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The effects of the noradrenaline reuptake inhibitor atomoxetine on circadian rhythms in mice

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Saileog O'Keeffe

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Abstract

Circadian rhythms are patterns in behavioural, physiological and many others parameters that recur approximately every twenty four hours. Dysfunction of the circadian system is being linked to a number of common illnesses. The psychiatric condition Attention Deficit/Hyperactivity Disorder (ADHD) can be characterised by an early onset of sleep problems and breakdown of stable circadian rhythms which recent studies have shown.

The drug atomoxetine used in this research project is used in the treatment of ADHD. It acts primarily through the noradrenergic system and is not a psychostimulant like the other mainstays of ADHD management such as Ritalin and Amphetamine. The main aims of this research project are to examine if acute treatments with atomoxetine produces alterations in the circadian rhythms in mice. In addition to examine whether treatment with these drugs alters cellular activation in brain areas implicated in the circadian timekeeping system. The effects of atomoxetine on locomotor circadian rhythms in mice were examined under free-running conditions (to look at the endogenous circadian clock without influence of environmental factors like light).

Results have shown that atomoxetine produces a novel resetting of circadian time in mice, producing significant phase delays in animals in constant light and phase advances in animals in constant dark. Atomoxetine also altered the expression of c-Fos and clock genes in the master circadian clock. As there is evidence that ADHD might be associated with misalignment of the circadian clock with the environment, Atomoxetine might ameliorate some of the symptoms of ADHD by resetting the clock.

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Abbreviations

ADHD - attention-deficit hyperactivity disorder

AMPK - adenosine monophosphate-activated protein kinase

AVP - arginine vasopressin

BPD - bipolar disorder

CREB - cAMP response element-binding protein

CRY – *cryptochrome*

CSNK1E - casein kinase 1 epsilon

CT - circadian time

DA - dopamine

DAB – diaminobenzidine

DAT - DA transporter

DD - dark dark

DSPS - delayed sleep phase syndrome

FASPS - familial advanced phase sleep syndrome

GABA - gamma-amino butyric acid

GRP - gastrin-releasing peptide

GSK3 - glycogen synthase kinase-3

HTI - hypothalamictubero infundibular

IGL - intergeniculate leaflet

I.P – intraperitoneal

KD - knock down

KO - knock out

LD - light dark

LL - light light

mRNA - messenger ribonucleic acid

NAc - nucleus accumbens

NET - norepinephrine transporter

NGS - normal goat serum

NPY - neuropeptide Y

OTC3 - organic cation transporter 3

PACAP - pituitary adenylate cyclase-activating peptide

PB - phosphate buffer

PER - period

PFA - paraformaldehyde

PFC - pre frontal cortex

RHT - retino-hypothalamic tract

SAD - Seasonal affective disorder

SCN - suprachiasmatic nucleus

SHR - spontaneously hypertensive rat

SNc - substantia nigra pars compacta

SNRI - selective norepinephrine reuptake inhibitor

SSRI - selective serotonin reuptake inhibitor

SUMO - small ubiquitin-related modifier protein

5-HT - serotonin

VIP - vasoactive intestinal polypeptide

VTA - ventral tegmental area

WKY - Wistar-Kyoto

ZT - Zeitgeber time

1. Introduction

1.1 Circadian Rhythms and SCN

The study of circadian and other biological rhythms is called chronobiology. Circadian rhythms are endogenous cycles that recur approximately every twenty four hours in relation to behaviour, physiology and other parameters. Circadian rhythms are present in all eukaryotic organisms from unicellular fungi and bacteria to plants, animals and humans (Jud et al, 2005; Reppert and Weaver, 2002). A range of biological processes are regulated by circadian clocks such as the sleep-wake cycle, body temperature, blood pressure and hormone secretion (Honma and Hiroshige, 1978; Kondratov et al, 2007). Jurgen Ashcoff was a key figure in the development of chronobiology as a field of study as he was the first to investigate the circadian clock of humans. He built an underground bunker, where he was able to research human subjects in isolation from time cues. A range of experiments were carried out for over 20 years and Ashcoff and his colleague Wever strongly established that human behaviour, cognition and physiology were controlled by endogenous circadian oscillators (Aschoff, 1960). The bunker was used for experiments looking at the human circadian system under constant conditions and under different entrainment. He also discovered while experimenting on himself that there was a difference in temperature over a 24-hour period in humans (Foster and Roenneberg, 2008; Daan, 2001).

In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) (Moore, 1983). The SCN is a bilateral structure located in the anterior part of the hypothalamus, above the optic chiasm (Welsh, 2009). The SCN is a heterogeneous structure and has been subdivided into the ventrolateral region and the dorsomedial region. The ventrolateral or core region receives heavy retinal input and shows low amplitude of circadian oscillations in clock

gene expression, whilst the dorsomedial or shell region shows high amplitude of circadian oscillations. The dorsal SCN is rich in vasoactive intestinal polypeptide (AVP) neurons, while the core region is rich in vasopressin (VIP) and gastrin releasing peptide (GRP; Yan, 2009). VIP is a major neurotransmitter of SCN neurons and assists in SCN function. The neurons are found in the core of the SCN and are retinorecipient. They are activated by light allowing the resetting of the circadian clock (Reghunandanan and Reghunandanan, 2006).

Studies have shown that ablation of the SCN resulted in arrhythmicity in various physiological and behavioural processes (Stephen and Zucker, 1972; Moore and Eichler, 1972; Yan, 2009). Transplanting fetal SCN tissue to SCN-lesioned animals restored rhythmicity corresponding to the donor and not the host animal (Ralph et al, 1990). The master clock controls peripheral clocks that are located in other areas of the brain and peripheral organs (Reppert and Weaver, 2002). Individual SCN neurons are able to generate self sustained oscillations (Walsh et al, 1995). Within the SCN there are 20,000 neurons that generate a coherent circadian rhythm of neuronal firing, usually synchronized to the light/dark cycle by retinal input, but can persist in complete darkness (Welsh, 2009). The SCN neuronal firing rhythms are vital for transmitting circadian timing signals to the peripheral clocks (Schwartz et al, 1987). Electrical activity in neurons is produced by the opening of membrane spanning ion channels, resulting in a voltage change of the neurons. The voltage threshold, also known as the firing threshold will cause neuron membrane voltage to depolarize, and the cell then has an influx of calcium due to the opening of Ca^{+2} channels. The increase of calcium in the cell releases neurotransmitters and neuropeptides to be released from the pre synaptic terminal of the neuron. The firing rate is dependent on Na^{+}, and the SCN cells randomly generate neuronal firing at varies times of the

day. The firing rate is high during the day and low at night in both diurnal and nocturnal mammals (Green and Gillette, 1982; Groos and Hindriks, 1982). The firing rate from the SCN serves to translate the transcription/translation feedback loop that takes place in the SCN to overt behavioural output (Stillman et al, 2007).

The circadian clock can not only generate its own rhythms but can also be entrained by the environmental light-dark (LD) cycle. Light is perceived from melanopsin ganglion cells in the retina, and then the photic information is transmitted to the SCN clock directly via the retino-hypothalamic tract (RHT) (Gooley et al, 2001; Challet, 2007). Glutamate is the major neurotransmitter of the RHT, and substance P and pituitary adenylate cyclase-activating peptide (PACAP) are two neuropeptides present in the RHT, which help modulate the entrainment process (Rosenwasser, 2009). There are also two indirect pathways which are involved in supplying retinal input to the SCN. The intergeniculate leaflet (IGL) pathway is the first pathway, which receives input from the same retinal cells whose axons compose the RHT (Challet, 2007; Reppert and Weaver, 2001). The second indirect pathway from the retina to the retinorecipient SCN is routed by the serotonergic raphe nuclei. Neurotransmitters in both the IGL and raphe pathways appear to play a role in mediating non photic phase shifts, for example those caused by behavioural arousal (Reppert and Weaver, 2001).

Light triggers intracellular signalling events in SCN neurons. Studies have been carried out to examine signal transduction events that occur in SCN neurons after neurochemical stimulation. Studies reveal that the signal transduction pathways activated by the neurotransmitter glutamate receptor activation depend on the circadian time at which the stimulus is applied. Light exposure

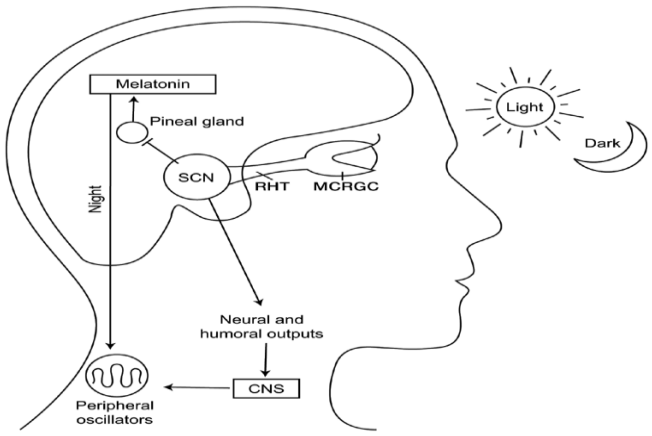
during the early subjective night causes a phase delay, via glutamate acting at *N*-methyl-D-aspartate receptors (Colwell and Menaker, 1992). The effects of glutamate appear mediated by release of intracellular calcium through ryanodine receptors (Ding et al, 1998). This causes downstream actions of calcium which result in activation of calcium/calmodulin kinase, MAP kinase, phosphorylation of cAMP response element-binding protein (CREB); and the induction of gene expression via calcium/cAMP response elements (Obrietan et al, 1998; Gillette and Tischkau, 1999). CREB phosphorylation is controlled by the circadian clock and the ability of light or glutamate receptor activation to induce CREB is restricted to a nocturnal zone of sensitivity (Ding et al, 1997). Therefore one of the outputs of the circadian clock is the ability to adjust its responsiveness to inputs at specific times of the circadian cycle (Gillette and Tischkau, 1999). Light exposure during the late subjective night in vivo or the glutamate receptor activation in vitro, result in phase advances. Glutamate then appears to activate nitric oxide production, activate soluble guanylyl cyclase, increase cGMP, activate cGMP-dependent protein kinase, and phosphorylate CREB (Ding et al, 1997).

1.2 **Entrainment of the clock**

The circadian clock not only can generate its own rhythms but can also be entrained by Zeitgebers, which are external cues such as photic stimuli (eg. the LD cycle) or non- photic stimuli such as feeding times and temperature. Light is the most powerful synchronizer of the SCN clock (Challet, 2007). Circadian rhythms vary between species as diurnal animals are active during the light phase, whereas nocturnal animals are inactive during the light phase. Zeitgeber time (ZT) 0 is defined as the start of the light phase and ZT12 corresponds to the end of the light phase, and the start of twelve hours of darkness.

Phase shifts can occur in circadian rhythm and are defined as the resetting of the organisms internal rhythm due to external stimulus such as a light pulse, causing phase advances or phase delays (Jud et al, 2005). In nocturnal and diurnal animals light pulses carried out in the early night produce phase delays, while light pulses administered in the late night produce phase advances (Sumova and Illnerova, 2005). However a light pulse administered during the subjective day has little or no effect in nocturnal animal, but diurnal animals respond to light pulses during the subjective day. For animals that are kept in constant light (LL), their SCN clock can be reset by dark exposure. Dark pulses in nocturnal rodents produce phase advances during most of the subjective day whereas phase delays result due to dark pulses in the late subjective night and early day (Challet, 2007).

Figure 1: Entrainment of the sleep/wake cycle by light



(Drake, 2010)

LL or constant darkness (DD) allows an animal to express a free running rhythm in the absence of external time cues, with a lengthened period in LL and low amplitude of activity that results in arrhythmic behavioural rhythms in mice (Sudo et al, 2003). In DD, mice internal rhythm is usually shorter than twenty four hours. Usually, free running animals no longer have a period length equal to that of twenty four. Due to this, time is no longer expressed in ZT but is represented in circadian time (CT) units, where CT12 is defined as the onset of activity in free-running conditions.

Whether the input is photic or non-photic, it reaches the clock through neurotransmitters in nerve terminals. Neurotransmitters play a very vital role in the SCN. The presence of neurotransmitters in the afferent and efferent projections of the SCN is as equally important for the entrainment of the clock and for the control of overt rhythms. Neurotransmitters and neuropeptides like glutamate, gamma-amino butyric acid (GABA), neuropeptide Y (NPY), serotonin, vasoactive intestinal peptide (VIP), and arginine vasopressin (AVP) have all been implicated in the functioning of the SCN (Reppert and Weaver, 2002). AVP plays an important role in circadian

time-keeping as it controls the circadian rhythm of food and water intake. The neuropeptide VIP, acting through $VPAC_2$ receptor contributes in the resetting of light and the maintenance of ongoing rhythmicity of the SCN (Reghunandanan and Reghunandanan, 2006). The principal neurotransmitter involved in expressing photic information to the SCN is glutamate. Direct secretion of glutamate results from light stimulation of the retina, from the RHT into the ventral VIP-containing part of the SCN (Ebling, 1996). Glutamate as a transmitter at RHT/SCN synaptic connections plays a vital role in mediating photic regulation of circadian rhythmicity. GABA is an important neurotransmitter for regulating SCN function. The key role of GABA involves the coupling between the ventral and dorsal parts of the SCN. Activation of GABA receptors during the middle of the subjective day produces a phase advance in nocturnal species, whereas during the subjective night GABAergic stimulation produces a phase in nocturnal species (Challet, 2007).

In SCN, the individual clock cells must become synchronized for a coordinated output signal. Gap junction communication and neurotransmitter based interactions are mechanisms to help with synchronization. Studies have indicated the presence of functional gap junctions in the SCN. Research carried out by Shinohara and colleagues reported transfer of Lucifer Yellow between adult rat SCN neurons and inhibition of this transfer by the GABA receptor agonist muscimol (Shinohara, 2000). GABA activity is usually inhibitory, but studies carried out by Wagner and colleagues reported that GABA excited SCN neurons during daytime, whereas at night GABA inhibited SCN electrical activity (Wagner et al, 1997). GABA could serve two roles, firstly to translate a rhythm in ionic concentrations for example intracellular chloride into a rhythm in excitability in individual clock neurons, or secondly it could provide a mechanism to increase the amplitude of rhythmicity in a population of SCN neurons. Research carried out

using electrical activity rhythms of SCN clock cells in culture showed that GABA is an important neurotransmitter for synchronizing SCN neurons. A change in phase shifts and entrainment of clock cells in culture is caused by GABA (Liu et al, 2000). All acute responses to GABA were inhibitory in the clock cell culture system, but neuronal inhibition alone was inadequate to cause a phase shift. GABA plays an important role in synchronizing the collective SCN in vivo, but the neuropeptide arginine vasopressin (AVP) may also be involved. AVP is one of the principal neuropeptides in the SCN. AVP neurons occupy a large part of the SCN, mostly in the dorsomedial part of the SCN. AVP is synthesized and secreted by the SCN in a circadian pattern. AVP has an important excitatory role by activating V1a receptors to increase the amplitude of firing rates in the SCN during subjective day and enhance SCN output. It is also an important factor in feeding times (Ingram et al, 1998). A coupled multi-oscillatory system provides entrainment of the circadian clock (Liu et al, 1997). If some of the oscillators in the network are phase shifted, this results in the whole network resynchronising to a new phase. Therefore only a subpopulation of clock cells is needed to react to a specific entraining stimulus to cause a phase shift to the whole network. The stable phase of the SCN clock maintained in light-dark conditions is possibly a balance between internal synchronization, for example via GABA and phase-shifting responses to light such as glutamate. For the SCN to function it requires accurate organisation between the intercellular synchronizing processes and the intracellular molecular loops (Liu et al, 2000; Reppert and Weaver, 2001).

The polypeptide VIP is one of the main neurotransmitters of SCN neurons and participates in SCN function. These SCN neurons are retinorecipient and are found in the core of the SCN. They are activated by light, and exogenous application of VIP can reset the circadian clock in a

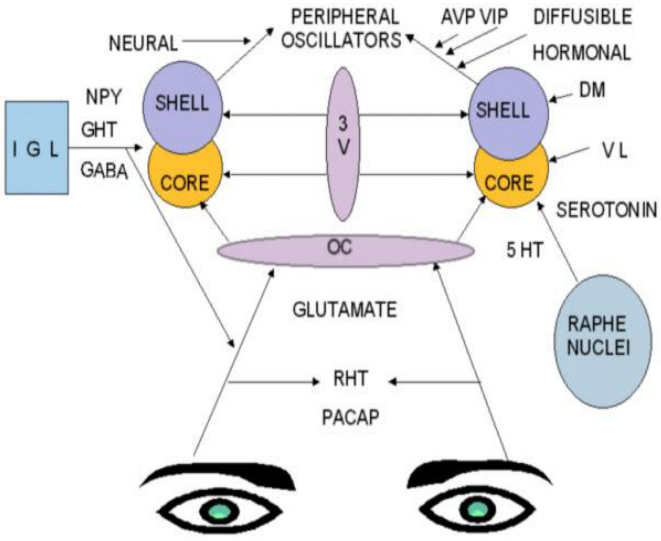
manner similar to that of light application, both in vitro and in vivo (Reghunandanan and Reghunandanan, 2006). Research carried out has shown that VIP is essential for the coordination of the daily rhythms in behaviour and physiology at the level of biological clock in mice (Aton et al, 2005). VIP has two important functions in the SCN, circadian rhythmicity in a subset of neurons and maintenance of synchrony between intrinsically rhythmic neurons (Reghunandanan and Reghunandanan, 2006).

Other neurotransmitters and neuropepides also play a role in the entrainment of the circadian timing system. For example, Neuropeptide Y (NPY) is important in the circadian timing system as it conveys non photic cues to the SCN of nocturnal species. NPY is released from projections in the IGL into the SCN. Serotonin is another essential neurotransmitter within the circadian timing system. The SCN and the IGL receive serotonergic projections from the midbrain raphe nuclei. The 5HT receptor agonists either *in vitro* or *in vivo*, are found to cause phase shifts of the SCN when administered at times which light does not cause phase shifts in the circadian cycle. In vitro studies have shown that serotonin causes a phase advance in the circadian pacemaker during the day and delays it at night, an action comparable to that of GABA (Reghunandanan and Reghunandanan, 2006).

Melatonin, a hormone secreted at night by the pineal gland can also phase shift the master clock. The SCN controls circadian rhythmicity of the pineal gland for its secretion of melatonin, therefore melatonin is thought to play a feedback role on the SCN (Challet, 2007). The SCN contains a high density of melatonin receptors such as MT_1 and MT_2. Melatonin function in the SCN has been established in several rodent studies. Melatonin injections administered at subjective dusk in mice produced phase advances (Benloucif et al, 1996). In addition, melatonin can entrain free running activities in rodents. The mechanisms behind these effects are created by

inhibiting neuronal firing, which might be essential for defining SCN sensitivity to entraining stimuli. In humans this might contribute to the regulation of sleep. Research studies carried out in human's show that phase shifts of major physiological factors, such as core temperature, endogenous melatonin and sleep timing, occur after melatonin administration. Phase delays are observed after morning administration, whereas phase advances occur after evening administration. Timed administration of melatonin helps readjustment after acute phase shifts occur in the light dark schedule that occur in jet lag and shift work. Melatonin administration can also entrain free running circadian rhythms in blind people (Sack, 2000) perhaps by acting in the SCN to synchronize circadian rhythms (Ekmekcioglu, 2006).

Figure 2: Afferent inputs and efferent pathways of the SCN

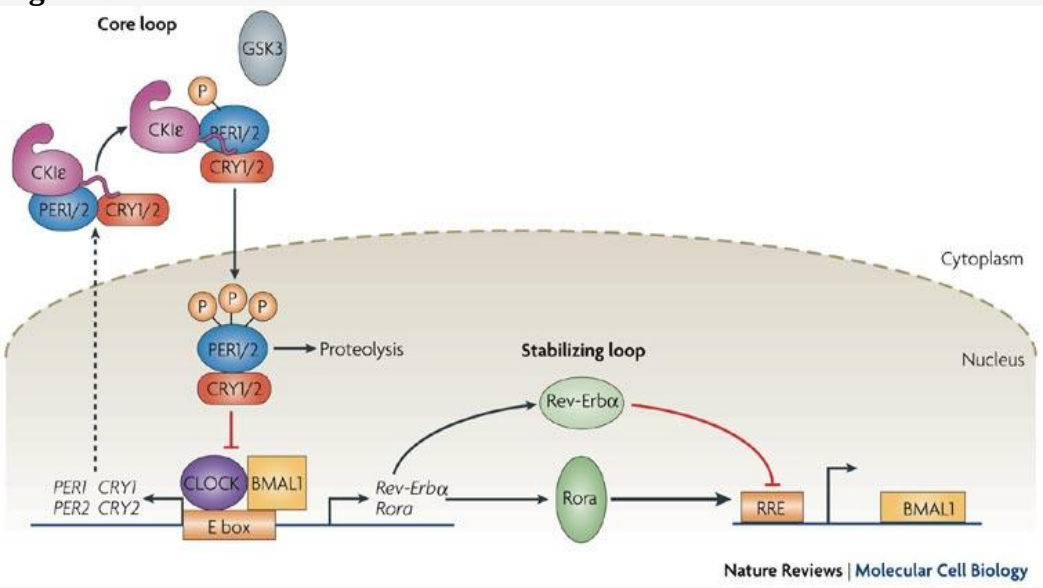


(Reghunandanan and Reghunandanan, 2006)

1.3 Clock genes as transcription factors

The first circadian rhythm mutants to be isolated were the *frequency* mutants in the fungi *neurospora* and the *period* mutants in the fruitfly *Drosophila* in the early 1970's. Research carried out firstly on the fruit fly *Drosophila melanogaster* has helped in finding out the mechanisms of the mammalian clock (Young and Kay, 2001). Genes in the fly circadian clock and the transcriptional feedback loop that takes place are similar to that of mice and humans (Reppert and Weaver, 2002). The SCN endogenously generates circadian rhythm by a molecular oscillator that consists of positive and negative transcription and translation feedback loops involving a set of clock genes and clock-controlled output genes that link the oscillator to clock-controlled processes. The following mammalian clock gene products are involved in this process, CLOCK, BMAL1, the period proteins PER1, PER2, PER3, and the cryptochromes CRY1 and CRY2 and casein kinase 1 epsilon (CSNK1E) (Eismann et al, 2010).

