reppert and Weaver, 2002). The master circadian clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus with other oscillators present throughout the brain and periphery (Guilding and Piggins, 2007). The molecular basis of circadian rhythm generation consists of positive and negative transcriptional/translational feedback loops of “clock” genes and their protein products (Dibner et al., 2010).

Numerous pharmacological agents are known to impact upon the molecular circadian clock (e.g. Uz et al., 2005) and the dopaminergic and noradrenergic systems are implicated in circadian clock functioning (McClung et al., 2005; Wongchitrat et al., 2009). MPD and ATO have both also been shown to exert effects upon the mammalian circadian system (chronic MPD treatment produces a circadian locomotor rhythm in arrhythmic SCN-lesioned rats (Honma and Honma, 1992)) as well as on rodent diurnal rhythms (Algahim et al., 2009, 2010), whilst time-of-day effects on behavioural sensitization to MPD have also been reported (Gaytan et al., 2000).

Recently it has been shown that ongoing MPD treatment of mice via drinking water produces phase delays and lengthened free running rhythms and also alters electrical discharge rhythms of SCN neurons (Antle et al., 2012). Further, acute ATO treatment has been shown to phase-shift the rodent locomotor rhythm and to alter circadian clock gene product expression (O’Keeffe et al., 2012).

Given the deficits of the circadian clock in ADHD and the interactions of ADHD medication with sleep, it could be postulated that the therapeutic properties and/or the adverse side effects of these drugs in part could involve modulation of the circadian clock. Our hypothesis for the current study was that in the light of data concerning phase-shifting effects of both MPD and ATO in rodents and the key role of the

A  
PER1  
ZT2  
Sal  
MPD  
ATO  

PER2  
ZT14  
Sal  
MPD  
ATO  

B  

Fig. 1 – Modulation of clock gene products and c-Fos in the SCN by methylphenidate and atomoxetine. A: Photomicrographs illustrating the expression of PER1, PER2, CLOCK, and c-Fos in the SCN. The SCN is delineated by the dashed line and the scale bar is 100 μm. B: Expression profiles of CLOCK, PER1, PER2 and c-Fos in the SCN in the saline (SAL), methylphenidate (MPD) and atomoxetine (ATO) groups. * represents \( P < 0.05 \) for pairwise comparison between the value in the appropriate treatment group at that time-point compared to the value in the saline control group at the same time-point. The arrow represents the time of the daily injection.
circadian clock gene cycle in determining behavioural rhythms, both ATO and MPD would produce altered diurnal expression profiles of clock gene product expression in one or more brain areas implicated in circadian timing. As such, this study represents the first analysis of the effects of MPD and ATO on rhythmic clock gene product expression.

Table 1 – Summary of the acrophase (peaks of expression) of clock gene products and c-Fos across the brain areas examined in the study in the three treatment groups as indicated by co-sinor analysis with a 24 h sinusoidal wave. n.s. indicates that no significant 24 h fit for expression was detected by co-sinor analysis.

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2. Results

2.1. Diurnal clock protein expression in the hypothalamus

In the SCN, rhythmic PER1 expression was unaltered by either MPD or ATO treatment (Fig. 1). There was a significant main effect of treatment on PER2 expression ($F_{2,70} = 34.9, P < 0.05$) and a significant interaction between time and treatment ($F_{6,70} = 5.6, P < 0.05$) and post hoc testing showed that both MPD and ATO treatment reduced SCN PER2 expression ($P < 0.001$; Fig. 1). SCN CLOCK expression was arrhythmic and unaltered by methylphenidate or atomoxetine, nor was there any effect of treatment on SCN c-Fos expression. Co-sinor analysis reveals that for PER2 in the SCN the MPD treated animals show an acrophase at ZT2 (compared to ZT14 in saline animals) and ATO treated animals do not show rhythmic PER2 expression (Table 1).