Figure 3: Mammalian circadian clock model



Gallego and Virshup, *Nature Reviews Molecular Cell Biology* **8**, 139–148 (February 2007).

The major transcriptional activator consists of a dimer between CLOCK and BMAL1, which binds to E-box sequences in the promoters of many genes including PER and CRY genes. Throughout the day, the PER and CRY proteins build up in the cytoplasm, where they are phosphorylated by casein kinase 1 ε and glycogen synthase kinase-3 (GSK3). mPER and mCRY proteins translocate into the nucleus and the mCRY acts as a negative regulator as it directly interacts with CLOCK/BMAL and it inhibits transcription, which in turn closes the negative feedback loop. At the end of the circadian cycle, the PER and CRY proteins are degraded in a CKI-dependent manner, which releases the repression of the transcription and allows the next cycle to start (McClung, 2007). These clock genes are entrained to the light-dark cycle with CLOCK: BMAL peak during the light period and PER: CRY being high during the dark period in diurnal and nocturnal animals (Sukumaran et al, 2010). In the positive feedback loop CLOCK-BMAL1 heterodimers activate transcription of the orphan nuclear receptor gene Rev-Erbα. The REV-ERBα protein then suppresses *Bmal1* transcription by using Rev-Erb/ROR response element in its promoter. This results in Bmal1 RNA levels falling and mCry and mPer RNA levels rising. The mCRY proteins enter the nucleus to inhibit mPer and mCry transcription as it acts on the CLOCK-BMAL1, mCRY protein also obstructs Rev-Erbα transcription which results in activation of *Bmal1* transcription (Preitner et al, 2002). Another core member of the mammalian circadian clock is neuronal PAS-domain protein 2 (NPAS2). NPAS2 can be compared to CLOCK, displaying similar activities but differs in tissue distribution. NPAS2 can heterodimerize with BMAL1, and bind to E-box motifs, where its transcription activates circadian genes (Rana and Mahmood, 2010).

The positive and negative feedback loops described above are responsible for producing messenger ribonucleic acids (mRNAs) from the *Per, Cry, Rev-erbα* and *Bmal1* genes across

circadian phases. In the SCN, *Per, Cry* and *Rev-erbα* all show peak during the light phase, with *Bmal1* producing an opposite phase with peak around 12 hours later. In other regions of the brain and peripheral tissues, these rhythms are delayed by several hours. Since a simple transcriptional feedback loops leads to mRNA oscillations with a period much smaller than 24 hours, other mechanisms have been added onto this simple loop model that allows that allows the 24 hour environment period to be slowed down. These mechanisms act at different levels and involve post-transcriptional processing of the mRNAs, translation, post-translational processing of the proteins and nuclear translocation. Each of these mechanisms can contribute to a delay between the activation and repression of transcription that is required to keep the period at about 24 hours (Harms et al, 2004; Rana and Mahmood, 2010). A model of the SCN clockwork based on the interacting positive and negative feedback loops suggests that, at the beginning of the circadian day (CT 0), *mPer* and *mCry* transcription is driven by the build up of CLOCK: BMAL1 heterodimers. The circadian oscillations of *mPer* and *mCry* RNA levels in the SCN display similar yet different temporal profiles, with the *mPer1* RNA rhythm peaking from CT 4 to 6, *mPer3* peaks between CT 4 and 9 (Shearman et al, 2000), *mPer2* at CT 8 (Bae et al, 2001), and *mCry1* at CT 10. By midpoint of the circadian cycle (CT 12), the mPER and mCRY proteins are synchronously expressed in the nucleus where the mCRY proteins shut off CLOCK: BMAL1 mediated transcription. Simultaneously, mPER2 enhances *Bmal1* transcription, which then leads to peak *Bmal1* RNA levels from CT 15 to CT 21 (Bunger et al, 2000). It is thought that the *Bmal1* RNA rhythm drives a BMAL1 protein rhythm after a four to six hour delay. The restoration of BMAL1 levels at the end of the night most likely increases CLOCK: BMAL1 heterodimers at the appropriate circadian time to drive *mPer/mCry* transcription, resulting in the cycle starting again. It seems that the availability of BMAL1 is rate limiting for heterodimer

formation and is essential for the positive: negative feedback loop to reoccur for the start of a new circadian day (CT 0), (Reppert and Weaver, 2001).

Post-translational modifications which regulate the circadian clock include acetylation, phosphorylation, and ubiquitination. During phosphorylation, Casein kinase 1 epsilon (CK1ε), Casein kinase 1 delta (CK1δ), Casein Kinase 2 (CKII), glycogen synthase kinase-3 (GSK3) and adenosine monophosphate-activated protein kinase (AMPK) are vital factors which help regulate the core circadian proteins (Reghunandanan and Reghunandanan, 2006). Phosphorylation is required for the recruitment of ubiquitin ligases, which in turn mediate the polyubiquitylation and the subsequent degradation of these proteins in the proteasome. In mammals, the stability of PER1 and PER2 is regulated by either βTrCP1 or βTrCP2. CKI phosphorylates PER1 and PER2 and this phosphorylation leads to the recruitment of βTrCP which mediates the ubiquitylation and proteasomal degradation of these proteins (Eide et al, 2005; Shirogane et al, 2005). In the last decade sumoylation was discovered, which is a post translational modification involved in the regulation of various mechanism involved in the circadian clock. It is a reversible posttranslational modification in which a small ubiquitin-related modifier protein (SUMO) is covalently linked to lysine residues. It is controlled by an enzymatic pathway similar to the ubiquitin pathway (Reghunandanan and Reghunandanan, 2006).

mPER and mCRY outputs of the negative feedback loop are vital for sustaining a functioning circadian clock. Double knockout of either *Per1/Per2* or *Cry1/Cry2* causes behavioural arrhythmicity if the knockout mice are put in constant conditions. *mPer3* does not appear to be necessary in the functioning of circadian rhythmicity with only a modest period alteration in DD (Shearman et al, 2000; Bae et al, 2001). In the *Clock* mutant mouse it produces long period

locomotor activity that eventually causes arrhythmicity (Young and Kay, 2001). Research carried out on the clock genes *Per1* and *Per2* suggests involvement in photic entrainment of the circadian clock (Reppert and Weaver, 2001). The clock Per 1 and Per2 genes are vital in regulating circadian rhythms as animals deficient in both these genes show no circadian rhythm (Yan, 2009).

1.4 **How Clock cells generate circadian output**

AVP and GABA in the SCN are implicated in some output pathways. AVP in the SCN may play a part in driving rhythmic hypothalamo-pituitary adrenal axis activity in rats, regulating corticosterone release from the adrenal gland (Buijs et al, 1999). Also AVP function may cause activation of the hypothalamo-pituitary-gonadal axis (Palm et al, 1999). There seems to be a connection between the molecular oscillations in pacemaker cells, the clock-controlled production of AVP, and overt rhythms. GABA which is released from SCN terminals in the paraventricular nuclei region appears to be involved in regulating melatonin synthesis from the pineal gland by switching off a stimulatory signal emitted from the paraventricular nuclei. Increased SCN electrical activity seems to lead to increased GABA release, however further research is needed in relation to the link between the circadian clock and the mechanisms that regulate GABA release from the SCN (Reppert and Weaver, 2001). Humoral routes may also be involved in the SCN conveying information within the brain. Studies carried out by Silver and colleagues showed that a diffusible substance from transplanted fetal SCN tissue can restore weak circadian rhythmicity in locomotor activity in SCN-lesioned hamsters (Silver et al, 1996).

1.5 Peripheral clocks

Circadian clocks can be found in the brain outside the SCN and in peripheral cells throughout the body (Eismann et al, 2010). The eight known clock genes are expressed in almost every peripheral tissue. In a study carried out by Yamamoto and colleagues, mRNA expression of clock and clock-controlled genes were analyzed in mouse peripheral tissue. RT-PCR methods were carried out on mRNA from heart, lung, liver, stomach, spleen and kidney. Eight genes as follows *mBmal1*, *mNpas2*, *mRev-erbα*, *mDbp*, *mRev-erbβ*, *mPer3*, *mPer1* and *mPer2* showed robust circadian expressions of mRNAs in all peripheral tissues (Yamamoto et al, 2004). These genes should be considered to be core molecules playing an important role in the circadian clock. Circadian mRNA expression patterns were comparable in each tissue, implying, that there may be a unitary mechanism for resetting the peripheral clock. The peak transcript level of each circadian rhythm was as follows in *mBmal1* and *mNpas2*, the peak was in the subjective night at CT20-CT0, *mRev-erbα* the peak in subjective day at CT4-8, *mDbp* and *mRev-erbβ*, at CT8, *mPer3*, at CT8-12, *mPer1*, at CT12; and *mPer2*, at CT12-16. In the peripheral tissues mRNA peaks occurred approximately four hours later than those in the central pacemaker, SCN. In a study carried out on a mouse liver, the BMAL1 gene was knocked out. This resulted in the liver no longer being able to produce sufficient amount of glucose needed in the blood circulation, which resulted in hypoglycaemia. This indicates how important the liver peripheral clock is to the metabolic system (Lamia et al, 2008; Dibner et al, 2010).

Generally, peripheral cells can sustain cellular rhythms independently. Studies have shown that in cell culture there is rhythmicity in gene expression and functions exist in cells of peripheral tissues. Peripheral clocks can generate circadian oscillation in gene expression on their own, however the SCN plays a vital role in coordinating and synchronizing rhythmic behaviour all

through the body. The central clock and the light/dark cycle regulate and retrain oscillations in the clocks in the peripheral cells with the help of other factors such as food, stress and certain hormones (Sukumaran et al, 2010). For example, glucocorticoid secretion is controlled by the SCN with the help of the adrenal gland. It modulates gluconeogenesis, lipid metabolism, inflammation, and immune functions. Glucocorticoid mediates behavioural adaptations to external cues, as its level responds sensitively to stress and to circadian rhythm. The adrenal peripheral clock is vital in helping to generate glucocorticoid rhythms that synchronize peripheral clocks (Son et al, 2008).

1.6 Circadian Rhythm Sleep Disorders

In circadian rhythm sleep disorders there is a mismatch between circadian timing of sleep and the demands of the environment, which results in symptoms of disturbed sleep and impaired day time alertness. There are two types of circadian sleeping disorders - extrinsic and intrinsic disorders. Jet lag and shift work are examples of extrinsic disorders while delayed sleep phase syndrome (DSPS) and familial advanced phase sleep syndrome (FASPS) are examples of intrinsic disorders.

Jet lag is a circadian rhythm sleep disorder which occurs from travelling across multiple time zones and alters the body's circadian rhythm. Symptoms of jet lag disorder include difficulty in getting to sleep and also maintaining sleep, excessive sleepiness and gastrointestinal disturbances. Because environmental cues at the flight destination support phase adaptation of the circadian clock to local time, symptoms of jet lag disorder are usually temporary; however, measurements of hormone levels, sleep architecture and body temperature have indicated that a complete phase shift after a long-haul flight can take up to two weeks. The severity of jet lag disorder is largely dependent on what direction of travel takes place and the number of time zones crossed. Travelling west can be easier accommodated by the circadian system, as passengers can delay their onset of sleep instead of advancing sleep times, which is required when travelling east. This occurs because the human circadian system runs at an internal period (tau) which is slightly longer than 24 hours, a period that is favourable to phase delays in circadian timing system. Melatonin pills are used to mask systems until the individual's phase adjusts to the new time zone. Chronic jet lag can occur in airline flight crew who may experience five or six different time zones every month. Due to the short stay in each zone adjusting to the new time zone can cause even more difficulties (Monk and Welsh, 2003; Drake, 2010). A study carried out showed

that administration of a combination of morning intermittent bright light and afternoon melatonin along with a gradually advancing sleep schedule can advance circadian rhythms almost an hour a day, with very little circadian misalignment. This protocol might be applied before eastward jet travel or for delayed sleep phase syndrome to evoke a phase advance of the circadian clock (Revell, 2006).

Shift work is also another circadian rhythm sleep disorder. Shift work is defined as work primarily outside of normal daytime working hours. Shift workers are at risk of developing a circadian rhythm sleep disorder and it occurs in some individuals who work at night, start work early in the morning (4 to 7 a.m.) or work according to a rotating-shift schedule (Culpepper, 2010). Shift work has caused a greater incidence of many medical disorders, such as cardiovascular, gastro-intestinal, and neurological disorders. Memory, learning, alertness, and performance are affected by sleep deprivation, even in the absence of circadian misalignment (Culpepper, 2010). In studies carried out on nurses in Massachusetts doing rotating shift work, frequent lapses of attention, resulting in increased error rates were discovered (Gold et al, 1992). Bright light at work and complete darkness during sleep at day may be helpful to patients (Monk and Welsh, 2003).

Intrinsic sleep disorders may be exemplified by DSPS, in which patients have shifted circadian rhythms where one falls asleep at a later time, has difficultly waking up in the morning and has impaired alertness in the morning. DSPS is the most common intrinsic circadian rhythm sleep disorder, and onset usually occurs in adolescence. Genetic studies show that the H4 haplotype of the hPer3 gene has been implicated in the pathogenesis of DSPS (Archer et al, 2003; Ebisawa et al, 2001). Another intrinsic circadian rhythm sleep disorder is known as FASPS. Patients with FASPS fall asleep and wake much earlier and are associated with depression (Xu et al, 2005;

Monk and Welsh, 2003). Its prevalence is lower than DSPS and is occurs more in elderly patients.Genetic studies have shown that missence mutation in the hPer2 gene causes FASPS in some patients. Sequencing of this gene revealed a serine-to-glycine point mutation in the CKI binding domain of the hPER2 protein that resulted in hypophosphorylation of Per2 in vitro (Toh et al, 2001).

A third intrinsic circadian rhythm sleep disorder is the non 24hour sleep/wake syndrome also known as free running disorder. This disorder is diagnosed in around 2% of individuals with circadian rhythm sleep disorder and most often occurs in totally blind individuals with no light perception due to the absence of photo entrainment of the sleep/wake cycle. Without entrainment, the behavioural sleep/wake cycle continues with a period like that of the internal circadian period of slightly more than 24 hours, resulting in a small but repeated off-setting of sleep/wake times compared with the 24-hour day/night cycle.

The final intrinsic circadian rhythm sleep disorder is irregular sleep/wake syndrome. Individuals with irregular sleep/wake rhythm experience disorganised and variable sleep and wakening times. Sleeping multiple times throughout the day and night also occurs. The prevalence of this disorder is 12% and occurs most frequently in the neurologically impaired who have damage to the SCN. This disorder is more associated with the elderly, with irregular sleep/wake rhythm due to the increasing prevalence of neurologic conditions such as dementia (Drake, 2010).

1.7 Circadian clocks in psychiatric disorders

Dysfunctions of biological rhythms are linked with mood disorders such as depression, bipolar disorder (BPD) and schizophrenia. Abnormal circadian rhythms in bodily functions such as temperature, norepinephrine, blood pressure and melatonin have been found in patients with depression or BPD (McClung, 2007). Circadian clock genes have been implicated in a variety of neuro-behavioural processes. Disruption of normal clock gene function in experimental animals alters sleep wake regulation, drug responses, and behaviour, while genetic polymorphisms and familial clock gene mutations are linked with sleep disorders, drug and alcohol addiction, and other psychiatric problems in human populations (McClung, 2007; Rosenwasser, 2010).

Schizophrenia is a chronic and worldwide illness that afflicts approximately 1% of the world's population. The syndromes of schizophrenia are often characterised by a classical clinical manifestation of psychotic symptoms such as delusions and hallucinations (Kilts, 2001). The first clinical symptoms of schizophrenia typically appear between 15 and 25 years of age in men, whereas women typically exhibit an age of onset about five years later than men (Szymanski et al, 1995). Unusually high dopamine activity in the mesolimbic pathway of the brain has been found in people with schizophrenia. The mainstay of treatment is antipsychotic medication; this type of drug primarily works by suppressing dopamine activity. Schizophrenia has been linked to polymorphisms in the PER1, PER3, CLOCK and TIMELESS genes (Mansour et al, 2006; Takao et al, 2007).

Seasonal affective disorder (SAD) is a unipolar disorder that develops from not adapting to changes in the environment. Characteristics of this disorder are depressive symptoms such as low

mood, difficulties concentrating and loss of energy or interest. These symptoms only occur throughout the winter months. Fatigue, hypersomnia, and weight gain can occur in some patients with others who report insomnia and weight loss. The prevalence of SAD is between 2 and 9%. The aetiology of SAD includes genetics, neurotransmitter function, melatonin and circadian phase shifts (Westrin and Raymond, 2007). Melatonin has being hypothesized to be involved in the development of SAD. Melatonin is a circadian hormone produced by the pineal gland and is involved in inducing sleep. Levels of melatonin are low during the day and high during the night in healthy individuals. Researchers have found irregular levels of melatonin in patients with SAD. When SAD patients were compared with healthy controls, it was found that the SAD patients had consistently higher daytime melatonin levels during the winter months. High daytime melatonin levels would be expected to produce the symptoms of excessive daytime sleepiness and the lack of motivation that is seen in SAD sufferers. Melatonin therapy in the evening cause phase advance and is one treatment used in SAD patients. This therapy helps patients have an earlier sleep onset and will help them wake earlier in the morning regulating their sleep/wake cycle (Lewy et al, 2006b).

The circadian phase shift hypothesis is linked with SAD. This is where the later dawn is thought to cause a delay in circadian rhythms and a disconnection between the molecular rhythms of the SCN and the sleep/wake cycle in SAD patients. Bright light therapy is the most effective treatment for SAD patients. Bright light therapy in the early morning can cause a phase advance in the circadian system and, therefore, help by realigning rhythms with the sleep/wake cycle (McClung, 2007; Lewy et al, 2006a). Genetic studies have focused on monoamine-related genes in SAD and seasonality. Promising candidate genes include *5 HT2A* (Enoch et al, 1999), and *5-*

HT2C (Praschak et al, 2005). Clock-related genes have also been investigated (Johansson et al, 2003). Seasonal affective disorder (SAD) has been associated with *CLOCK, BMAL1, PER3,* and *NPAS2* polymorphisms. Results from a study carried out by Johansson and colleagues showed that two circadian clock-related polymorphisms, *NPAS2 471 Leu/Ser* and *Period3 647 Val/Gly*, may be implicated in SAD but further research is needed. Partonen and colleagues found variations in the three circadian clock genes - Per2, Arntl, and Npas2 - which are associated with the disease, supporting the theory that the circadian clock mechanisms contribute to seasonal affective disorder (Johansson et al., 2003; Partonen et al., 2007).

Bipolar disorder is a psychiatric mood disorder which is characterised by alternating periods of mania and depression. Treatment taken for this disorder is usually with mood stabilisers such as lithium or valproate. Lithium slows down the abnormal fast circadian rhythms which occur in bipolar patients (McClung, 2007). Genetic studies have confirmed that BPD is highly heritable, with genetic influences explaining 60–85% of variance in risk (Smoller and Finn, 2003). BPD is thought to be more prevalent in patients that are born with an arrhythmic clock. Studies have found abnormal circadian rhythms in body temperature, norepinephrine, plasma cortisol, and melatonin in bipolar patients (McClung, 2007). A significant association of *BMAL1* and *TIM* gene single nucleotide polymorphisms (SNPs) with bipolar disorder has been discovered by a study on a family based sample of bipolar patients (Mansour et al, 2006). An independent haplotype analysis confirmed the *BMAL1* association with bipolar disorder. The same study found a new association with *PER3* gene SNPs (Nievergelt et al, 2006).

The first studies to be carried out on the associations between attention-deficit disorder (ADHD) and *CLOCK* took place in 2008.This study suggested that a polymorphism (rs1801260) at the 3'-untranslated region of the *CLOCK* gene is a direct or linked contributing factor in ADHD (Kissling et al, 2008) and this finding was confirmed in a latter study (Xu et al, 2010).

1.8 Attention-deficit disorder (ADHD)

Attention-deficit hyperactivity disorder (ADHD) is a clinically heterogeneous neuropsychiatric condition characterised by inattention, impulsivity and hyperactivity (Heal et al, 2009). Children with ADHD have increased risk for low education; social dysfunction, being involved in crime (Rosler et al, 2004) and drug abuse (Bymaster et al, 2002). ADHD is the most common psychiatric disorder diagnosed in children, with a prevalence rate of 3-7% in children and adolescents (Nair et al, 2006) and around 2.5% in adults worldwide (Berman et al, 2009). DSM-IV guidelines state that diagnosis of ADHD requires the presence of six or more of nine symptoms of inattention and six or more of nine symptoms of hyperactivity and impulsivity that have persisted for six or more months.

The etiology of this disease is not well understood. Findings suggest that genetic factors play a role in the pathogenesis (Biederman et al, 2005) and its pathophysiology involves dysfunction in the central dopaminergic and norepinephrine systems (Swanson et al, 2005). Genetic studies have shown that ADHD is transmitted in families. Heritability data from twin studies of ADHD estimate that the heritability of ADHD to be, on average, 0.76. This indicates genes do have a role to play in the etiology of this disease. Further to this, adoptive relatives of ADHD children are less likely than biological relatives to have this disorder (Biederman et al, 2005). Studies

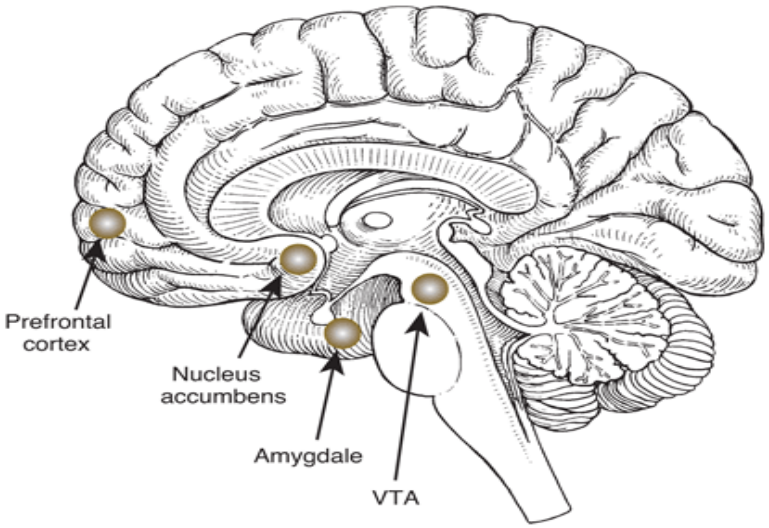
carried out have shown that ADHD patients are more likely to carry DRD4 7-repeat allele than control subjects. Therefore the DRD4 maybe a susceptibility gene for ADHD, but further studies will need to be carried out on this gene and others as no single gene has been proven as the main factor in this multifactorial disorder (Faraone et al, 2001).

1.9 ADHD and Pharmacological Treatment

Dysfunctions of catecholamine - mainly dopamine (DA) and norepinephrine systems - have been proposed to be involved in ADHD (Swanson et al, 2005). In the pre frontal cortex (PFC), dopamine controls the flow of information from other areas of the brain. Reduced dopamine in the PFC can lead to a decline in attention, problem solving, memory, and psychomotor and are thought to contribute to ADHD.

There are four major dopaminergic pathways in the brain; the mesolimbic, mesocortical, nigrostriatal and hypothalamic tubero infundibular pathway. The mesolimbic pathway projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and is involved in addiction, reward and feeding behaviour. The mesocortical pathway projects from the VTA to the cerebral cortex; the PFC regulates information, selective attention and memory in this part. The nigrostriatal system starts in the substantia nigra pars compacta (SNc) and projects to the striatum and regulates motor functions. The hypothalamictubero infundibular (HTI) pathway, which begins in the arcuate nucleus of the hypothalamus and the immediate periventricular nucleus projects mostly to the pituitary gland and is involved in the secretion of prolactin and luteinising hormone.

Figure 4: dopamine system



(Wand)

The mesolimbic and mesocortical pathways are thought to be involved in ADHD. Mesolimbic DA disorder is thought to be involved in ADHD patients. The subject wants instant reward, rather than having patience to wait for a bigger, but delayed, reward. The subject's impulsivity may be controlled by the nucleus accumbens. The nigrostriatal pathway is also thought to be involved in the pathology of ADHD in relation to hyperactivity. The DA transporter (DAT), an important regulator of DA neurotransmission, is lowered in adult ADHD patients by methylphenidate in the striatum (Kooij and Glennon, 2007). Norepinephrine plays a role in attention, and the maintenance of arousal, a cognitive function which is deficient in ADHD (Biederman et al, 1999). Studies that have been carried out have shown that increasing levels of norepinephrine can reduce the levels of impulsivity in ADHD (Robinson et al, 2008).

The main treatments for ADHD have been psychostimulant drugs, such as methylphenidate (Ritalin) which has been used for over forty years (Biederman et al, 2005) and d-amphetamine. Methylphenidate and amphetamine treatment is 70-80% effective in treating ADHD patients. It reduces ADHD core symptoms and produces clinical improvements. If the patient does not

respond to the psycostimulant, using an alternative psycostimulant can increase the response rate to 90% (Heal et al, 2009). Methylphenidate is known to inhibit dopamine (DAT) and norepinephrine (NET) transporters (Han and Gu, 2006; Bymaster et al, 2002), therefore enhancing catecholamine neurotransmission (increasing levels of dopamine and noradrenaline) in different cortical and subcortical regions (Seu et al, 2008).

In the last decade, research was carried out in developing a non-stimulant alternative for treating ADHD patients, because some patients did not respond or could not tolerate stimulants. Also stimulant drugs may cause side effects such as weight loss, delayed growth and tics (Biederman, 2005; Faraone et al, 2005; Popper, 2000). The pharmacological profile of methylphenidate is similar to that of cocaine, and could suggest potential for abuse (Kollins et al, 2002). Drugs such as the stimulant methylphenidate that act on the DAT increase dopamine in the nucleus accumbens and striatum associated with abuse potential (Carboni et al, 2006).

Atomoxetine is a non-stimulant drug and is a highly selective inhibitor of the noradrenergic transporter (NET) and was approved in the United States in 2002 for the treatment of ADHD (Newcorn et al, 2008). Atomoxetine has a response rate of 50-60% in ADHD patients (Michelson et al, 2002). It is taken as a once daily dose in the morning. Atomoxetine has a different pharmacological profile, acting primarily through the noradrenergic system. Atomoxetine increases noradrenaline concentrations in multiple brain regions implicated in the actions of ADHD drugs but it only increases dopamine concentrations in the PFC. Since atomoxetine does not increase dopamine in the nucleus accumbens, the region which is associated with psycostimulation and rewarding behaviours, atomoxetine would not be expected to have the same drug abuse danger found with psycostimulants (Bymaster et al, 2002; Swanson et al, 2006; Heal et al, 2009).

Reboxetine is another non-stimulant, NET inhibitor drug used in the treatment of depression. It is rapidly absorbed in humans, with an elimination half life of 13 hours, making reboxetine suitable for twice daily dosing (Kasper et al, 2000; Dostert et al, 1997). Reboxetine acts on NET increasing norepinphrine which helps to restore the balance of this neurotransmitting agent inside and outside cells in the nervous system.

Developments of animal models which resemble the clinical characteristics of ADHD are essential to optimise and validate pharmaceutical drug research. Therefore, the assessment of which animal model has the greatest validity is of vital importance. Rat and mouse models have been used in ADHD research. Rat models include spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) ADHD models, and mouse models are usually based on so-called knockout technology such as mice models *DAT, NK1* receptor and organic cation transporter 3(OTC3) knockouts (Thome and Reddy, 2009).

The SHR which was developed in the 1960s is the most widely studied animal model used for ADHD. The SHR model parallels ADHD in humans since they display hyperactivity, impulsivity and poor performance (Davids et al, 2003; Boix et al, 1998). The DAT knockout (KO) and the DAT knock down (KD) mice were developed to better understand ADHD pathophysiology. The DAT-KO and DAT-KD mouse model showed that downregulated DAT results in an attenuated reuptake of DA leads to hyperactivity due to the increased extracellular levels of DA (Kooij and Glennon, 2007). The DAT-KO model has disadvantages as an ADHD model due to the fact that DAT the possible primary target is missing therefore research into treatments using psychostimulants on DAT is hindered.

The DAT-KD mouse model used by Zhuang and colleagues overcame some of the problems seen with the DAT-KO. The DAT-KD does not have growth retardation and is not associated

with premature death seen in the DAT-KO animals. The DAT-KD model is a knock down of the gene for the dopamine transporter. This mutation resulted in only 10% of dopamine transporters in the animal brain, which resulted in an increase in extracellular dopamine levels in the brain. This meant that psychostimulants targeting the DAT could be tested (Zhuang et al, 2001). The DAT-KO and DAT-KD animal models both present hyperactivity and impulsivity. Predictive validity in these models was demonstrated as amphetamine inhibited increased activity behaviour in DAT-KO and DAT-KD while methylphenidate lowers activity in the DAT-KO as well (Kooij and Glennon, 2007).

1.10 ADHD and Circadian Rhythms

Many common psychiatric diseases are characterised by an early onset of sleep problems and breakdown of stable circadian rhythms (McClung, 2007). A rapidly evolving research area is ADHD and circadian rhythmicity which may have important clinical implications. ADHD patients with irregular sleeping patterns, such as difficulty falling asleep at night and wakening in the morning are suggestive of a delayed phase in circadian organisation of sleep and wakening. Adult patients with ADHD are more at risk for general physical health problems that may be related to disorder of biological rhythms (Boonstra et al. 2009).

The emerging field of the role of circadian rhythms in ADHD was the focus of a study carried out by Kooij and colleagues (2009). Based on the observation that many adult patients with ADHD suffer from a troubled sleep pattern, Kooij further studied whether such patients prefer mornings or evenings regarding their daily activity patterns using standardised questionnaires and actigraphy using actiwatches. The actiwatches were worn by the individuals taking part in

the study over a prolonged period of time; they registered activity and rest patterns. Results confirmed that adults with ADHD prefer evening. About 80% of adult patients with ADHD exhibit a chronically delayed sleep pattern. Additionally, melatonin expression levels were significantly altered (Kooij et al, 2009). As discussed previously the psychostimulant drug methylphenidate has been found to improve circadian related sleep problems in ADHD patients. The number and total duration of nocturnal awakening decrease using methylphenidate, improving the sleep quality. (Boonstra et al, 2007).

A study comparing the effects of atomoxetine and methylphenidate on sleep in children with ADHD showed that patients who received twice daily doses of atomoxetine had shorter sleep onset latencies than patients who received twice daily doses of methylphenidate. Methylphenidate did however decrease night time awakening more than atomoxetine (Sangal et al, 2006). However, questions remain on the impact of stimulant drugs such as methylphenidate on the timing and regularity of the circadian motor activity levels. A study carried out in 2008 showed that children with ADHD that took methylphenidate were found to have significant reductions in sleep duration and significant increases in sleep-onset latency during treatment with stimulant medication (Corkum et al, 2008).

Another study was carried out examining measurable changes in circadian rhythms in children with ADHD that were been treated by methylphenidate. Actigraphy was used in this study to monitor circadian rhythms in children as it is found to be a valid and reliable way to examine changes in sleep and circadian activity rhythms in children. The results suggest that strength of the circadian rhythm is reduced and the timing of the daily peak activity level was delayed in the children receiving methylphenidate. These changes in the circadian rhythm may account for the

delayed sleep-onset and symptoms of insomnia experienced at bedtime by a large percentage of children with ADHD being treated with methylphenidate (Ironside et al, 2010).

Molecular studies have also established phase delay in circadian organisation in ADHD patients. Melatonin is used to treat circadian rhythm sleep disorders. Melatonin is a hormone which is secreted at night by the pineal gland and is involved in the sleep/wake cycle (Garidou et al, 2002; Vivanco et al, 2007). The SCN manages the circadian rhythmicity of pineal synthesis of melatonin (Challet, 2007). Levels of melatonin are low throughout the day with a sharp rise in evening. However studies have shown that the increase of melatonin at night time is delayed in ADHD children (O'Brien et al, 2003). This can provide further evidence of a circadian phase delay in patients with ADHD (Van Veen et al, 2010). Studies show that the length of time it takes to get to sleep is reduced when a patient who is taking methylphenidate is administered melatonin (Tjon Pian et al, 2003).

Abnormal sleep patterns due to dysfunction of circadian timing may be the primary cause of ADHD, which then would lead to secondary symptoms of inattention, hyperactivity and impulsivity that certain researchers have proposed (Boonstra et al, 2007).

As there is evidence that ADHD might be associated with misalignment of the circadian clock with the environment, atomoxetine might ameliorate some of the symptoms of ADHD by re-aligning the clock.

1.11 Aims of this Project

- To examine the effects of non- psycho stimulant ADHD medication on circadian rhythms in mice. Atomoxetine is used as alternatives to psycostimulant medication in the treatment of ADHD will be monitored to see any effects it will have on circadian rhythms. Reboxetine, another NET inhibitor, will also be examined for comparison.

- To examine the effects of atomoxetine on locomotor circadian rhythms in mice under free- running conditions of constant light or constant darkness.
- Examine if acute treatments with atomoxetine/reboxetine during the subjective day (CT6) or subjective night (CT18) produces any alternations in the circadian rhythms in mice.

- Examine if treatment with atomoxetine/reboxetine alters c-Fos and clock gene expression in the master circadian clock (SCN) of mice.

- The pharmacological nature of atomoxetine actions will be looked at, using the antagonist prazosin to see if it blocks the effects of atomoxetine. Prazosin is an alpha adrenergic blocker.

2. Materials & Methods

2.1 Animals and housing

The animal model used in this study is the C57BL/6 strain of mouse. This study requires the use of an animal model as we monitor rhythmicity at the whole animal level and C57/BL6 mice are now well characterised from a chronobiological aspect. Mice are key models in behavioural pharmacology, and the pharmacokinetics of drugs that are used in this study are well characterised in this species. Thirty-two, male C57BL/6 mice (3-5 months old; 24-30g) were used for all studies carried out. They were obtained from Harlan Laboratory a designated breeding establishment (Leicester, UK). The animals were individually housed in polypropylene cages (33cm long x 15cm wide x 13cm high). The cages were equipped with steel running wheels (11.5cm diameter) and their daily rhythms of activity were constantly recorded via microswitches connected to a data acquisition system computer using the Chronobiology Kit by Stanford System (Santa Cruz, California). The lighting conditions were adjusted via a timer on the outside of the cabinet therefore not requiring opening the cabinet unnecessarily. The inside of the cabinets were black and non- reflective in order to minimise reflection of light and guarantee similar lighting conditions for each cage. Food and water were accessible *ad libitum.* Animals were housed at an ambient temperature $23 \pm 2\,^{\circ}C$ and a constant humidity of 40%. The luminance levels in the individual cages were on average 200 lux and were ventilated via axial fans to prevent the build up of pheromones. All protocols were approved by the Research Ethics Committee at the National University of Ireland, Maynooth and licensed by the Department of Health and Children. All animals will be treated in accordance with the Cruelty to Animals Act, 1876 and the SI No.17 – European Communities (Amendment of Cruelty to Animals Act, 1876)

regulations, 1994 (European Directive 86/609/EC). To minimise the numbers of animals used immunohistochemical analysis was undertaken on brains from the same animals used for behavioural monitoring.

2.2 Drugs

2.2.1. Atomoxetine

The noradrenaline reuptake inhibitor Atomoxetine Hydrochloride was provided by Tocris, UK. Atomoxetine was dissolved in a 0.9% isotonic saline solution (0.9% NaCl). Atomoxetine was administered at a dose of 3mg/kg (Swanson et al, 2006; Bymaster et al, 2002; Tzavara et al, 2006, and Heal et al, 2009). The injections were administered intraperitoneal (i.p) and were all equalised to the same volume of 0.12 mls. Control injections of 0.12 mls of saline were also used.

2.2.2. Reboxetine

Another noradrenaline reuptake inhibitor reboxetine was purchased from Tocris, UK. Reboxetine was dissolved in 0.9% isotonic saline solution (0.9% NaCl). Reboxetine was administered at a dose of 20mg/kg (Cryan et al, 2004; Wasylewska et al, 2006). The injections were administered intraperitoneal (i.p) and all injections had a volume of 0.125 mls. Control injections of 0.125 mls of saline were also used.

2.2.3. Prazosin

Prazosin 100mg was obtained from Tocris, UK. Prazosin was firstly dissolved in 10mls of DMSO (Sigma) (10mg/ml). 1ml of this stock was than diluted with 9mls of 0.9%NaCl to give 1mg/ml. Each injection volume was 0.1ml. 0.1mg/animal works out at 4mg/kg for 25g mouse.

2.3 Experimental procedure for behavioural analysis

2.3.1 Atomoxetine administered in LL

Design

In the first and third part of this experiment a within subject design was used. The independent variable was the drug treatment which was either an atomoxetine injection or a saline injection, which were administered two weeks apart. The dependent variables were the magnitude of the phase shift and change in the rhythm amplitude and free running period.

In the second part of the experiment, a between subjects design was used. The independent variable was the drug treatment. The mice were divided into two groups; the treatment groups (n=5) were administered atomoxetine and the control group (n=3) were administered saline. The dependent variables were the magnitude of the phase shift and change in the rhythm amplitude and free running period.

Procedure

Animals were housed in a light–dark cycle (12:12 LD) for two weeks and were then exposed to constant light (LL) for the duration of the study. Animals free ran in LL for two weeks before any treatments were administered. The locomotor activity was continually monitored and recorded for the duration of this study. The aim of the first part of the experiment is to determine whether administration of atomoxetine effects locomotor activity rhythms when administered during the animal's inactive phase (CT6) compared to the control saline. The mice free ran in LL for two weeks before each animal was administered with atomoxetine (3mg/kg; i.p), during their inactive period (CT6). CT12 is defined as the onset of their activity. The CT6 point was

extrapolated for each animal individually, from an actogram of their baseline times of activity onset. The animals free ran for another two weeks and then received an injection of saline. The saline injection was again administered at CT6 and the animals free ran for a further two weeks.

In the second part of the experiment the aim was to determine whether atomoxetine affects the locomotor activity rhythm when administered near the end of the animal's active phase at CT18. The within groups design divided the eight mice into a treatment group (n=5) and a control group (n=3). CT18 was calculated by extrapolating the time of the activity onset from the actogram. The activity of the mice was than monitored for ten days.

In the final part of the experiment the aim was to investigate whether atomoxetine administered near the beginning of the animal's active phase at CT13 affects the locomotor activity rhythms. CT13 was calculated by extrapolating the time of the activity onset from the actogram. The activity of the mice was than monitored for ten days.

Data analysis

Circadian activity was monitored using the Chronobiology kit (Stanford Systems), which plotted actograms of circadian patterns of activity. Phase shifts were calculated on the actogram by using a line that best fit through the onset of activity, for one week prior to the treatment and to one week post treatment. The difference between these two lines was calculated and the phase shifts were calculated independently by three researchers and then averaged to minimize any experimenter bias.

The chi squared procedure was used to assess any possible changes in the rhythmicity, for example the peak period or the rhythms amplitude, caused by the administration of either

atomoxetine or saline. The period and amplitude for one week prior to treatment and to one week post treatment were compared.

Statistical analysis

In the first and third part of the experiment a paired t-test was used to assess the difference between the phase shifts caused by i.p administration of atomoxetine compared to that of the control saline. Also a paired t-test was used to examine any differences in the period and amplitude of rhythm, pre treatment and post treatment. An independent t-test was used in the second part of the experiment to examine the phase shifts observed in the in the atomoxetine treatment group compared to that seen in the control group. A Wilcoxon Signed rank test was used to compare the period and amplitude of the rhythms, pre-treatment and post-treatment, as these results were not normally distributed. A paired t-test was then used to assess the effect of a single i.p. administration of atomoxetine at CT6 compared to the effect of administration of atomoxetine at CT18. Bonferroni corrections for multiple comparisons were applied as appropriate.

2.3.2 Atomoxetine administered in DD

Design

This experiment was a within subject design like in the previous experiment. The independent variable was the drug treatment which was either an atomoxetine injection or a saline injection, which were administered two weeks apart. The dependent variables were the magnitude of the phase shift and change in the rhythm amplitude and free running period.

In the second part of the experiment, a between subjects design was used. The independent variable was the drug treatment. The mice were divided into two groups; the treatment groups (n=5) were administered atomoxetine and the control group (n=3) were administered saline. The dependent variables were the magnitude of the phase shift and change in the rhythm amplitude and free running period.

Procedure

Animals were housed in a light–dark cycle (12:12 LD) for two weeks and were then exposed to constant darkness (DD) for the duration of the experiment. Animals free ran in DD for two weeks before any treatments were administered. The locomotor activity was continually monitored and recorded for the duration of this study.

The aim of the first part of the experiment is to determine whether administration of atomoxetine effects locomotor activity rhythms when administered during the animal's inactive phase (CT6) compared to the control saline. The mice free ran in DD for two weeks before each animal was administered with atomoxetine (3mg/kg; i.p), during their inactive period (CT6). The CT6 point was extrapolated for each animal individually, from an actogram of their baseline times of

activity onset. The animals free ran for another two weeks and then received an injection of saline. The saline injection was again administered at CT6 and the animals free ran for a further two weeks.

In the second part of the experiment the aim was to determine whether atomoxetine affects the locomotor activity rhythm when administered during the animal's active phase at CT18. The within groups design divided the eight mice into a treatment group (n=5) and a control group (n=3). CT18 was calculated by extrapolating the time of the activity onset from the actogram. The activity of the mice was than monitored for ten days.

Data analysis

Circadian activity was monitored using the Chronobiology kit (Stanford Systems), which plotted actograms. Phase shifts were calculated on the actogram by using a line that best fit through the onset of activity, for one week prior to the treatment and to one week post treatment. The difference between these two lines was calculated and the phase shifts were calculated independently by two researchers and then averaged to minimize any experimenter bias. The chi squared procedure was used to assess any possible changes in the rhythmicity, for example the peak period or the rhythms amplitude, caused by the administration of either atomoxetine or saline. The period and amplitude for one week prior to treatment and to one week post treatment were compared.

Statistical analysis

In the first part of the experiment a paired t-test was used to assess the difference between the phase shifts in DD caused by i.p administration of atomoxetine compared to that of the control saline. Also a paired t-test was used to examine any differences in the period and amplitude of rhythm, pre treatment and post treatment. An independent t-test was used in the second part of the experiment to examine the phase shifts observed in the in the atomoxetine treatment group compared to that seen in the control group. A wilcoxon signed rank test was used to compare the period and amplitude of the rhythms, pre-treatment and post-treatment, as these results were not normally distributed. A paired t-test was then used to assess the effect of a single i.p. administration of atomoxetine at CT6 compared to the effect of administration of atomoxetine at CT18.

2.3.3 Reboxetine administered in LL

Design

In this experiment a simple crossover design is used, half the subjects get a control treatment followed by an experimental treatment, while the other half get the treatments followed by the control. The independent variable was the drug treatment which was either a reboxetine injection or a saline injection, which were administered two weeks apart. The dependent variables were the magnitude of the phase shift and change in the rhythm amplitude and free running period.

Procedure

Animals were housed in a light–dark cycle (12:12 LD) for two weeks and were then exposed to constant light (LL) for the duration of the experiment. Animals free ran in LL for two weeks before any treatments were administered. The locomotor activity was continually monitored and recorded for the duration of this study.

The aim of this experiment was to determine whether administration of another noradrenaline reuptake inhibitor reboxetine effects locomotor activity rhythms when administered during the animal's inactive phase (CT6) compared to the control saline. The mice free ran in LL for two weeks before reboxetine (n=4) (20mg/kg; i.p) and saline (n=4) was administered, during their inactive period (CT6). The CT6 point was extrapolated for each animal individually, from an actogram of their baseline times of activity onset. The animals free ran for another two weeks and then a cross over took place, with the previous mice who received reboxetine than receiving saline (n=4) and the previous mice who received saline two weeks previous being administered with reboxetine (n=4). The injection was again administered at CT6 and the animals free ran for a further two weeks.

Data analysis

Circadian activity was monitored using the Chronobiology kit (Stanford Systems), which plotted actograms. Phase shifts were analysed on the actogram by using a line that best fit through the onset of activity, for one week prior to the treatment and to one week post treatment. Then the difference between these two lines was calculated and the phase shifts were calculated independently by two researchers and then averaged to minimize any experimenter bias. The chi squared procedure was used to assess any possible changes in the rhythmicity, for example the peak period or the rhythms amplitude, caused by the administration of either reboxetine or saline. The period and amplitude for one week prior to treatment and to one week post treatment were compared.