In the dorsomedial hypothalamus (DMH), there were no main effects of treatment nor significant time x treatment interactions for PER1, PER2 or CLOCK expression (Fig. 2A). c-Fos expression was altered by treatment ($F_{2,70} = 6.2, P < 0.05$) and there was also a time x treatment interaction ($F_{6,70} = 3.9, P < 0.05$), with c-Fos levels in the MPD treated animals significantly higher at ZT2 than in saline controls (Fig. 2A). Co-sinor analysis shows that for CLOCK, PER1 and c-Fos the peak of the expression is altered to occur earlier in the lights on phase in both MPD and ATO treated animals (e.g. to ZT2 from ZT8 for CLOCK and PER1 expression; Table 1).

There was a significant main effect of treatment on PER1 expression in the paraventricular nucleus of the hypothalamus (PVN; $F_{2,70} = 24.3, P < 0.05$; Fig. 2B). Post hoc testing revealed a general upregulation of PVN PER1 in MPD treated animals and a downregulation in ATO treated ones ($P < 0.001$). There was also a significant main effect of treatment on PVN PER2 expression ($F_{2,70} = 161.4, P < 0.001$) and post-hoc testing demonstrated a reduction in PER2 expression by MPD and ATO (both $P < 0.001$). CLOCK was not altered by treatment in the PVN, but there was a significant time x treatment interaction for c-Fos expression ($F_{6,70} = 4.1, P < 0.01$). Co-sinor analysis (Table 1) indicates changes in the time of peak expression or loss of significant 24 h fit for the ATO and MPD treated groups for PER1, PER2, CLOCK and c-Fos expression.

2.2. Diurnal clock protein expression in the limbic forebrain and cerebral cortex

In the hippocampus there were no significant treatment effects or time x treatment interactions in the CA1, CA3 or dentate gyrus (DG) for PER1, PER2 and CLOCK (Fig. 3). There were main effects on c-Fos expression in the CA1 ($F_{2,70} = 35.4, \text{mod} P < 0.05$), CA3 ($F_{2,70} = 34.3, \text{mod} P < 0.05$) and DG ($F_{2,70} = 23.8, \text{mod} P < 0.05$). Post-hoc testing revealed that the MPD treated group showed elevated hippocampal c-Fos (most notably at ZT2, 24 h after the last treatment), whilst there were no significant differences between the ATO and the saline groups (Fig. 3).

Similar to the hippocampus, there were no significant effects of treatment or treatment x time interactions for PER1, PER2 or CLOCK in either the basolateral amygdala (BLA) or the central nucleus of the amygdala (CEA; Supplementary Fig. 1). There was a significant main effect of treatment on c-Fos expression in the BLA ($F_{2,70} = 12.9, P < 0.05$) and in the CEA ($F_{2,70} = 8.2, P < 0.05$). In both CEA and

Fig. 2 – Modulation of clock gene products and c-Fos in the DMH and PVN by methylphenidate and atomoxetine. Expression profiles of PER1, PER2, CLOCK and c-Fos in the dorsomedial nucleus of the hypothalamus (DMH; A) and the paraventricular nucleus (PVN, B) in the three treatment groups. * represents $P < 0.05$ for pairwise comparison between the value in the appropriate treatment group at that time-point compared to the value in the saline control group at the same time-point. The arrow represents the time of the daily injection.
BLA the MPD treated group shows significant elevation of c-Fos expression at ZT2, similar to the effect observed in the hippocampus.

In the cortical areas examined, again there were no effects of treatment on PER1, PER2 or CLOCK expression (Supplementary Fig. 2). There were significant main effects of treatment on c-Fos expression in the PLC, ILC and CC ($F_{2,70} = 18.9, P < 0.05; F_{2,70} = 19.0, P < 0.05; F_{2,70} = 17.6, P < 0.001$ respectively). Post-hoc analysis revealed that MPD increased c-Fos expression in all three regions of the cerebral cortex in comparison to the saline controls ($P < 0.001$).

2.3. Diurnal clock protein expression in the striatum and ventral tegmental area

There was a main effect of treatment on PER2 expression in the caudate putamen (CPu; $F_{2,70} = 31.5, P < 0.05$) and post-hoc analysis demonstrated that both ATO and MPD significantly increased PER2 expression (Fig. 4A). There were no significant effects of treatment on PER1 or CLOCK in the CPu, although there was a significant effect of treatment on c-Fos expression ($F_{2,70} = 47.7, P < 0.05$) with both MPD and ATO treatment groups showing elevation of c-Fos expression. From the Cosinor analysis the timing of peak expression of CLOCK in the CPu was earlier in both the MPD and ATO groups than the saline controls, whilst the timing of the PER2 was later in the MPD group than the saline controls (Table 1).