Statistical analysis

In this experiment a paired t-test was used to assess the difference between the phase shifts in LL caused by i.p administration of prazosin compared to that of the control saline in relation to atomoxetine. Also a paired t-test was used to examine any differences in the period and amplitude of rhythm, pre treatment and post treatment.

2.3.4 Prazosin administered prior to atomoxetine in LL

Design

In this experiment a simple crossover design is used, half the subjects get a control treatment followed by an experimental treatment, while the other half get the treatments followed by the control. The independent variable was the drug treatment which was either a prazosin injection or a saline injection prior to atomoxetine, which were administered two weeks apart. The dependent variables were the magnitude of the phase shift and change in the rhythm amplitude and free running period.

Procedure

Animals were housed in a light–dark cycle (12:12 LD) for two weeks and were then exposed to constant light (LL) for the duration of the experiment. Animals free ran in LL for two weeks before any treatments were administered. The locomotor activity was continually monitored and recorded for the duration of this study. The aim of this experiment was to determine whether administration of the alpha-1 receptor prazosin 15 minutes prior to the administration of atomoxetine, blocked the effect of the atomoxetine when administered during the animal's inactive phase (CT6). Prazosin belongs to the class of alpha adrenergic blockers.

The mice free ran in LL for two weeks, before prazosin (n=4) and saline (n=4) were administered 15 minutes prior to an injection of atomoxetine given to each animal (n=8), during their inactive period (CT6). The animals free ran for another two weeks and then a cross over took place, with the previous mice who received prazosin than receiving saline (n=4) and the previous mice who received saline two weeks previous being administered with prazosin (n=4),

before all mice received another injection of atomoxetine (n=8) 15 minutes later.. The injection was again administered at CT6 and the animals free ran for a further two weeks

Data analysis

Circadian activity was monitored using the Chronobiology kit (Stanford Systems), which plotted actograms. Phase shifts were analysed on the actogram by using a line that best fit through the onset of activity, for one week prior to the treatment and to one week post treatment. Then the difference between these two lines was calculated and the phase shifts were calculated independently by two researchers and then averaged to minimize any experimenter bias. The chi squared procedure was used to assess any possible changes in the rhythmicity, for example the peak period or the rhythms amplitude, caused by the administration of either reboxetine or saline. The period and amplitude for one week prior to treatment and to one week post treatment were compared.

Statistical analysis

In this experiment a paired t-test was used to assess the difference between the phase shifts in LL caused by i.p administration of prazosin compared to that of the control saline in relation to atomoxetine. Also a paired t-test was used to examine any differences in the period and amplitude of rhythm, pre treatment and post treatment.

2.4 Immunohistochemistry

The following standard immunohistochemistry protocol was used for the analysis of immediate early and clock genes in the SCNs of animals.

1. Mice were anesthetized using chloroform before cervical dislocation took place. Mice brains were removed (from the LD, LL and DD experiments) and stored in 4% paraformaldehyde (PFA) (Aldrich) for two days and then transferred to 30% sucrose (Sigma). Following fixation, brains were sectioned 30µm using a freezing stage microtome. Sections were placed in phosphate buffer (Sigma) (0.1M) with two drops of (0.01%) sodium azide (Sigma) and stored at 4°C for up to three week. Sodium azide inhibits bacterial growth.

2. The sections were washed in (0.1M) PB and PBX which contained (0.03% triton X-100 Sigma).

3. Sections were then washed in PB and H_2O_2 (Sigma) which stops enzymes and reduces non- specific background staining. Further PB and PBX washes were carried out.

4. The sectioned were blocked in 5% normal goat serum (NGS) (Sigma) for an hour.

   The sections were then incubated with a primary antibody (rabbit polyclonal) diluted in PBX and 2% NGS overnight or 48 hours. Primary antibodies (rabbit polyclonal IgG) used were c-Fos (1:2000), Per2 (1:500), and B-MAL1 (1:500) (Santa Cruz) and a goat polyclonal anti-CLOCK antibody (Santa Cruz; 1:500).

5. Sections were washed again with PB and PBX and were then incubated with the secondary antibody biotinylated goat anti rabbit (Jackson Immuno research Labs) diluted

1:400 in PB and 2% (NGS) for 70 minutes. For anti-CLOCK an anti-goat biotinylated secondary was used in rabbit serum.

6. Sections were incubated with vectastain ABC solution (Vector) Rabbit IgG for 90 minutes.

7. Immunoreactivity was visualized by diaminobenzidine (DAB) reaction, made up with DAB (Aldrich), sodium acetate (Sigma), nickel sulphate (Sigma), glucose (Merck) and ammonium chloride.

The brain sections were mounted on gelatinized slides. The slides were put through a serious of ethanol and dehydration steps and were then cover slipped.

2.4.1. Atomoxetine at CT6 in Light/dark

Procedure

Eight mice were group housed under a light dark cycle (12:12), and received an i.p dose of atomoxetine (n=4) or saline (n=4) at CT6, followed by removal at CT8 and fixation of their brains for immunohistochemistry to examine if treatment with atomoxetine alters c-Fos gene expression in the SCN of mice.

Data analysis

Brain sections were examined under a microscope connected to a computer monitor. The numbers of immunoreactive c-Fos, PER2, BMAL1 or CLOCK cells per SCN were counted by an observer blind to the treatment. The SCN was divided into three anatomical regions for counting, the rostral SCN, mid SCN and the caudal SCN.

Statistical Analysis

An independent t-test was used to examine the expression of c-Fos in the SCN observed in the atomoxetine treatment groups compared to the saline control groups.

2.4.2 Atomoxetine/Reboxetine at CT6 in LL/DD

Procedure

Eight mice were group housed under LL, and received an i.p dose of atomoxetine (n=4) or saline (n=4) at CT6, followed by removal at CT8 and fixation of their brains for Immunohistochemistry. The antibodys c-Fos, CLOCK, PER2 and BMAL1were tested, to examine if treatment with atomoxetine alters gene expression in the SCN of mice.

Data analysis

Brain sections were examined under a microscope connected to a computer monitor. The numbers of immunoreactive cells per SCN were counted by an observer blind to the treatment. The SCN was subdivided into three sections again; the rostral SCN, mid SCN and the caudal SCN.

Statistics

Independent t-test‘s was used in this experiment to assess the difference between the number of immunoreactive cells per SCN caused by i.p administration of atomoxetine compared to that of the control saline.

2.4.3 Prazosin prior to atomoxetine in LL

Procedure

Eight mice were group housed under LL, and received either an i.p dose of prazosin (n=4) or saline (n=4) 15 minutes prior to an injection of atomoxetine at CT6, followed by removal of their brains at CT8 and fixation for Immunohistochemistry. Antibodies; c-Fos, CLOCK, PER2 and BMAL1 were used. The antagonist prazosin was used to see if it blocks the effects of atomoxetine in gene expression in the SCN in mice.

Data analysis

Again the brain sections were examined under a microscope connected to a computer monitor. The numbers of immunoreactive cells per SCN were counted by an observer blind to the treatment. The SCN was subdivided into three sections again; the rostral SCN, mid SCN and the caudal SCN.

Statistics

Independent t-test‘s was used in this experiment to assess the difference between the number of immunoreactive cells per SCN caused by i.p administration of prazosin/atomoxetine compared to that of the saline/atomoxetine.

3. Results

Mice were analysed in different photic conditions either LL/DD to allow the animals to express a free running rhythm in the absence of external time cues. Mice were examined with acute treatments of either atomoxetine/reboxetine during the subjective day (CT6) or subjective night (CT18) to see if it produced any alternations in the circadian rhythms in mice do to the time the drug was administered.

3.1 Behavioural analysis: Mice in Light/dark cycle and constant light (LL)

Behavioural analyses of mice were monitored using wheel running activity and their daily rhythms of activity were constantly recorded. The mice were first entrained under a light/ dark cycle (L/D 12:12) for two weeks and then were put into constant light (LL). After a week in LL mice locomotor activity developed a free running rhythm, with the average period increasing from 24.08 +/-0.30h to 25.10 +/-0.48h, (Figure1). LL also reduced the robustness of the rhythm, with the amplitude being reduced from around 772.31 (+/-103.97) to 575.5 (+/-30.25).

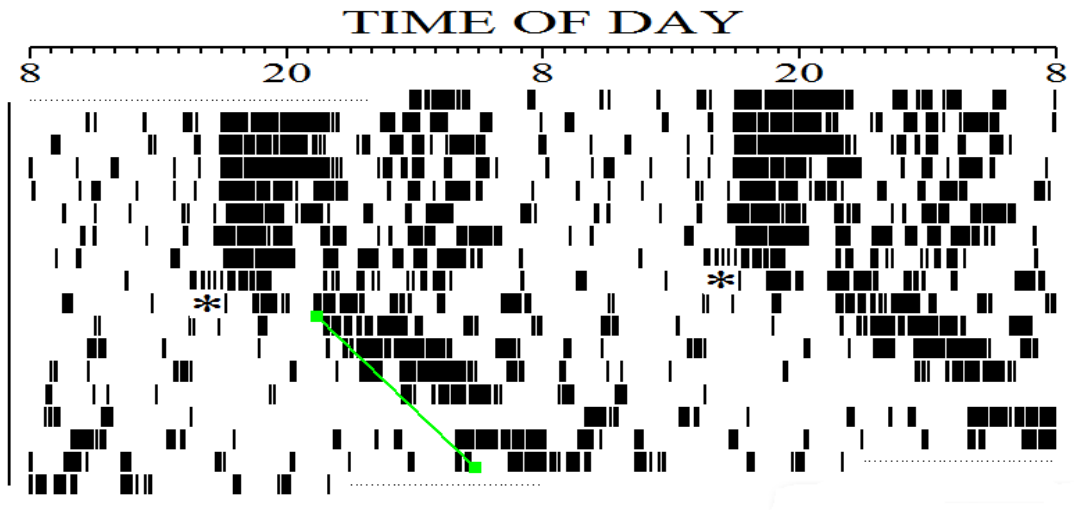


Figure 5: *An actogram which shows for 8 days light/dark cycle 12:12. When put into constant light conditions (LL) the mice started to free run. The green line represents the lengthening of the period after the introduction to LL.*

3.2 The effect of atomoxetine administration in LL

3.2.1 The effects of atomoxetine administration at CT6 in LL

A single i.p injection of atomoxetine and the control saline was administered at CT6, and the effect on their locomotor activity was assessed using a paired t-test. Atomoxetine produced a large phase delay (mean of -3.15+/- 0.27h) of the circadian locomotor rhythm in the mice compared with the saline control (-0.61+/-0.18), which was shown to be statistically significant (t=-6.89; P<0.001; Figure 6).

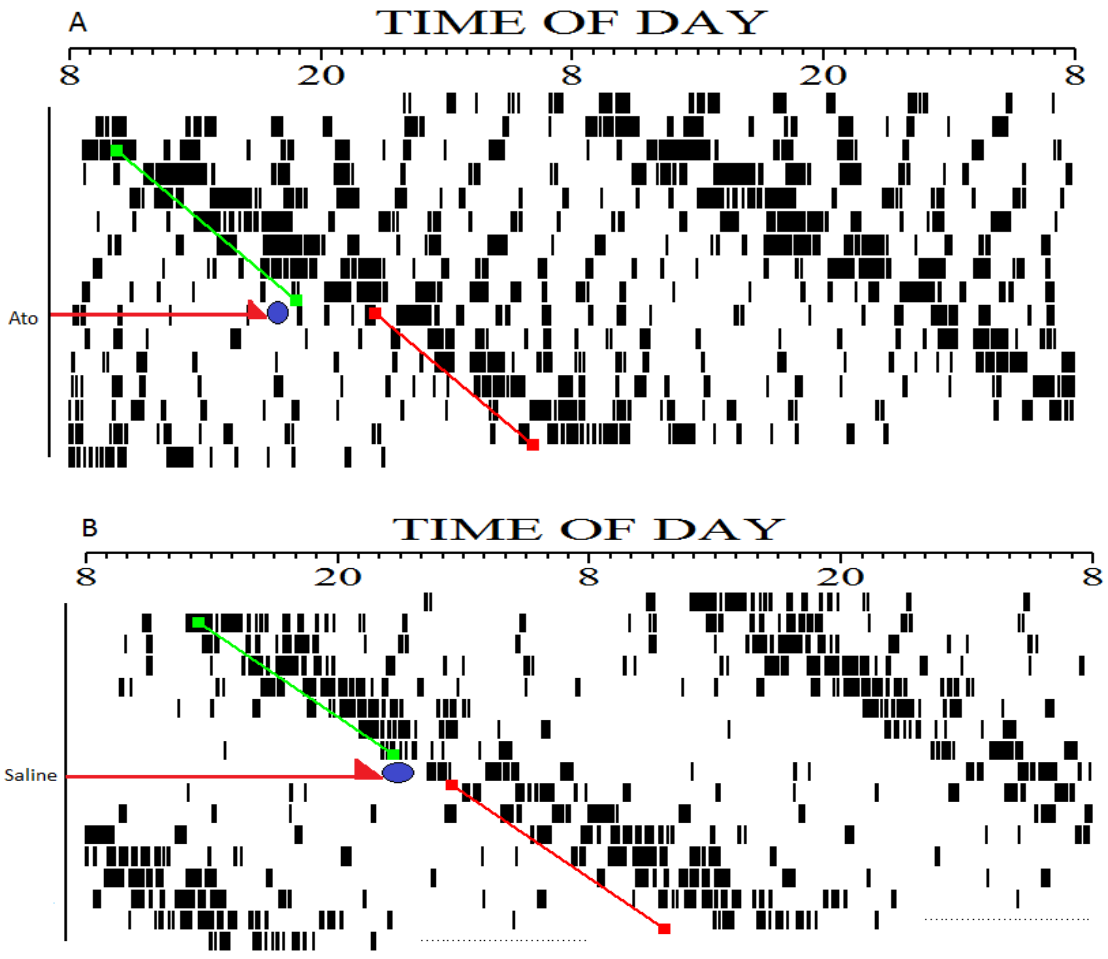


***Figure 6**: Double plotted actograms illustrating that administration of Ato at CT6 in LL induces large magnitude phase delays (A) whilst administration of saline does not (B). The green lines show the line of best fit through the onsets prior to treatment, the red lines after treatment.*

The animal's behaviour was observed post treatment to see if drug administration had any notable effect. No observable behavioural chances took place in either the saline or the atomoxetine treated animals at CT6 or CT18. Also the atomoxetine treatments did not induce wheel running activity in the period following treatment.

The effect of atomoxetine on the rhythmicity of the animal's locomotor activity was also assessed. A paired t-test was used to examine if there was a change in amplitude from pre-treatment (574.46 +/-30.35) to post treatment (555.96+/-27.73; Figure 7), and to examine if there was a change in the period from pre-treatment (25.96+/-0.18) to post treatment (22.22+/-0.43; Figure 8). There was no significant change in the amplitude (t=0.65; P =0.54), or the period (t=-0.651; P =0.539) found due to the administration of atomoxetine.

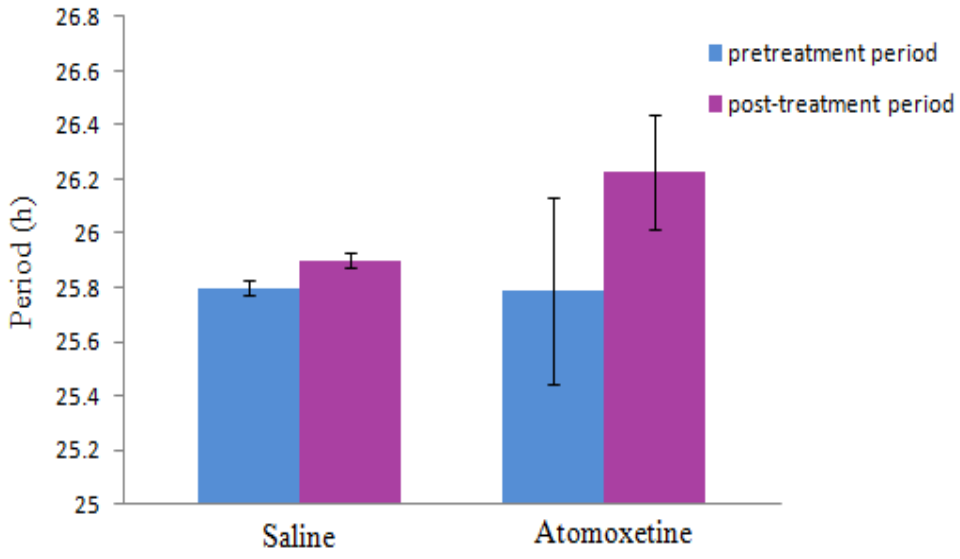


Figure 7: *The mean period pretreatment and post treatment with atomoxetine or saline at CT6.*

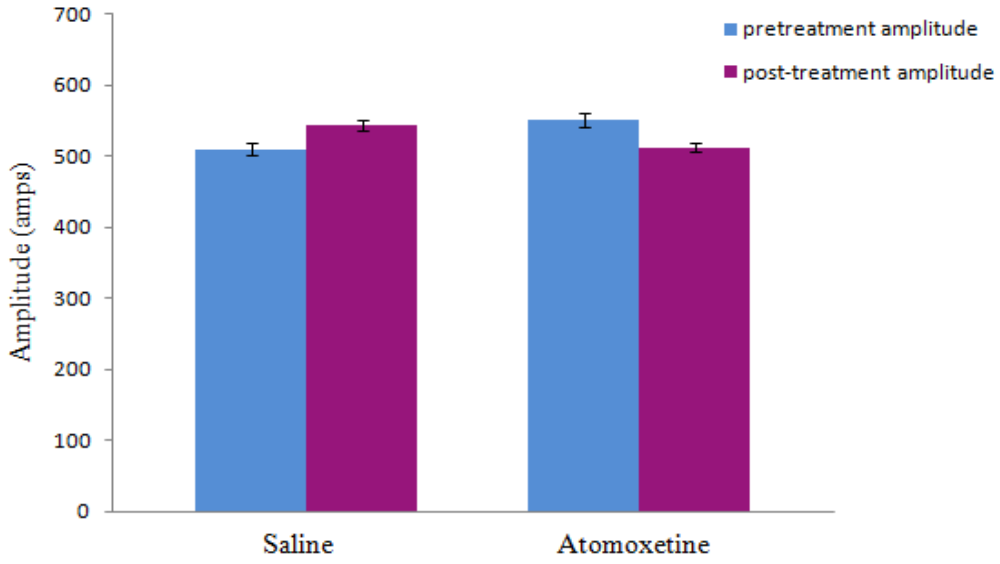


Figure 8: *The mean amplitude pre treatment and post treatment with atomoxetine and saline at CT6.*

3.2.2 <u>The effects of atomoxetine administration at CT18 in LL</u>

The effect of atomoxetine on circadian timing during the animal's late active phase at CT18 was then assessed using an independent t-test. A modest delay was observed in the atomoxetine group (-1.15h+/- 0.25h) relative to that of the saline group (-0.53h +/-0.35h; Figure 9), and this was found not to be statistically significant (t=-2.58; P =0.082). The effects of the administration of atomoxetine on the rhythmicity of the animal's circadian rhythm at CT18 was also measured. The data was not normally distributed therefore a wilcoxon signed rank test was used to see if there was a change in amplitude pre-treatment (631.00+/-195.72) to post treatment (552.06+/-92.45; Figure 10), and the period pre- treatment (25.79+/-0.35) to post treatment (25.73+/-0.76;

Figure 11). There was no significant change in the amplitude (P=0.893) or the period (P=0.225) as a result from atomoxetine treatment.