There was a main effect of treatment on PER1 expression in the ventral tegmental area (VTA; $F_{2,70} = 18.1, P < 0.05$), with post-hoc analysis indicating elevation of PER1 expression in the ATO group (Fig. 4B). There was no significant effect of treatment on PER2 or CLOCK expression in the VTA. There was a main effect of treatment on c-Fos in the VTA ($F_{2,70} = 25.8, P < 0.001$). Post-hoc analysis showed this to be a significant increase in c-Fos in the MPD treatment group (Fig. 4B).
In both the core and the shell of the nucleus accumbens there were no significant effects of treatment on PER1, PER2 or CLOCK expression (Supplementary Fig. 3). Similar to the hippocampus, amygdala and cortical areas, where there was also no effects on clock gene product expression, the only effects of treatment were observed on c-Fos expression in both the AcbC and AcbSh (F2,70 = 7.0, P < 0.05 and F2,70 = 11.2, P < 0.05 respectively) with MPD inducing higher levels of expression (Supplementary Fig. 3A).

3. Discussion

In this study we have shown that daily administration of both MPD and ATO exerts effects on diurnal patterns of expression of clock gene proteins and the immediate early gene c-Fos throughout the mouse brain. These data complement recent data from behavioural analysis that demonstrate that MPD and ATO cause changes in both circadian and diurnal rhythms in rodents (Algahim et al., 2009; O’Keeffe et al., 2012; Antle et al., 2012). The modulation of daily clock gene profiles examined in the current study might provide a molecular correlate of the behavioural effects previously described. The present results, taken in the context of previous work, further appears to strengthen the contention that commonly used ADHD medications can impact on the circadian system and such effects may be of importance in both the therapeutic and the side effect profile of these agents.

3.1. Methylphenidate and atomoxetine effects in the hypothalamus.

The effects of both MPD and ATO on PER2 expression in the SCN suggests that they modulate the master circadian clock’s function as PER2 is a core component of the molecular clockworks. An effect on the SCN clock gene cycle would be in keeping with some previous reports: daily methylphenidate treatment leads to the development of anticipatory locomotor rhythm in the rat (Algahim et al., 2009,2010), a finding that is congruent with our finding that daily MPD alters the acrophase of the PER2 SCN rhythm. Our present findings also appear to be consistent with the findings of Antle et al. (2012) who reported that methylphenidate treatment induces phase delays under a light/dark cycle and induces lengthening of the free-running period in mice, as well as delaying the peak in maximal SCN electrical activity. It is not immediately apparent from the current study why PER1 and PER2 expression are differentially regulated by both methylphenidate and atomoxetine in the SCN, although such differences in regulation have been described in a number of previous studies and may be due to regulation by different intracellular mechanisms (Coogan et al., 2011; Bendova and Sumova, 2006). There may also be a differential response to MPD or ATO in cells expressing PER2 and not PER1, but previous evidence has suggested that cells expressing PER1 also express PER2 (LeSauter et al., 2003). Effects of acute ATO on SCN CLOCK and c-Fos expression have been reported (O’Keeffe et al., 2012), although we did not observe any significant changes in CLOCK expression in the current study.
Dopamine has been implicated in SCN function and the wider circadian system, including in the entrainment of the master pacemaker and in the regulation of light input to the retina (Mcllglung et al., 2005; Witkovsky, 2004). Further dopaminergic agents have previously been shown to regulate clock gene expression in a number of studies (Manev and Uz, 2006). There has been less research on the roles of noradrenaline in circadian processes, although there is a circadian rhythm of noradrenaline release in the SCN independently of light input (Cagampang et al., 1994). It is therefore possible that since MPD is an inhibitor of both dopamine and noradrenaline re-uptake by DAT and NET (Madras et al., 2005), and ATO selectively inhibits NET mediated noradrenaline removal (Bymaster et al., 2002), the subsequent increases in synaptic dopamine and noradrenaline could impact upon such aspects of the circadian system. Moreover, since the SCN is the site of the master circadian pacemaker, and PER2 is a core component of the molecular mechanism that drives circadian rhythms (Dibner et al., 2010), any alteration in its rhythm could have knock on effects upon not only the other core clock genes and their protein products but also the expression of clock controlled genes and downstream physiological and behavioural processes.

The regulation of clock gene protein products by MPD and ATO was also region specific. For example, in the hypothalamus, differential effects were observed between the SCN, PVN and DMH. There appears to be region, drug and protein specificity in the effects observed, and given reciprocal connections between these regions it is not possible to ascribe order effects to the results observed (e.g. are the effects seen in the PVN and DMH just secondary to actions on the SCN?). Both the DMH and PVN have been strongly implicated in circadian function and serve as important efferent regions of the SCN. As many of these processes are dysregulated in ADHD (e.g. Baird et al., 2011), and are also known to be regulated by both MPD and ATO (Boonstra et al., 2007; Sofuoglu et al., 2009), it appears plausible that MPD and ATO action on circadian processes in the DMH and PVN may be of consequence in such physiological events.

### 3.2. Methylphenidate and atomoxetine effects in the limbic forebrain and cerebral cortex

PER2, CLOCK and c-Fos were not rhythmically expressed in all the hippocampal and amygdala regions examined of the saline treated mice, although studies have reported some of these proteins to be rhythmic in these regions (Wyse and Coogan, 2010; Feillet et al., 2008; Lamont et al., 2005). The analysis of clock protein expression at four six hourly time-points across the cycle may not provide sufficient temporal resolution to detect significant rhythmicity in these areas. Another possible explanation is the effects of daily saline injections in the control group compared to expression patterns in treatment naive animals reported in other studies. MPD treatment resulted in the loss of diurnal variation in PER1 expression in the CA1 and CA3 regions, an effect also observed on per gene expression in the hippocampus of rats undergoing withdrawal after chronic morphine treatment (Li et al., 2009). MPD also caused an increase in neuronal activation throughout the limbic forebrain as indicated by an increase in c-Fos expression, whereas this effect was only observed in DG after atomoxetine treatment. These data indicate that areas that show neuronal responses to ADHD medication do not necessarily then also show alterations in clock gene product expression. Alterations of circadian processes in both the hippocampus and amygdala have been associated with altered cognition and emotional regulation (Gerstner and Yin, 2010; Pantazopoulos et al., 2011), processes that are of obvious importance in the pharmacological management of ADHD by MPD or ATO.

In the cerebral cortical areas examined, MPD did not impact upon circadian clock protein expression whilst ATO altered the timing of the CLOCK expression profile. MPD treatment did however increase c-Fos expression in all three regions of the cerebral cortex examined, thus demonstrating again that whilst MPD does not affect c-Fos expression in the cerebral cortex it does cause a widespread increase in neuronal activation throughout the region. A key factor in the greater effects of ATO upon cerebral cortex circadian clock protein expression observed could be an increased abundance of NET compared to DAT, which has been observed in the prefrontal cortex (Koda et al., 2010). Furthermore, ATO has been shown to also increase dopamine levels also in the prefrontal cortex, due to the non-selective uptake of dopamine by NET, which is blocked by ATO (Koda et al., 2010; Bymaster et al., 2002). The frontal cortex is known to be involved in working memory, attention, impulse control and other executive functions (Neelstler and Carlezon, 2006) and actions of methylphenidate or atomoxetine on circadian processes in cortical areas may be of particular importance in addressing core symptoms of ADHD.

### 3.3. Methylphenidate and atomoxetine effects in the striatum and ventral tegmental area

MPD exerted a more widespread effect upon striatal protein expression than ATO, as might be expected given its pharmacological mode of action. MPD also increased c-Fos expression in the CPu and AcbSh, which is in agreement with the previous reports of the effects of MPD on c-fos gene expression in these areas (Steiner et al., 2010). ATO does not increase extracellular noradrenaline levels in the striatum (Koda et al., 2010) due to a lack of NET as demonstrated by minimal immunoreactivity fibres (Schroeter et al., 2000) and this could therefore underlie the lesser effects of ATO upon striatal circadian clock protein expression in comparison to methylphenidate. In the VTA both MPD and ATO treatments altered PER1 expression whilst MPD increased the degree of neuronal activation as judged by c-Fos staining. The modulation of circadian processes in the mesostriatal pathway by MPD and/or ATO could exert effects on cognitive and behavioural processes implicated in the actions of these drugs in ADHD management.