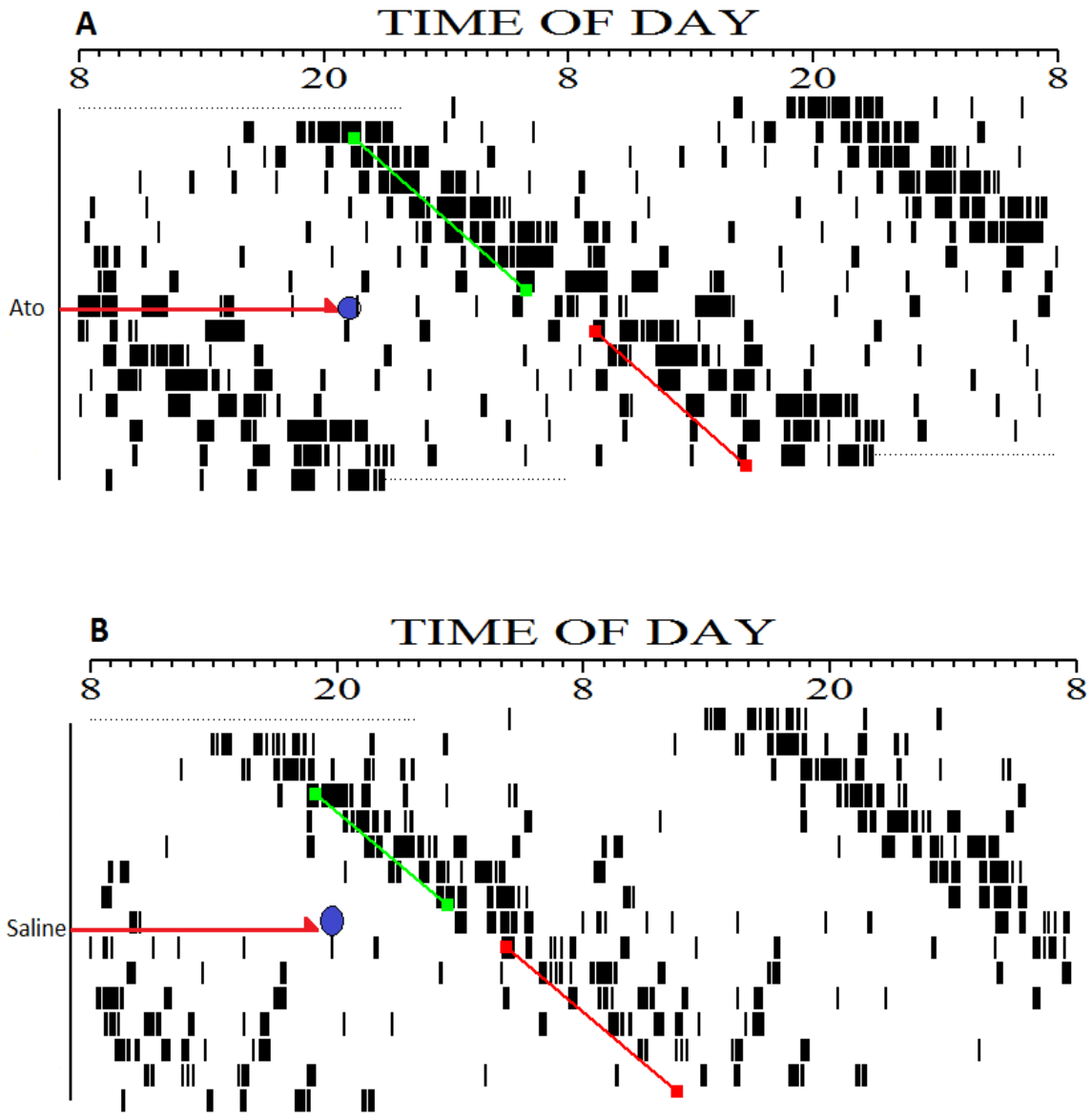


Figure 9*: Double plotted actograms illustrating that administration of Ato at CT18 in LL induces a slight phase delays (A) whilst administration of saline does not (B). The green lines show the line of best fit through the onsets prior to treatment, the red lines after treatment.*

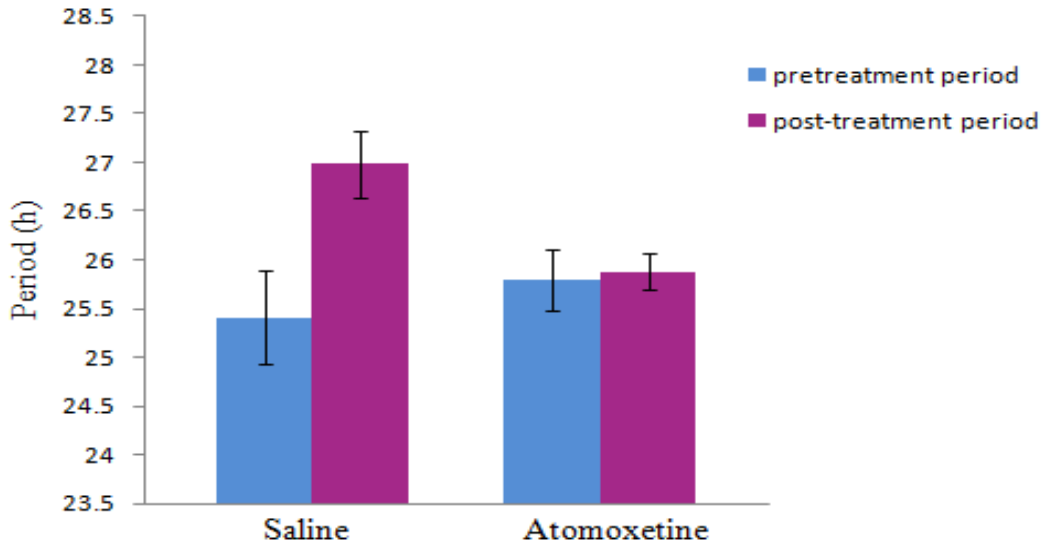


Figure 10: *The mean period pre treatment and post treatment with atomoxetine and saline at CT18.*

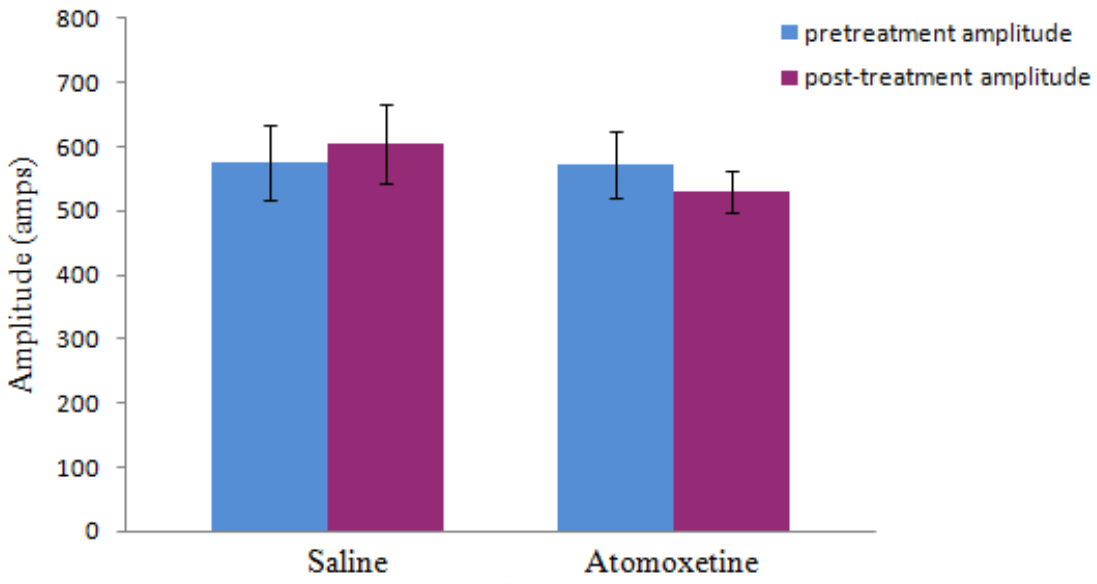


Figure 11: *The mean amplitude pre treatment and post treatment with atomoxetine and saline at CT18.*

3.2.3 The effects of atomoxetine at CT6 compared to administration at CT18 in LL

The final part of the experiment was examining the effect of a single i.p. administration of atomoxetine at CT6 compared to the effect of atomoxetine administration at CT18 using a paired t-test. Atomoxetine administered at CT6 causes a large phase delay of the circadian rhythm (-3.15+/- 0.27h) compared to the effect of atomoxetine at CT18 (-1.15h+/- 0.25h), as discussed previously. The difference between atomoxetine at CT6 v CT18 was found to be statistically significant (t=-4.365; P <0.011; Figure 12).

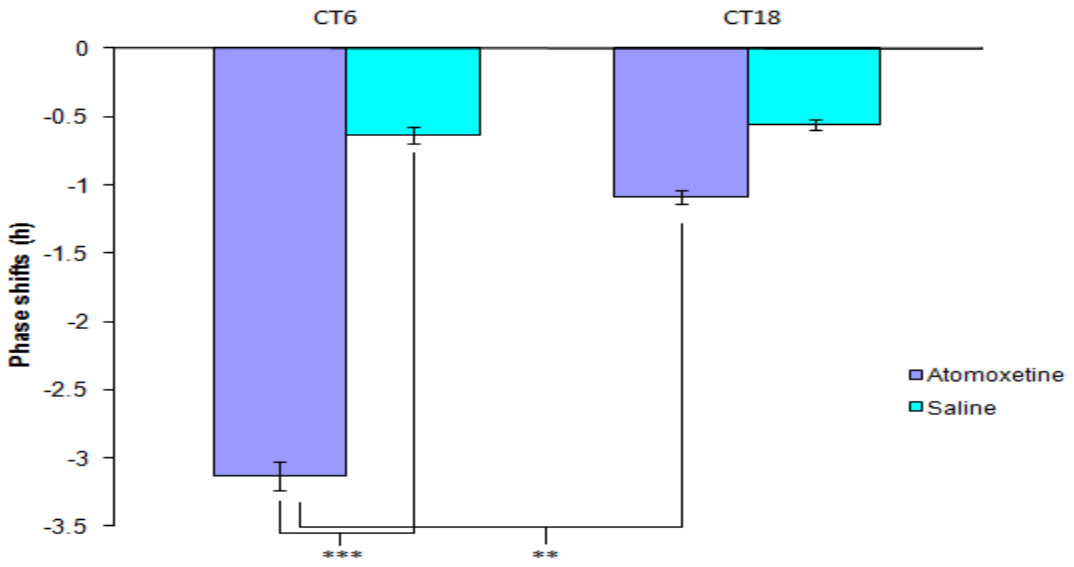


Figure 12*: Bar graphs illustrating the effects of atomoxetine on behavioural circadian rhythms in animal's free running in LL. Ato treatment at CT6 induces significant phase-delays, whilst Ato treatment during the subjective night phase (CT18) does not. *** denotes P<0.001, ** denotes P<0.01.*

In summary results show that atomoxetine produces large phase-delays of the circadian rhythm when administered to animals in LL in the subjective day, but not the night. Atomoxetine did not have any significant effect on the amplitude or free running period of the rhythms when administered at either CT6 or CT18.

3.2.4 The effects of atomoxetine administration at CT13 in LL

The effect of atomoxetine on circadian timing during the animal's the early active phase at CT13 was then assessed using an independent t-test. A slight delay was observed in the atomoxetine group (-0.64h+/- 0.21h) relative to that of the saline group (-0.21h +/-0.17h), but this was found not to be statistically significant (t=-2.67; P=0.092; Figure 13 and 14.

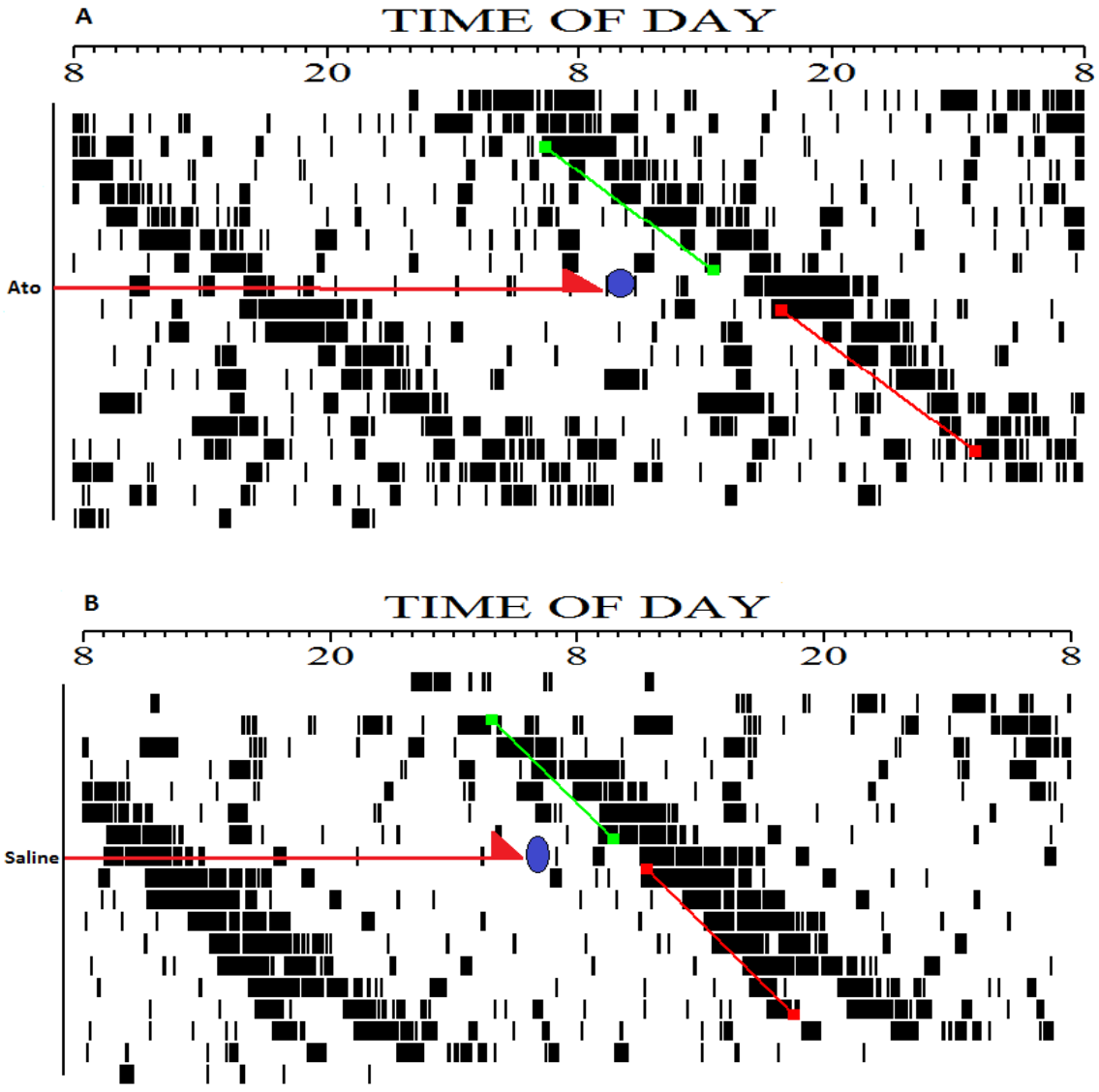


***Figure 13**: Double plotted actograms illustrating that administration of Ato at CT13 in LL induces a slight phase delays (A) whilst administration of saline does not (B). The green lines show the line of best fit through the onsets prior to treatment, the red lines after treatment.*

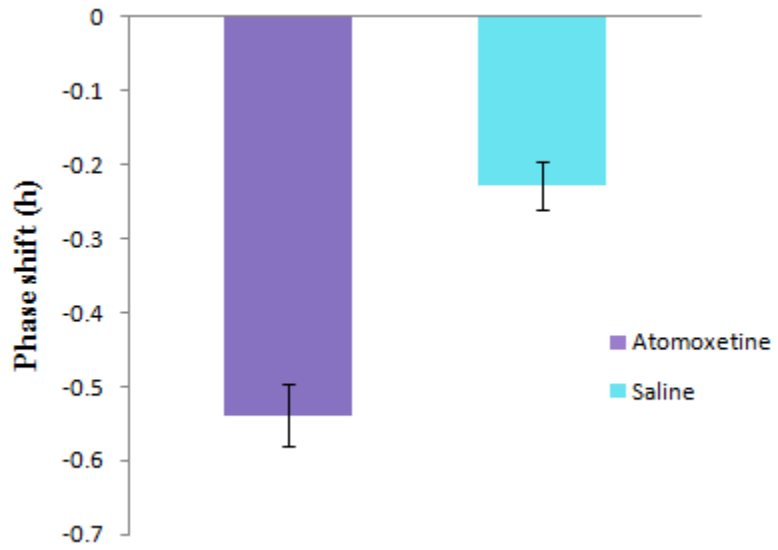


Figure14*: Bar graph illustrating the effects of atomoxetine at CT13, on behavioural circadian rhythms in animals free running in LL.*

There were no significant effects of treatment on either free-running period (Figure 15) or amplitude of rhythm (Figure 16).

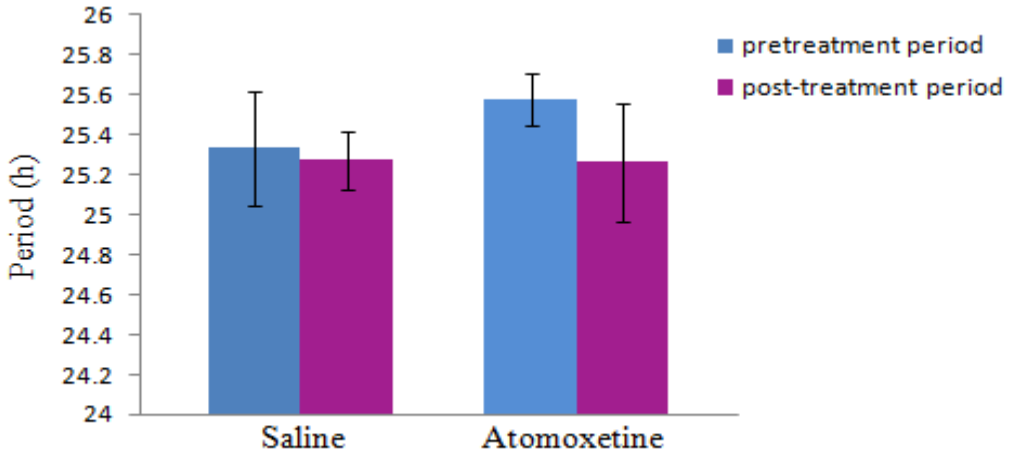


Figure 15: *The mean period pre treatment and post treatment with atomoxetine and saline at CT13.*

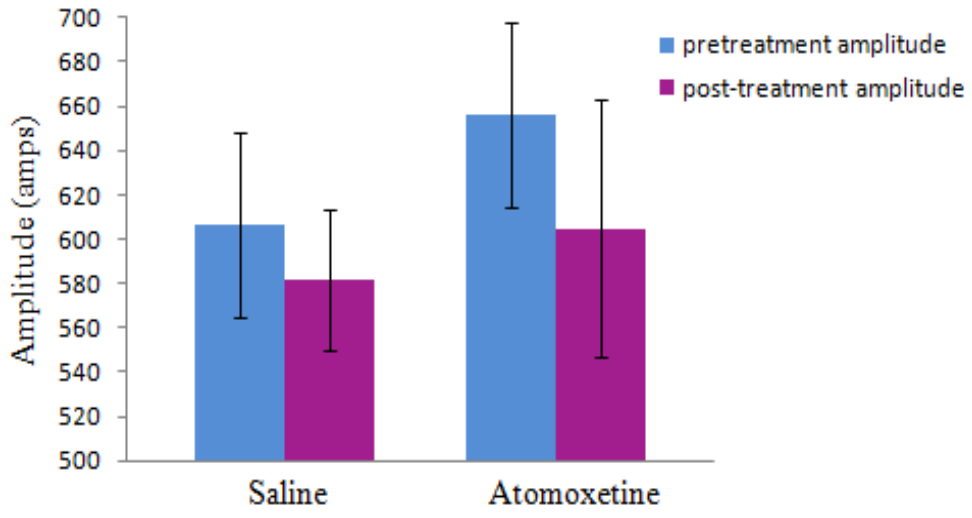


Figure 16: *The mean amplitude pre treatment and post treatment with atomoxetine and saline at CT13.*

3.3 The effect of atomoxetine administration in DD

3.3.1 The effects of atomoxetine administration at CT6 in DD

In order to examine the role of photic background may play in determining the effect of atomoxetine; a group of mice were free-run in constant darkness (DD). A single i.p injection of atomoxetine and the control saline was administered at CT6 in DD, and the effect on their locomotor activity was assessed using a paired t-test. Atomoxetine elicited a modest phase advance (0.96+/- 0.22h) in the circadian rhythm in the mice compared with the saline control (0.09+/-0.18), which was shown to be statistically significant ($t = -6.379$; $P<0.001$; Figure 17).

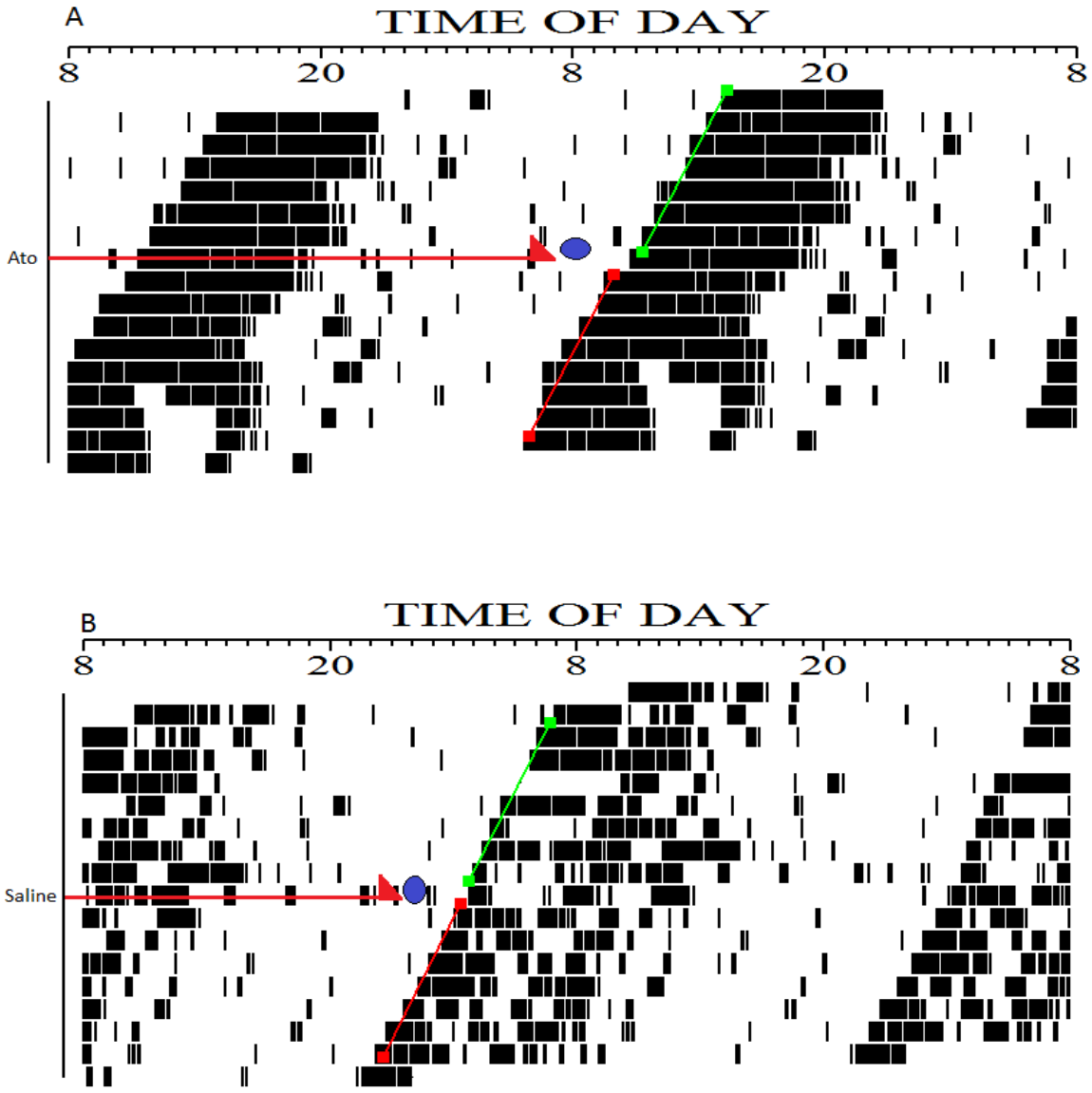


***Figure 17**: Double plotted actograms illustrating that administration of Ato at CT6 in DD induces a modest phase advance (A) whilst administration of saline does not (B). The green lines show the line of best fit through the onsets prior to treatment, the red lines post treatment.*

The effect of atomoxetine on the rhythmicity of the animal's locomotor activity in DD was also assessed. A paired t-test was used to examine if there was a change in amplitude from pre-treatment (1112.6 +/-30.35) to post treatment (954.86+/-27.73), and to examine if there was a change in the period from pre-treatment (23.56+/-0.18) to post treatment (23.51+/-0.43). There

was no significant change in the amplitude (t=0.69; P =0.534; Figure 18), or the period (t=-0.631; P=0.552; figure 19) found due to the administration of atomoxetine.

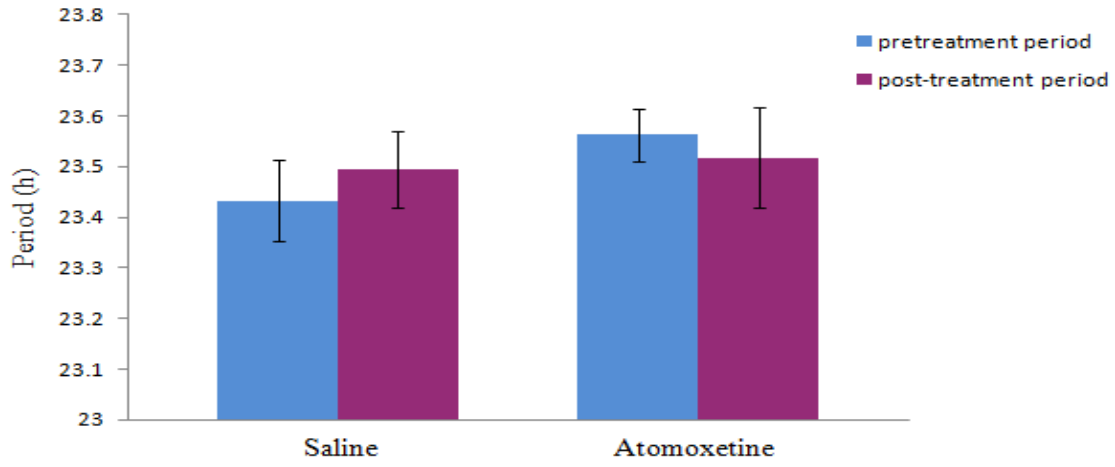


Figure 18: *The mean period pretreatment and post treatment with atomoxetine or saline at CT6.*

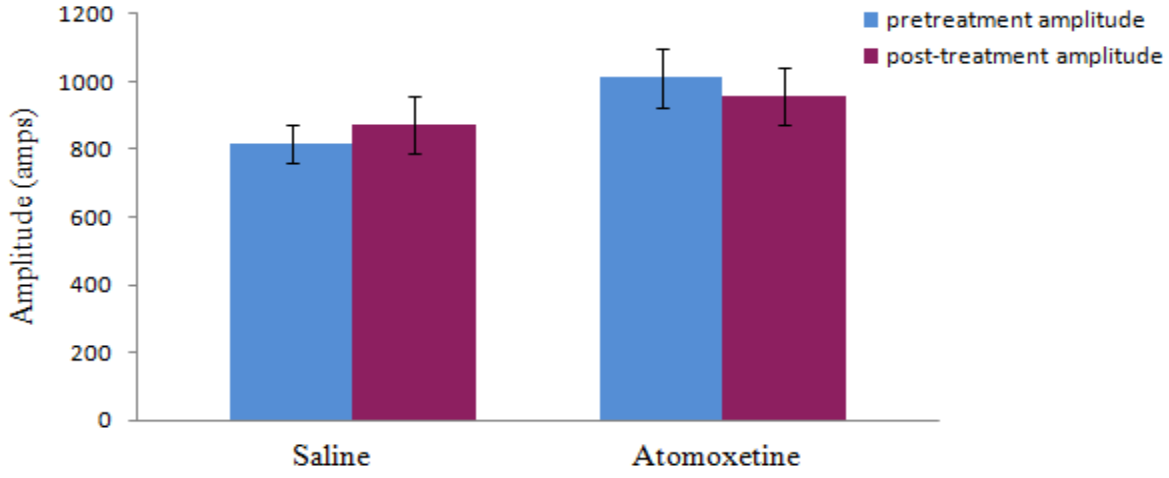


Figure 19: *The mean amplitude pretreatment and post treatment with atomoxetine or saline at CT6.*

3.3.2 <u>The effects of atomoxetine administration at CT18 in DD</u>

The effect of atomoxetine on circadian timing during the animal's active phase at CT18 was then assessed using an independent t-test. A slight advance was observed in the atomoxetine group (0.23+/- 0.10h) relative to that of the saline group (0.12h +/-0.13h), and this was found not to be statistically significant (t=2.58; P =0.093; Figure 20).

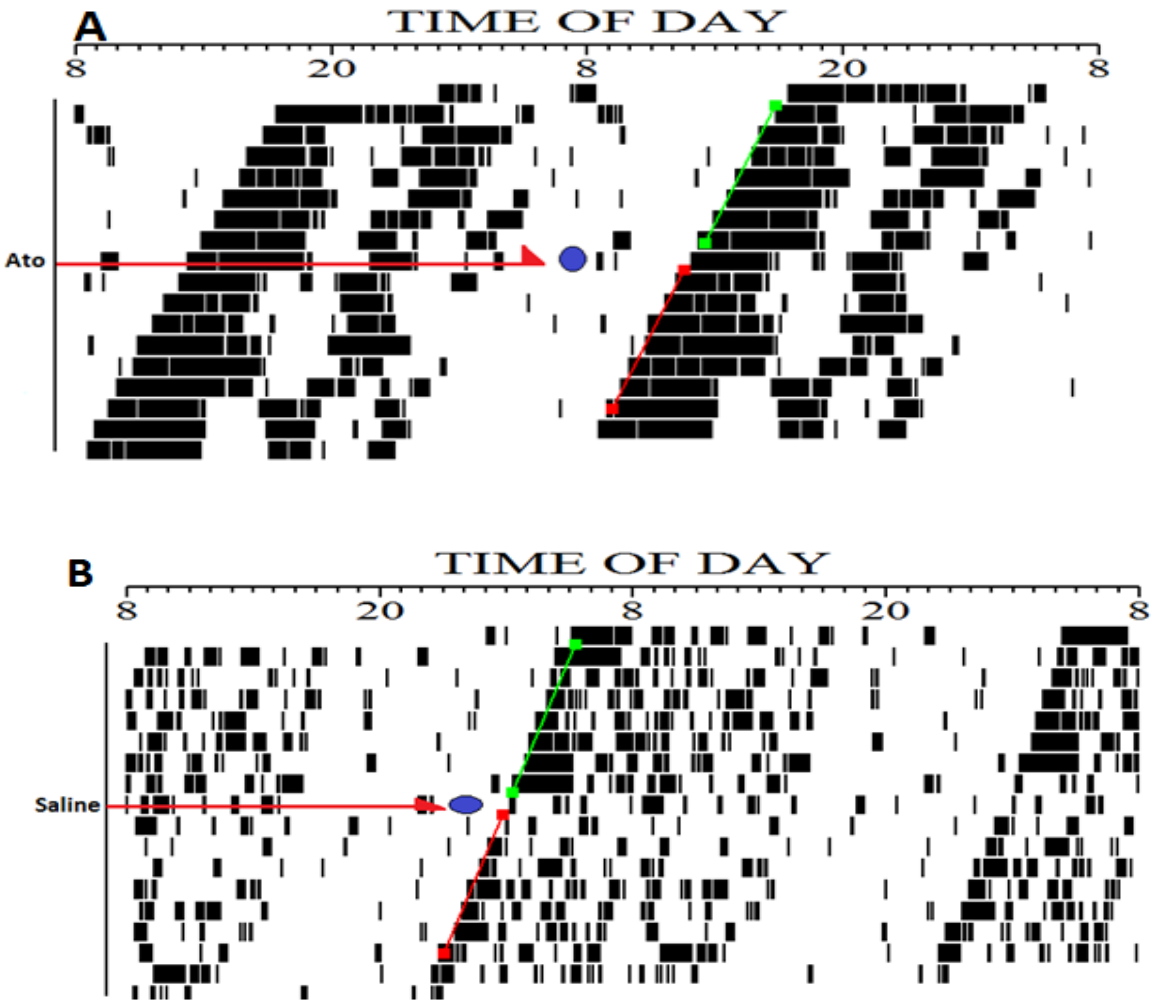


***Figure 20**: Double plotted actograms illustrating that administration of Ato at CT18 in DD induces little phase advance (A) as does the administration of saline (B). The green lines show the line of best fit through the onsets prior to treatment, the red lines post treatment.*

The effects of the administration of atomoxetine on the rhythmicity of the animal's circadian rhythm at CT18 was also measured. The data was not normally distributed therefore a wilcoxon signed rank test was used to see if there was a change in amplitude pre-treatment (754.8+/-195.72) to post treatment (738.9667+/-92.45; Figure 21), and the period pre-treatment (23.58+/-0.35) to post treatment (23.75+/-0.76; Figure 22). There was no significant change in the amplitude (P=0.893) or the period (P=0.525) as a result from atomoxetine treatment.

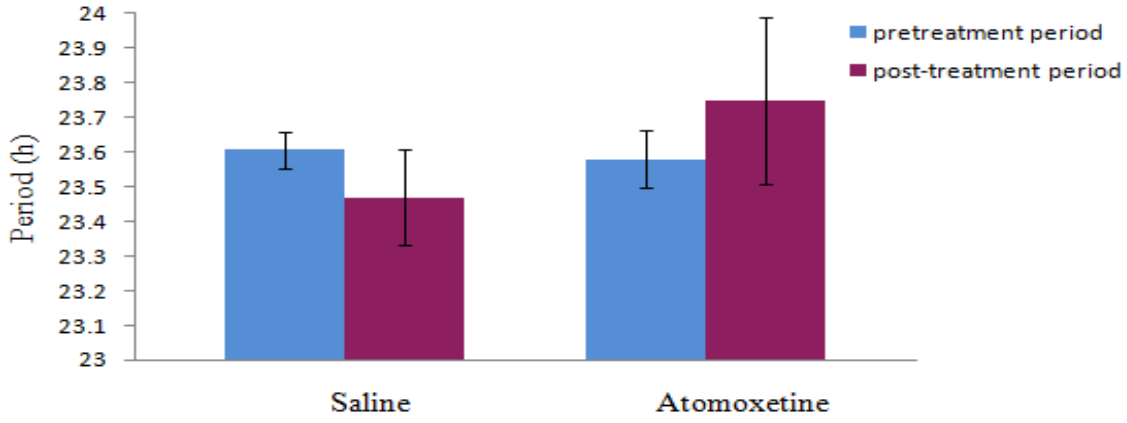


Figure 21: *The mean period pretreatment and post treatment with atomoxetine or saline at CT18.*

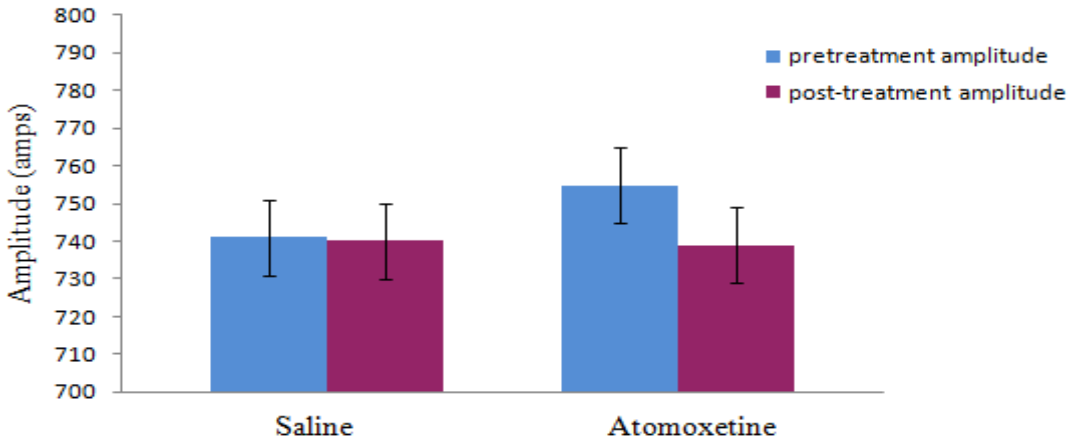


Figure 22: *The mean amplitude pretreatment and post treatment with atomoxetine or saline at CT18.*

3.3.3 The effects of atomoxetine at CT6 compared to administration at CT18 in DD

The final part of this experiment was examining the effect of a single i.p. administration of atomoxetine at CT6 compared to the effect of atomoxetine administration at CT18 using a paired t-test. Atomoxetine administered at CT6 causes a modest phase delay of the circadian rhythm (0.96+/- 0.22h) compared to the effect of atomoxetine at CT18 (0.23+/- 0.10h), as discussed previously. The difference between atomoxetine at CT6 v CT18 was found to be statistically significant (t=2.65; P=0.046; Figure 23).

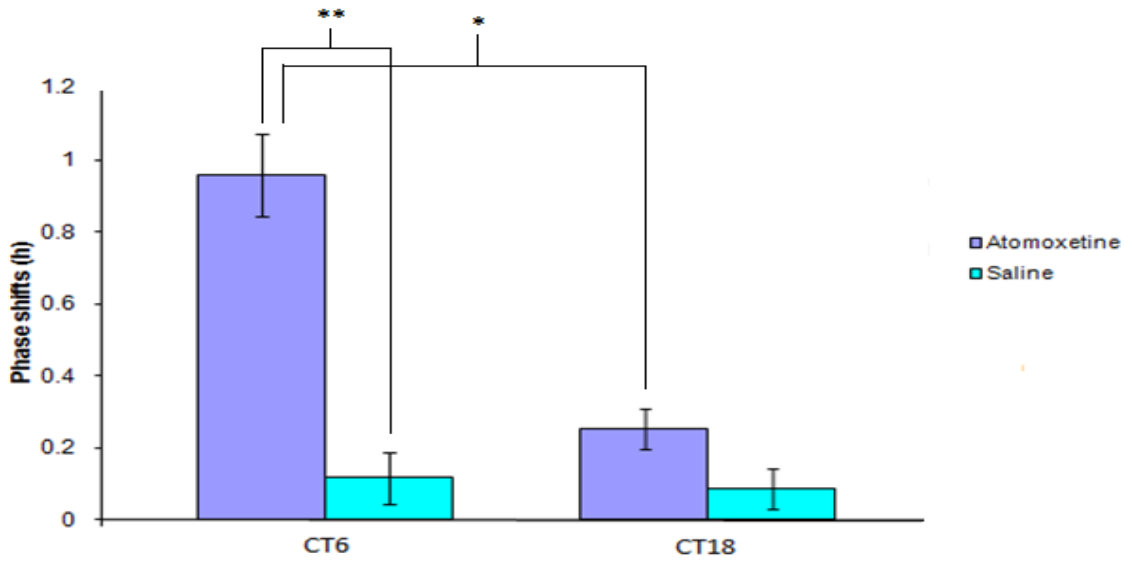


***Figure 23**: Bar graphs illustrating the effects of atomoxetine on behavioural circadian rhythms in animal's free running in DD. Ato treatment at CT6 induces significant phase advances, whilst Ato treatment during the subjective night phase (CT18) does not. ** denotes P<0.01, * denotes P<0.05.*

3.4. The effect of reboxetine administration in LL

A single i.p injection of the noraderenaline reuptake inhibitor reboxetine and control saline was administered at CT6, and the effect on their locomotor circadian activity was assessed using a paired t-test. Administration of reboxetine resulted in a moderate phase delay (-2.08+/- 0.27h) in the circadian rhythm of the mice compared with the saline control (-0.24+/-0.18; Figure 24, 25), which was shown to be statistically significant (t=-4.89; P<0.01).

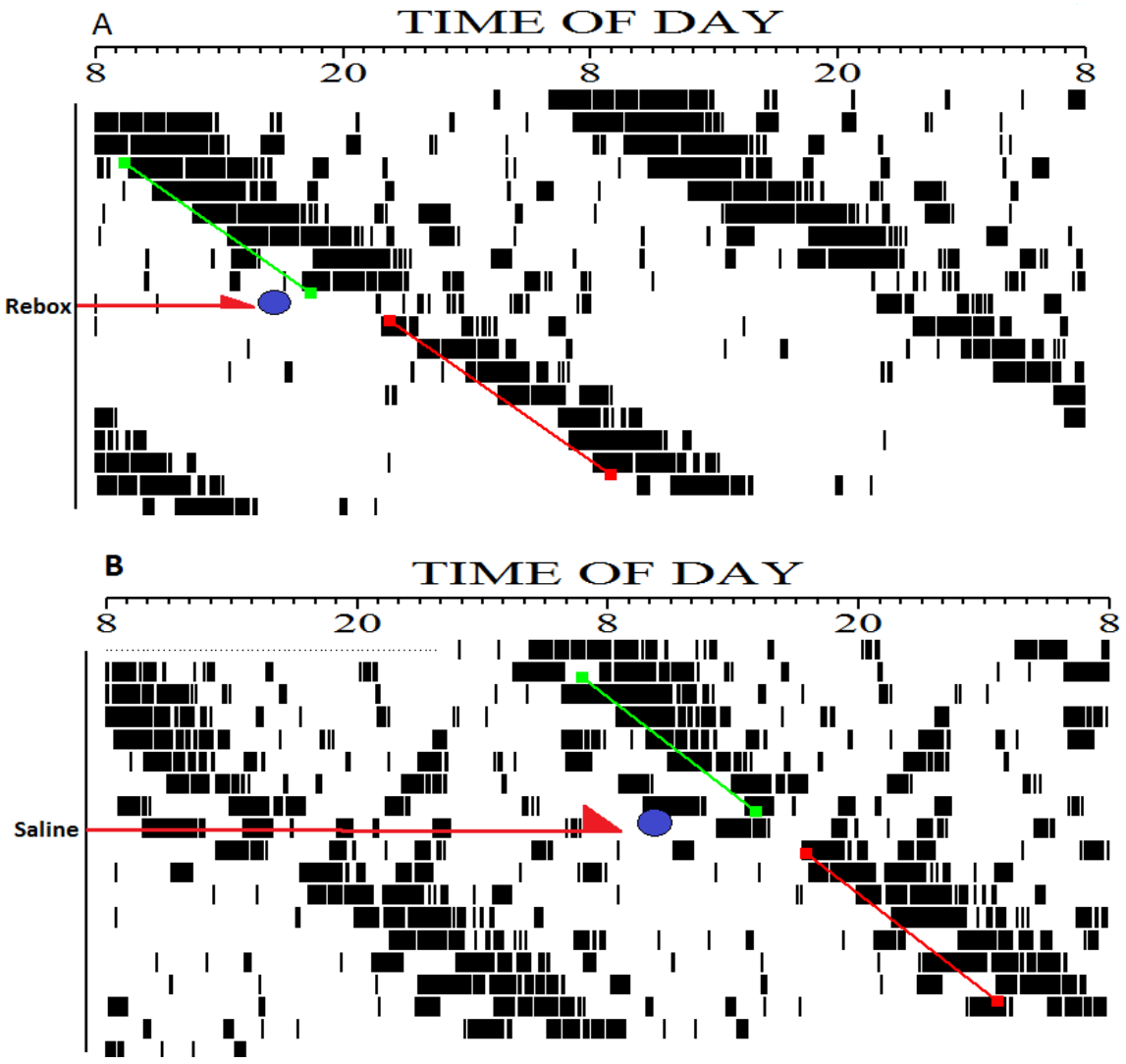


***Figure 24**: Double plotted actograms illustrating that administration of Reboxetine at CT6 in LL induces large magnitude phase delays (A) whilst administration of saline does not (B). The green lines show the line of best fit through the onsets prior to treatment, the red lines after treatment.*

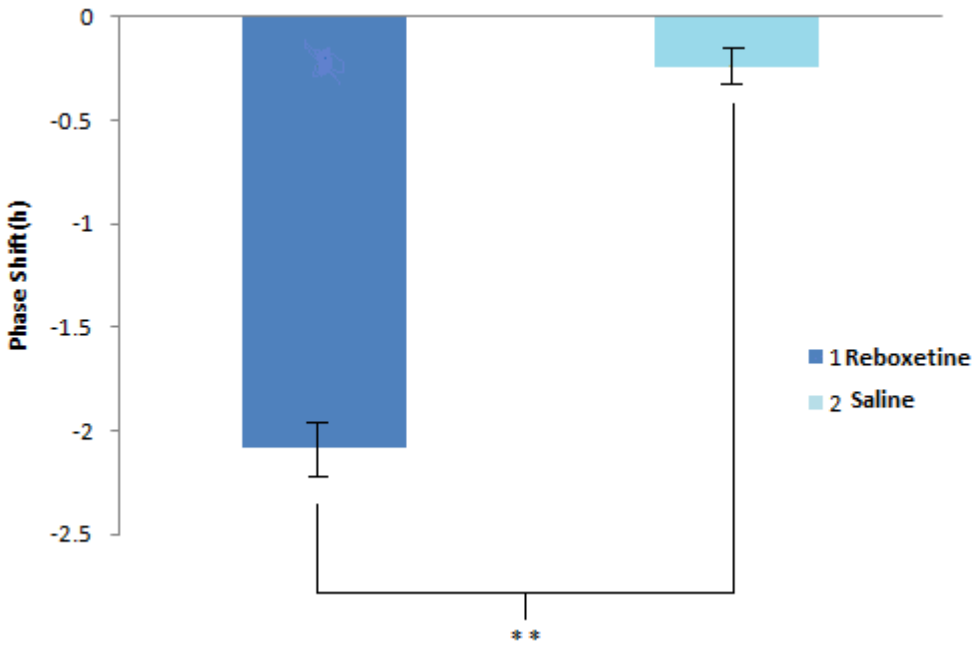


Figure 25*: Bar graph illustrating the effects of reboxetine at CT6, another specific noradrenaline reuptake inhibitor, on behavioural circadian rhythms in animals free running in LL.**denotes P<0.01.*

The effect of reboxetine on the rhythmicity of the animal's locomotor activity was also assessed. A paired t-test was used to examine if there was a change in amplitude from pre-treatment (623.75 +/-25.35) to post treatment (589.45+/-28.63; Figure 25), and to examine if there was a change in the period from pre-treatment (26.343+/-0.19) to post treatment (26.192+/-0.33; Figure 26). There was no significant change in the amplitude (t=0.74; P =0.69), or the period (t=0.662; P=0.63) found due to the administration of reboxetine.