### 3.4. Ongoing versus acute effects of methylphenidate and atomoxetine on clock gene product expression

In the current study we have utilised a daily treatment regime of daily i.p. injections of the drugs/saline at a set time...
of day. We chose such a treatment regime as it allowed us to control the time of drug treatment, something which is not possible with other routes (e.g. via drinking water, in which case the drug will be delivered according to the normal drinking rhythm as in the study of Antle et al., 2012). Such a treatment regime raises the possibility that effects observed at the various time points are not due to the ongoing nature of the treatment, and rather are elicited acutely in response to the last treatment. Given that both atomoxetine and methylphenidate have relatively short half-lives in humans and rodents (24 h; Coghill and Seth, 2006; Cui et al., 2007; Thai et al., 1999; Mattiu et al., 2003), it is interesting to note that for methylphenidate treated animals, a number of brain areas examined show elevation of c-Fos at ZT26, a full 24 h following the final treatment, suggesting that at least in part the effects of the treatment regimes observed in this study is due to the ongoing nature of the treatment. In these types of experiments there is the complicating interaction between the time course of drug induced effects, the ambient photic environment and the underlying chronobiological rhythms of the factors being examined. Future studies may investigate the effects of single treatments of atomoxetine and methylphenidate on 24 h profiles of clock genes in the brain.

A separate issue is the timing of the doses at ZT2 in our study. Previous studies examining methylphenidate modulation of diurnal rhythms have dosed at a similar time point (ZT1; Algahim et al., 2009, 2010), whilst the study of O’Keeffe et al. (2012) who examined effects of acute atomoxetine treatment on circadian parameters found that treatment during the subjective day elicited phase shifts, whilst those during the active night phase did not. Changes in clock gene product expression levels at any given time point may be due to an acute up/down-regulation, or via a phase shift of the underlying rhythmic expression and possibly to drug-related behavioural activation/depression which in turn might alter the photic exposure of the animal (e.g. by the animal showing greater activity in the light on phase, during which it would normally be behaviourally quiescent). Equally any behavioural activation induced by drug treatment may act itself as a non-phototic type zeitgeber to feedback on the clock and alter rhythmicity. Interestingly, Schaap and Meijer (2001) demonstrated that light and behavioural activity have opposing actions on SCN neuronal function. It may be the case, especially for methylphenidate, that drug application induces behavioural activation in the lights on phase (which would be expected to inhibit SCN neuronal excitability) but that such an activation may also increase light perception (in a phase when animals would normally often be asleep with closed eyes), which would have the effect of increasing SCN neuronal activity. Therefore the effects of any behavioural activation and increased light exposure may cancel each other out, in which case the changes in SCN PER2 expression observed might be explained by a direct action of the drug on clock mechanisms, rather than being mediated indirectly through changes in behavioural state and light perception. Further experiments that evaluate such behavioural effects in parallel with changes in clock gene product expression would be very useful in addressing such an issue.

Whether the timing of treatment used in our present study recapitulates the clinical usage of these drugs is difficult to interpret in terms of the use of a nocturnal rodent in our study, and the lack of clarity on the mechanism that determine day/night activity. For example, the SCN rhythms of both diurnal and nocturnal species are very similar (Ramanathan et al., 2010). Typically patients taking either methylphenidate or atomoxetine will take a number of doses a day (due to their relatively short half lives), or be treated with slow release formulations of methylphenidate starting in the morning, and so it is likely that patients are exposed to these drugs through the lights on phase, during which the SCN appears to be in a similar phase in diurnal and nocturnal species. Further work examining the phase-dependency of methylphenidate’s and atomoxetine’s effects on circadian processes would clarify such questions.