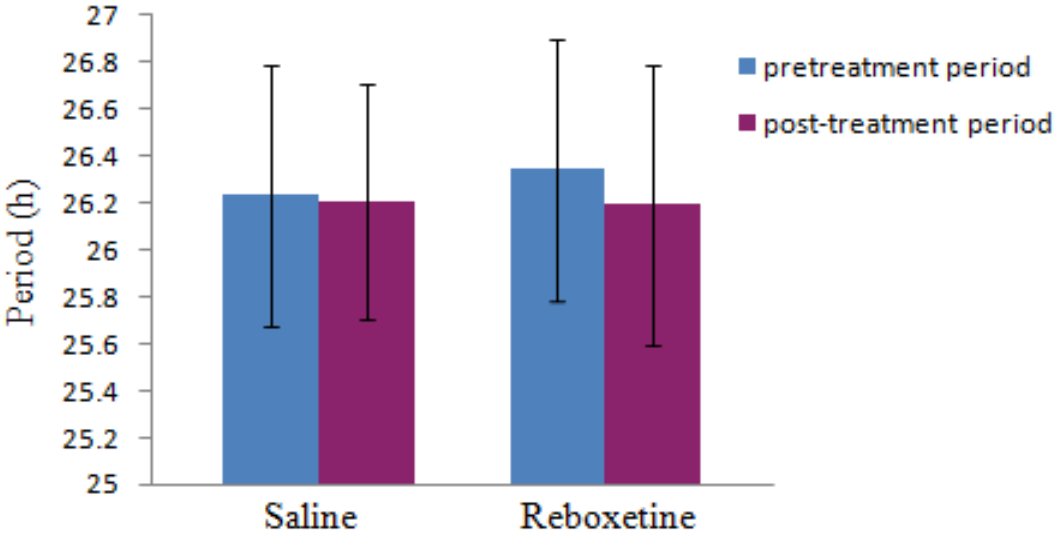


Figure 26: *The mean period pretreatment and post treatment with reboxetine or saline at CT6.*

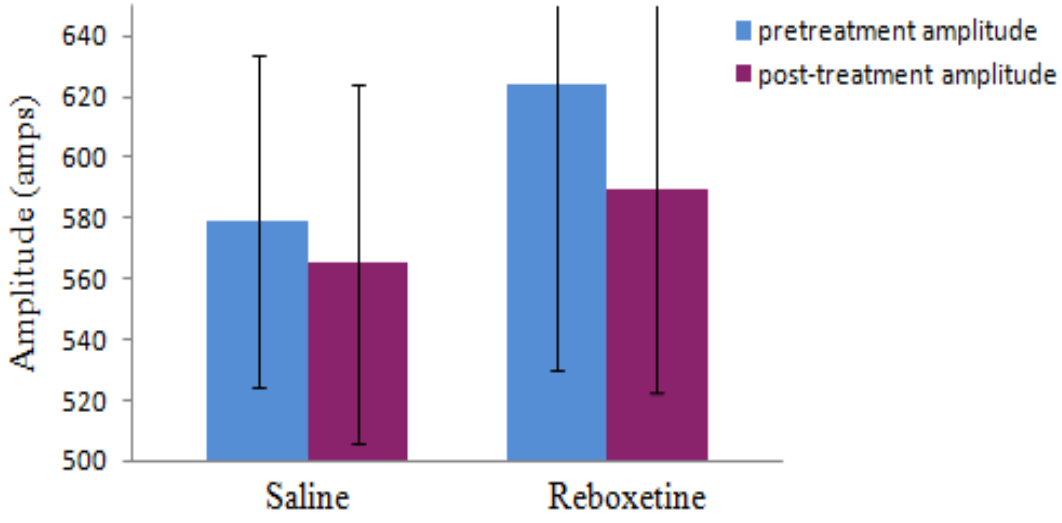
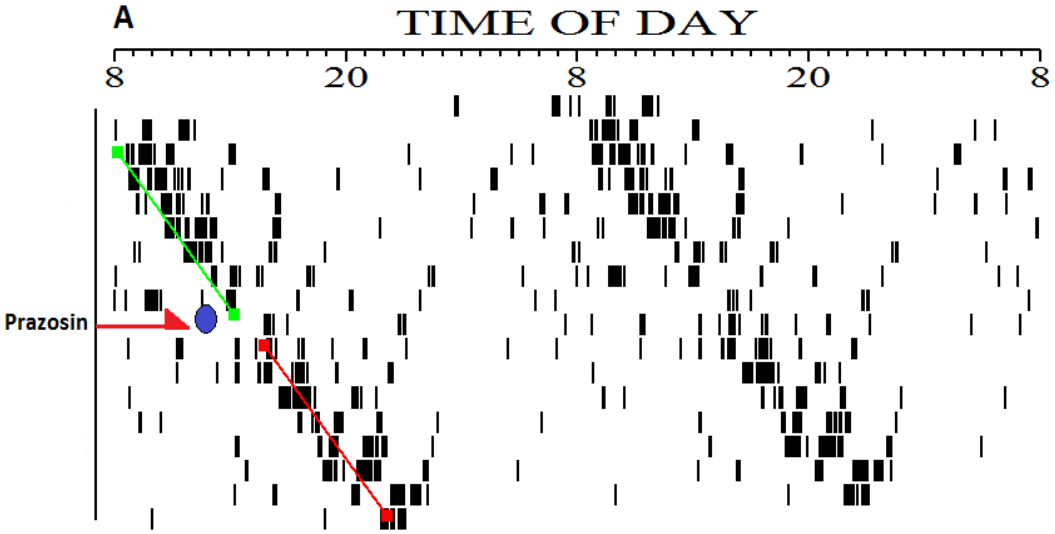


Figure 27: *The mean amplitude pretreatment and post treatment with reboxetine or saline at CT6.*

3.5. The effect of prazosin on atomoxetine at CT6 in LL

Prazosin, an alpha-2 adrenergic receptor antagonist was administered 15 minutes prior to atomoxetine in the treatment group, and in a control group saline was administered 15 minutes prior to atomoxetine at CT6 in LL. The effect on their locomotor circadian activity was assessed using a paired t-test. Administration of prazosin prior to receiving atomoxetine resulted in blocking the effect of atomoxetine previously seen in experiment 3.2.1 causing a delay of (-.447+/- 0.17h) in the circadian rhythm in the mice compared with the saline control (-2.26+/- 0.18; Figure 28), which was shown to be statistically significant (t=-4.89; P<0.01; Figure 29). There were no significant effects of prazosin/atomoxetine treatments on free running periods (Figure 30) or amplitudes of rhythms (Figure 31).



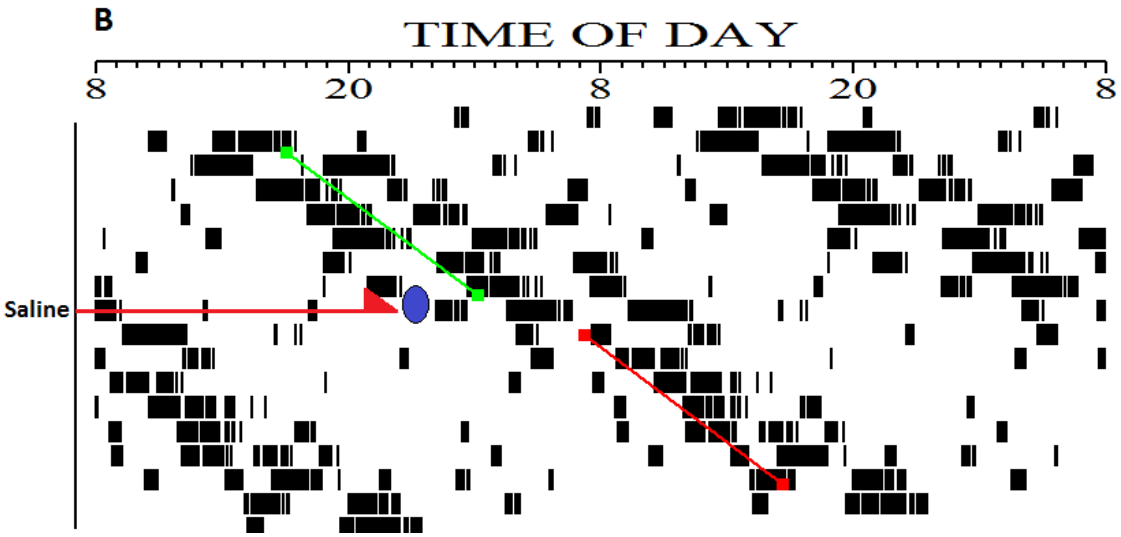


***Figure 28**: Double plotted actograms illustrating that administration of Prazosin prior to administration of atomoxetine at CT6 in LL blocks the effect of the drug as no large phase shift is seen (A) whilst administration of saline does not block atomoxetine and causes a large phase delay (B). The green lines show the line of best fit through the onsets prior to treatment, the red lines after treatment.*

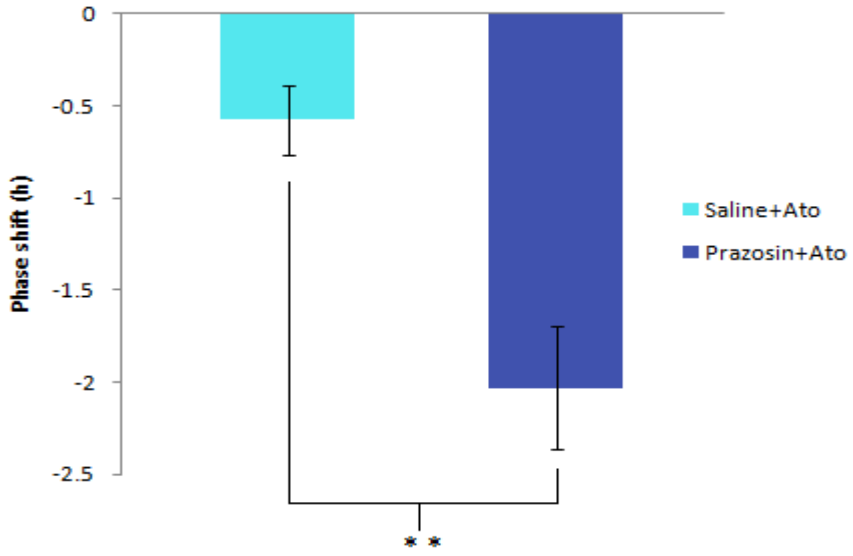


***Figure 29**: Bar graph illustrating the effects prazosin + Ato at CT6, compared to saline + Ato on behavioural circadian rhythms in animals free running in LL. The saline + Ato produced a large phase delay, this did not occur in prazosin. **denotes P<0.01.*

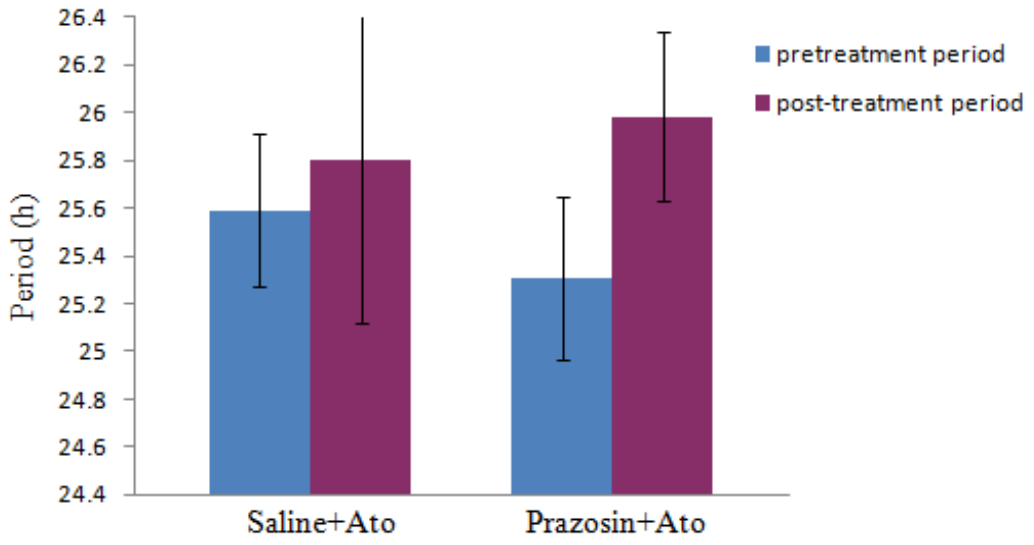


Figure 30: *The mean period pretreatment and post treatment with prazosin or saline, 15 minutes prior to atomoxetine at CT6.*

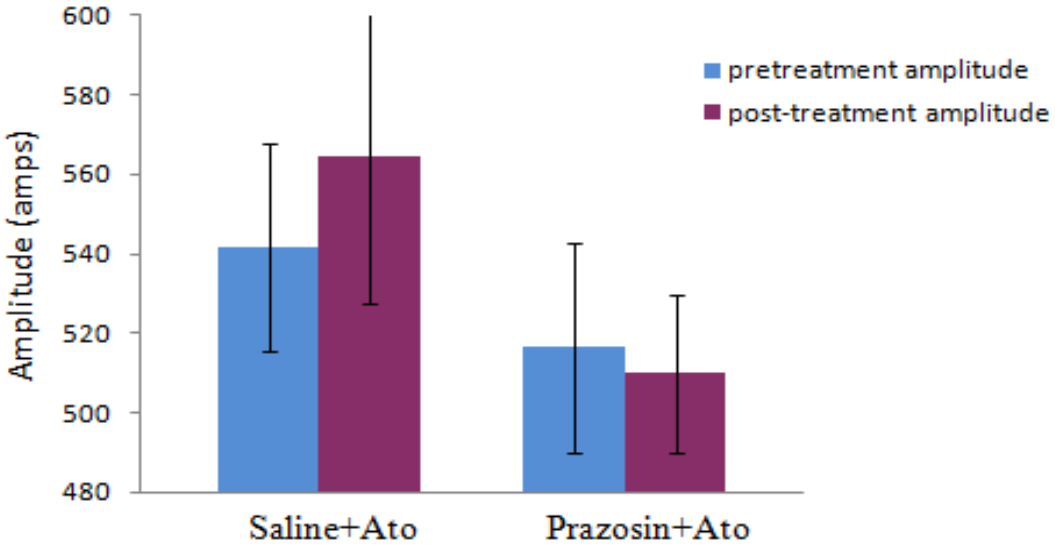


Figure 31: *the mean amplitude pretreatment and post treatment with prazosin or saline, 15 minutes prior to atomoxetine at CT6.*

3.6. Immunohistochemistry

3.6.1 Examine the effects of atomoxetine administered at CT6 in light/dark cycle, LL and DD on the expression of clock genes in the SCN.

In the first experiment here, atomoxetine or saline were administered at ZT6 in the light: dark cycle and immunohistochemistry was carried out to see if the expression of c-Fos in the SCN was different in the two treatments. An independent t-test was carried out and there was no statistical significance between the atomoxetine and the saline control, (t=0.870; p=0.418; Figure 32).

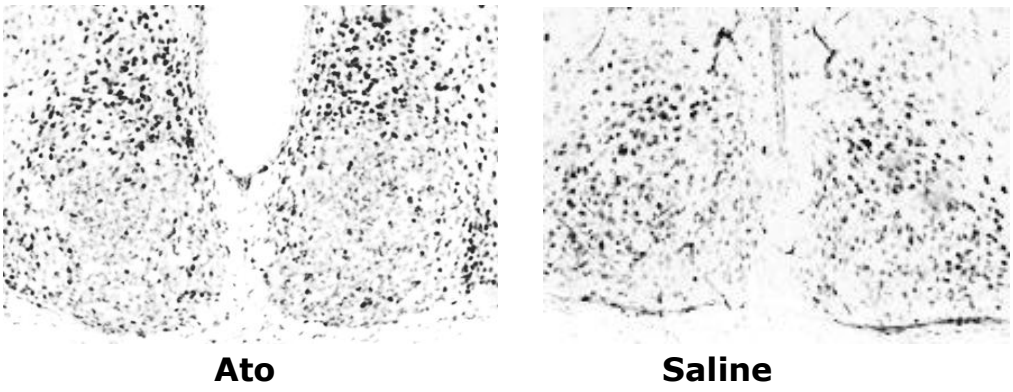


Figure 32: *photomicrograph illustrates the effects of atomoxetine treatment at CT6 on expression of c-Fos in the SCN in L: D. Scale bar = 100μm.*

Following on from the findings of behavioural phase-shifts elicited in LL, immunohistochemistry was carried to examine if treatment with atomoxetine alters clock genes expression in c-Fos, CLOCK, BMAL1 and PER2 in LL housed mice. Using an independent t-test it was found that atomoxetine at CT6 produces significant alteration in c-Fos ($p<0.001$) and CLOCK ($p<0.001$) in animals in LL, but not in BMAL1 or PER2 (Figure 33 and 34).

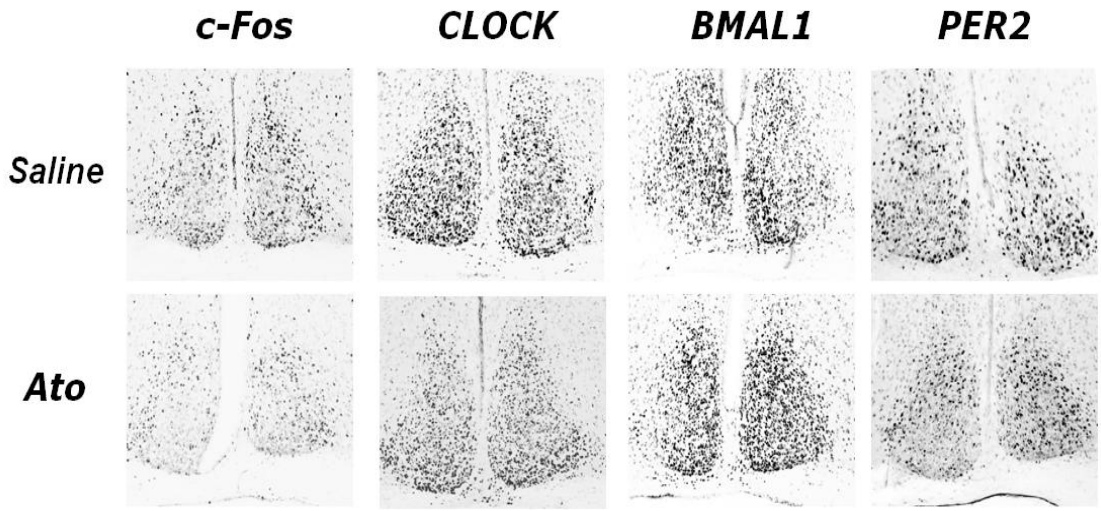


Figure 33: *photomicrographs illustrate the effects of atomoxetine treatment at CT6 on expression of c-Fos, CLOCK, BMAL1 and PER2 in the SCN in LL. Scale bar = 100μm.*

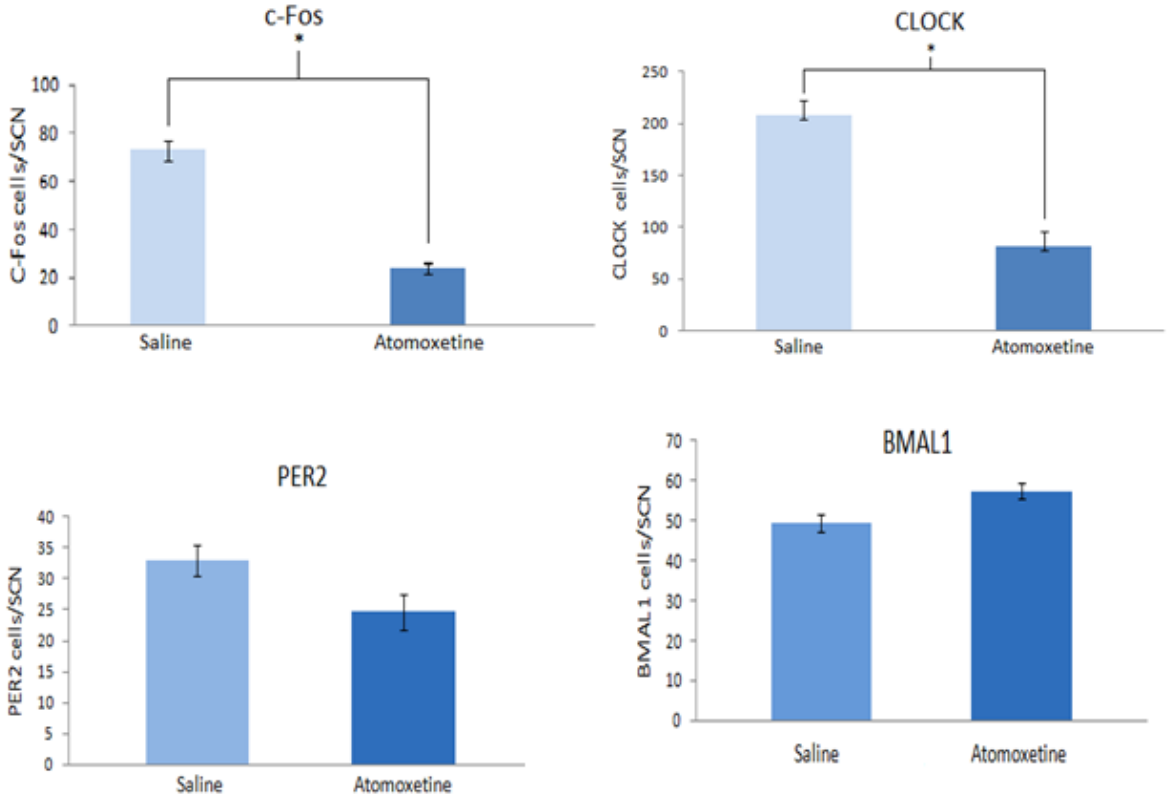


Figure 34: *Bar charts illustrating the effects of atomoxetine treatment at CT6 on expression of c-Fos, CLOCK, BMAL1 and PER2 in the SCN in LL. * denotes P<0.05.*

Immunohistochemistry was carried to examine if atomoxetine administration alters clock genes expression in c-Fos, CLOCK, BMAL1 and PER2 in DD. The numbers of immunoreactive clock cells per SCN were counted by an observer blind to the treatment. Using an independent t-test it was found that atomoxetine produces no significant alteration in any of the clock genes in constant darkness (Figures 35 and Table 1).