3.5. Conclusions

We have demonstrated widespread effects of both MPD and ATO on the expression of circadian clock gene products in the mouse brain in a neuroanatomically differentiated manner. The pharmacological modulation of the circadian clockwork observed could have implications for the regulation of various behaviours that are governed by these brain regions, and could help explain the wide variety of therapeutic and adverse side effects of these drugs. Having said this, it is important to note that the functional consequence of clock gene expression in extra-SCN sites remains largely unknown, and as such future studies investigating behavioural consequences of long-term MPD and ATO treatment on circadian parameters would be illuminating in light of the molecular data presented here. Further, assessment of the impact of these drugs upon the circadian rhythms in animal models of ADHD and in both healthy control and patient populations would contribute to our understanding of the therapeutic mechanisms of these drugs and may lead to a more efficacious therapeutic deployment of these drugs in the clinic (e.g. by examining effects of time of dosing on ADHD symptom relief).

4. Experimental procedures

4.1. Animals

Male C57BL/6J mice (n=75, 8 weeks, Charles River, St Germain sur L’Arbresle, France) were housed in cages of three, with ad libitum access to food and water, and constant temperature and humidity, and were habituated to the housing conditions prior to beginning the experiments. Male mice were used, as is common in chronobiology, to avoid any possible interactions between oestrous stage and drug effect. As such, effects reported in this study may not extrapolate to females. The mice were subject to a 12:12 light/dark cycle, with lights on at 6 am (designated Zeitgeber Time (ZT) 0), using standard fluorescent light bulbs (~100 lx at cage level). These experiments adhered to the guidelines outlined in the Animals (Scientific Procedures) Act, 1986, the European Communities Council Directive 86/609 and by the Research Ethics Committee, School of Medicine, Swansea University. All efforts were
made to minimise the animal numbers used and suffering experienced.

4.2. Drug treatments

The mice underwent daily treatment with either 2.5 mg/kg MPD (Methylphenidate hydrochloride, Sigma-Aldrich, UK), 2.0 mg/kg ATO (Tomoxetine hydrochloride, Tocris Bioscience, UK) or a 0.9% saline control. The injections were given in a volume of 5 ml/kg, in 0.9% saline and administered intraperitoneally, once a day at ZT2 for 7 days. The doses of both ATO and MPD are based on numerous previous studies showing efficacy of action at these concentrations and the relevance of these doses to those used in clinical settings (e.g. Bymaster et al., 2002; Alghim et al., 2009; O’Keeffe et al., 2012; Keck et al., 2012).

4.3. Tissue collection

On day 7 of treatment, 6 mice were killed from each treatment group at each of the 4 sampling time-points: ZT8, ZT14, ZT20 and ZT26. The ZT26 sampling was the last time point, occurring 24 h after the final treatment and 2 h after lights of the light/dark cycle. This procedure was carried out in a light-proof room and under red light for those mice sampled in the dark phase of the cycle. Mice were anaesthetised with chloral hydrate, prior to transcardial perfusion with 4% paraformaldehyde (PFA). Brains were removed and post-fixed in 4% PFA at 4 °C overnight, and stored in 0.1 M phosphate buffer (PBS, pH 7.4) at 4 °C. The brains were then cryoprotected in 30% sucrose at 4 °C and sliced into 30 μm sections using a freezing-stage sliding microtome (Leica, Germany).

4.4. Immunohistochemistry

Free-floating sections were processed for c-Fos, PER2, PER1 and CLOCK immunohistochemistry using a standard avidin-biotin complex (ABC/nickel-DAB protocol (e.g. Beynon et al., 2009). Primary antibodies used were c-Fos (sc-52; dilution 1:8000; rabbit polyclonal; Santa Cruz Biotechnology, UK), PER1 (sc-7724; dilution 1:500; goat polyclonal; Santa Cruz Biotechnology, UK), PER2 (PER21-A; dilution 1:1000; rabbit polyclonal; Alpha Diagnostic International, USA), PER1 (sc-7724; dilution 1:500; goat polyclonal; Santa Cruz Biotechnology, UK) and CLOCK (sc-6928; dilution 1:500; goat polyclonal; Santa Cruz Biotechnology, UK). The sections were allowed to react overnight, and stored in 0.1 M phosphate buffer (PBS, pH 7.4) at 4 °C. The brains were then cryoprotected in 30% sucrose at 4 °C and sliced into 30 μm sections using a freezing-stage sliding microtome (Leica, Germany).

4.5. Image analysis

A Zeiss Axioskop light microscope (Zeiss, Jena, Germany) with an Axiocam digital camera was used to examine the brain sections. Three to six images were quantitated per time-point, region and mouse. The cell/count per area was calculated using image analysis software (ImageJ 1.43a, National Institutes of Health, USA). As area was not calibrated in our image analysis we express the cell counts as arbitrary units. The images were adjusted for background, and a threshold value was set that was optimal for the visualisation of each antigen. This threshold was then applied to all brain areas examined for that antigen and cells expressing immunostain in excess of the threshold were counted as being immunopositive. There are important caveats to such analysis; such as once a cell express immunolabel above a certain threshold it is counted, irrespective of stain intensity, and there is no necessarily a linear relationship between antigen concentration and subsequent immunostaining (Rieux et al., 2002). However, such immunohistochemical approaches are widely used in chronobiology and are a mainstay for assessing clock gene protein product expression (e.g. Amir et al., 2004; Debruyne et al., 2006; Challet et al., 2012).

Anatomical regions were identified according to the stereotaxic coordinates defined in the mouse brain atlas (Paxinos and Franklin, 2004). Immunoreactivity was examined in the suprachiasmatic nuclei (SCN) at the mid-rostral level, the paraventricular nucleus of the hypothalamus (PVN), the dorsal medial nucleus of the hypothalamus (DMH), the basolateral (BLA) and central (CeA) amygdala, the hippocampus (CA1, CA3, dentate gyrus (DG)), the caudate putamen (CPU), the ventral tegmental area (VTA), the nucleus accumbens including the core (AcbC) and shell (AcbSh) regions, and the cerebral cortex including the prefrontal infralimbic cortex (ILC), the prefrontal prelimbic cortex (PLC) and the cingulate cortex (CC). The areas of interest within these structure analysed are shown in Figs. 1 and 3 and supplementary Fig. 4. These regions were chosen based on their roles in the circadian system (e.g. SCN, and its major efferent regions PVN and DMH, semi-autonomous oscillators in the hippocampus, amygdala (Guilding and Piggins, 2007), and/or involvement in ADHD and ADHD drug action (e.g. dopaminergic system, VTA, CPU, AcbSh and AcbC, prefrontal cortex implicated in executive dysfunction in ADHD (Coogan et al., 2012).

4.6. Statistical analysis

Average immunoreactivity was calculated per mouse, time-point, treatment group and brain region. SPSS (IBM Corporation, USA) was used to run 2-way analysis of variance (ANOVA), with zeitgeber time and treatment (Saline, MPD or ATO) as the factors, with Tukey post-hoc testing to describe specific pairwise effects within main effects. To correct for multiple hypothesis testing, results from 2-way ANOVAs were corrected in a stepwise manner with the Holm-Bonferroni method, and the P values cited in the results is the P value after such correction was applied. Twenty four hour Cosinor analysis was performed using statistical software (CircWave v1.4, Department of Chronobiology, University of Groningen, Netherlands), to test whether the data exhibited a significant diurnal rhythm. This programme determines if a statistically significant (P <0.05) sinusoidal wave can be fitted to the data, by automatically adding harmonics to the wave fit to best describe the data. The following function describes the wave-form:

\[
f(t) = a + \sum_{i=0}^{\infty} \left[ p_i \sin \frac{2 \pi t}{\tau} + q_i \cos \frac{2 \pi t}{\tau} \right]
\]
where $a$ is the average, $i$ is either 1, 2, 3, when 2 it describes the first harmonic, when 3 it describes the second harmonic. $p_i$ is the sine coefficient of the $(i-1)$th harmonic, $q_i$ is the coefficient of the $(i-1)$th harmonic; $t$ is the time-point value (modulo 1), $f(t)$ is the calculated function value at time point $t$, $a$ and $b$ are linear estimates for the sine and cosine contribution to a flat line (when $i=0$), the fundamental wave (i=1), first harmonic (i=2), second harmonic (i=3). For our analysis, optimal fits were obtained with the fundamental wave.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2013.03.038.

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