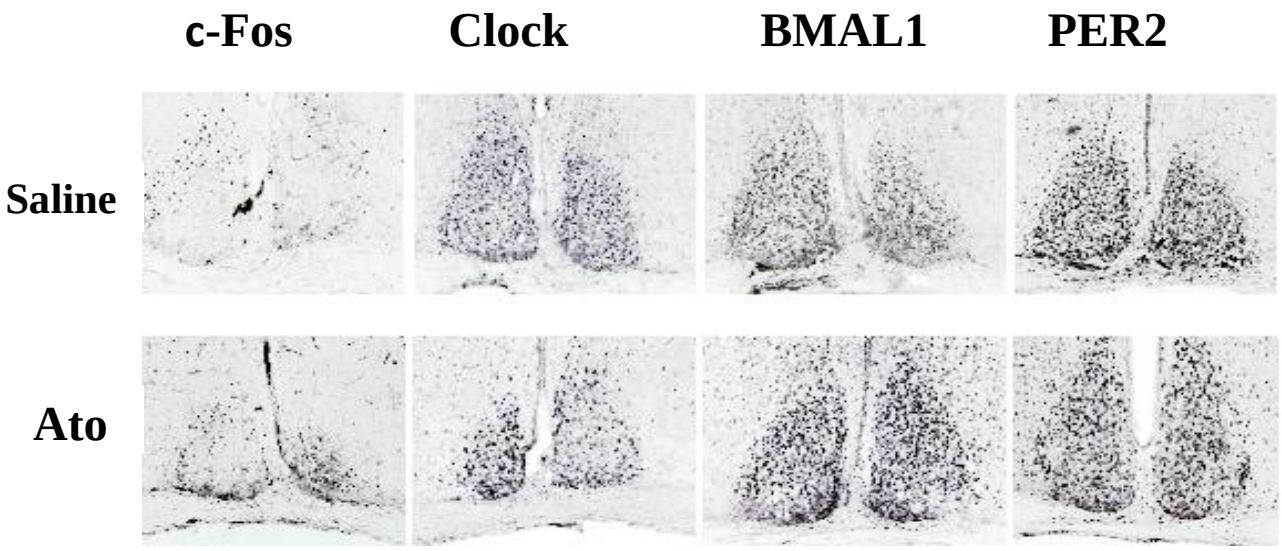


Figure 35: *The photomicrographs illustrate the effects of atomoxetine treatment at CT6 on expression of c-Fos, CLOCK, BMAL1 and PER2 in the SCN in DD. Scale bar = 100μm.*

	LD	LL	DD
C-Fos	**p= 0.42 (n.s)**	**p<0.001****	**p=0.081 (n.s)**
PER2	**-**	**p=0.072 (n.s)**	**p=0.702 (n.s)**
CLOCK	**-**	**p<0.001****	**p=0.901 (n.s)**
BMAL1	**-**	**p=0.576 (n.s)**	**p=0.363 (n.s)**

Table 1: *Summary of effects of Atomoxetine compared to saline under different photic conditions on c-Fos, PER2, CLOCK and BMAL-1 in the SCN.*

3.6.2 Effects of reboxetine administration at CT6 in LL on the expression clock genes in the SCN.

Immunohistochemistry was carried to examine if treatment with reboxetine at CT6 alters clock genes expression in c-Fos, CLOCK, BMAL1 and PER2 in LL housed animals. The numbers of immunoreactive clock cells per SCN were counted by an observer blind to the treatment. Using an independent t-test it was found that reboxetine produces significant alteration in c-Fos ($P<0.04$) and CLOCK ($P<0.005$) in animals in LL, but not in BMAL1 or PER2 (Figures 36, 37).

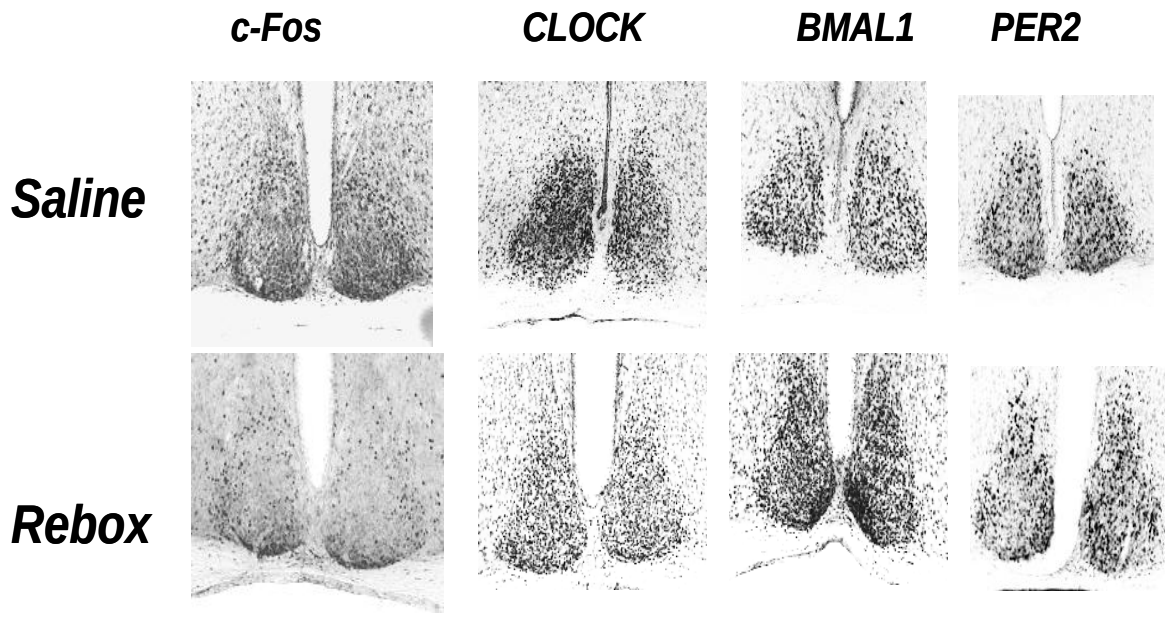


Figure 36: *The photomicrographs illustrate the effects of reboxetine treatment at CT6 on expression of c-Fos, CLOCK, BMAL1 and PER2 in the SCN in LL. Scale bar = 100 μm.*

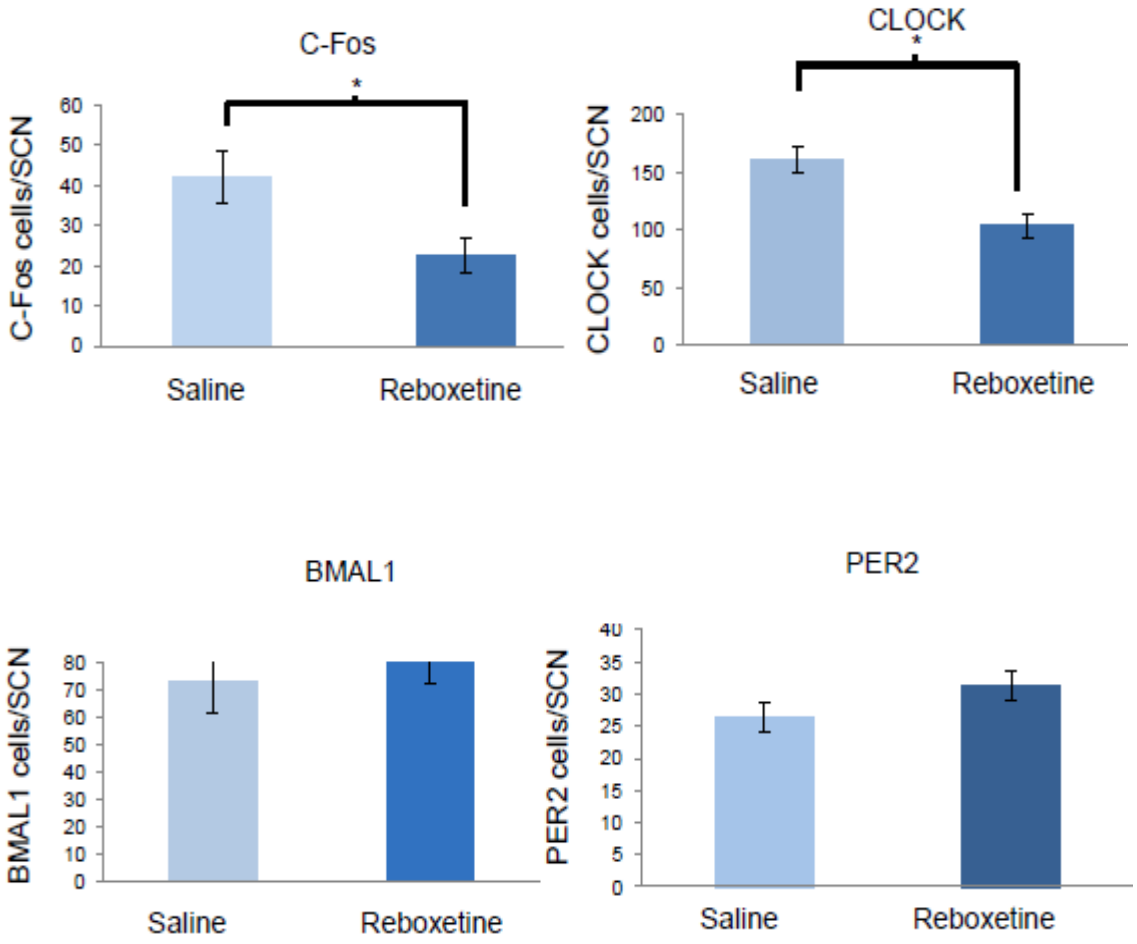


Figure 37: *Bar charts illustrating the effects of reboxetine treatment at CT6 on expression of c-Fos, CLOCK, BMAL1 and PER2 in the SCN in LL. * denotes P<0.05, **denotes P<0.01.*

3.6.3 The effects of prazosin being administered prior to atomoxetine injection at CT6 in LL.

Prazosin was administered 15 minutes prior to atomoxetine in the treatment group to see if it blocked the effect of atomoxetine in the expression of clock genes in the SCN, and a control group saline was administered 15 minutes prior to atomoxetine at CT6 in LL. Pre-treatment with prazosin resulted in significantly higher levels of immunostaining for c-Fos and Clock than in animals pre-treated with saline prior to atomoxetine treatment (Figures 38, 39 and Table 2), but there was no effect on BMAL1 nor on PER2.

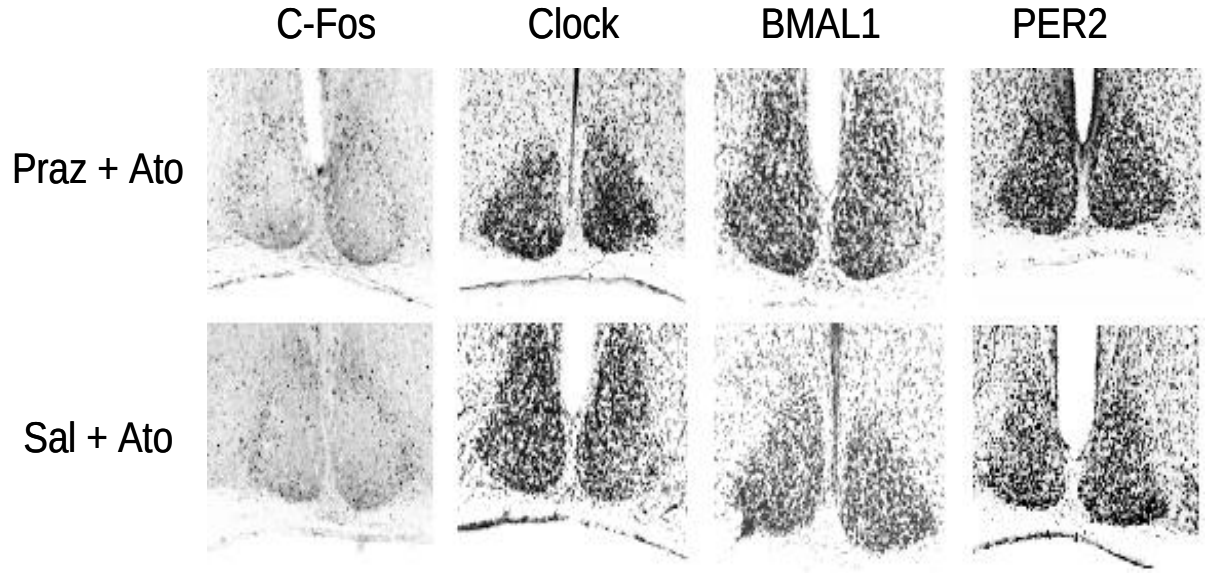


Figure 38: *The photomicrographs illustrate the effects of prazosin treatment on atomoxetine at CT6 on expression of c-Fos, CLOCK, BMAL1 and PER2 in the SCN in LL. Scale bar = 100μm.*

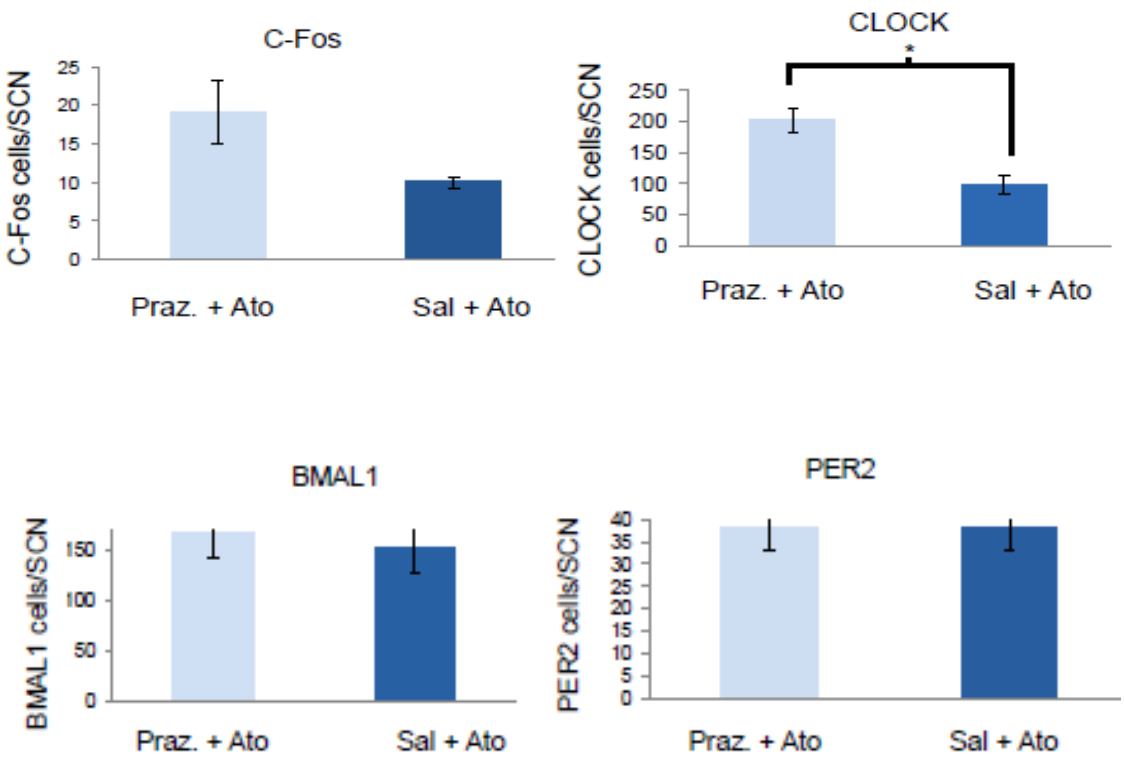


Figure 39: *Bar charts illustrating the effects of prazosin on atomoxetine treatment at CT6 on expression of c-Fos, CLOCK, BMAL1 and PER2 in the SCN in LL. * denotes P<0.05, **denotes P<0.01.*

LL	Rebox	Prazosin
c-Fos	p<0.04*	p=0.073 (n.s)
PER2	p=0.100 (n.s)	p=0.980 (n.s)
CLOCK	p<0.005**	p<0.006**
BMAL1	p=0.413 (n.s)	p=0.710 (n.s)

Table 2: *Summary of effects of reboxetine and prazosin compared to saline in LL on c-Fos, PER2, CLOCK and BMAL-1 in the SCN.*

4.0 Discussion

4.1 Current Study

The psychiatric condition ADHD can be characterised by an early onset of sleep problems and breakdown of stable circadian rhythms which recent studies have shown. The drug atomoxetine used in this research project is used in the treatment of ADHD. It acts primarily through the noradrenergic system and differs from catecholaminergic psychostimulant drugs such as Ritalin and Amphetamine. The current study aimed to examine if acute/chronic treatments with atomoxetine/ reboxetine produces alterations in the circadian rhythms in mice. In addition the study examined whether treatment with these drugs alters cellular activation in brain areas implicated in the circadian timekeeping system. The effects of atomoxetine on locomotor circadian rhythms in mice were examined under free-running conditions (to look at the endogenous circadian clock without influence of environmental factors like light). One advantage of animal studies over human studies is the great difficulty involved in obtaining free-running rhythms in human subjects.

Atomoxetine produced a large phase delay of the circadian rhythms when administered during the subjective day in LL (CT6). However when atomoxetine was administered during the early/late subjective night in LL (CT13 or CT18) it did not result in a significant response. During the animals late active phase (CT18) a modest phase delay took place relative to that of the saline group. In the early active phase (CT13) a slight delay was observed in the atomoxetine group relative to that of the saline group but was not significant. Atomoxetine treatment did not produce any behaviour changes and there was no marked wheel running activity in the 4 hours

following treatment. There was no effect on the free running period or the amplitude of the circadian rhythms in any of the experiments carried out in this study.

Therefore the time of treatment, appears to determine the effects of atomoxetine on the circadian clock, with administration of atomoxetine having maximum effect during the animals inactive phase CT6.

4.2 Phase shifts

At present there are no studies published concerning the direct effects of atomoxetine on circadian rhythmicity. The phase delay produced by the administration of atomoxetine during the subjective day, observed in this current study does not comply with other studies using non photic stimuli. This may be due to the fact atomoxetine a SNRI, inhibits NET, therefore increasing synaptic noradrenaline concentrations (Bymaster et al, 2002). Non photic stimuli will usually produce a phase advanced when presented during the subjective day and it will produce a phase delay when it is presented during portions of the subjective night (Mrosovsky et al, 1989; Reebs et al, 1989; Mrosovsky, 1996). Selective serotonin re-uptake inhibitors (SSRI) such as the antidepressant fluoxetine, produce phase advances in firing of the SCN neurons in rat slice culture in the mid subjective day. The phase shifts might differ due to the fact that SSRI blocks the serotonin (5-HT) transporter resulting in an increase of synaptic 5-HT (Sprouse et al, 1996). Administration of triazolam which is used in treating insomnia, produce phase shift advances, when administered during the mid subjective day to normal and blinded hamsters, that are housed under constant darkness (van Reeth et al, 1987). It is thought that triazolam may act through the non-photic input pathway on the SCN, as it produce phase shift advances of 90 min

when given during the mid subjective day, but no effect occurred when a light pulse was administered at the exact same time (Turek et al, 1986).

4.3 Norepinephrine system

Norepinephrine concentrations in the SCN over 24 hours, peak during the subjective day and is lower during the subjective night (Cagampang et al, 1994). As atomoxetine is a specific norepinephrine reuptake inhibitor it would be thought to be most effective when norepinephrine concentrations are high. Therefore findings in this project where atomoxetine administration during the subjective day produced the largest phase shifts correlate with this theory. This study demonstrates a link between norepinephrine and the circadian timing system which support previous studies carried out which have suggested a noradrenergic mechanisms for circadian regulation (Dwyer and Rosenwasser, 2000).

4.4 Photic and non photic conditions

In LL the circadian response is enhanced to pharmacological substances (Klemfuss and Kripke, 1994; Knoch et al, 2004); therefore the next experiment was carried out in DD to examine the effect LL conditions has on the magnitude of the phase shift that occurred.

In order to examine the role of photic background may play in determining the effect of atomoxetine; a group of mice were free-run in DD. When atomoxetine was administered in the subjective day (CT6) a modest phase advance occurred shown to be statistically significant compared to saline control. However when atomoxetine treatment was applied during the subjective night CT18 only a slight phase advance took place, again showing that the time of

drug treatment administration of atomoxetine is vital for phase shifts in the circadian timing system. The photic conditions under which subjects are maintained, appears to determine the effects of atomoxetine on the circadian clock as mice free running in LL has a greater phase shift than those in DD after treatment with atomoxetine.

Serotonergic input from the midbrain raphe nuclei is another important source of non-photic signaling to the SCN (Glass et al, 2003). Serotonin acts as an essential neurotransmitter in the brain. The ventral and ventromedial regions of the SCN have high concentration of serotonergic fibers (Cassone, 1988). Nonphotic serotonin input from the midbrain raphe nuclei (MRN) to the SCN is abolished by MRN lesioning (Meyer-Bernstein and Morin, 1996), where as serotonergic neuron fibers from dorsal raphe nuclei (DRN) innervates MRN and IGL, therefore indirectly affecting serotonin release from SCN (Glass et al, 2000; Glass et al, 2003). This serotonergic input from the MRN assists in regulating the entrainment of the circadian pacemaker to external zeitgebers (Meyer-Bernstein and Morin, 1997). Both the DRN and MRN appear to play a role in altering the SCN serotonin release, as electrical stimulation of either DRN or MRN increases serotonin release from SCN (Dudley et al, 1999).

Increased locomotor activity as in wheel running, also increase serotonin levels peaking during mid day (Dudley et al, 1998). The electrical stimulation of MRN acutely enhances serotonin release from the SCN (Dudley et al, 1999) producing phase advances (Glass et al, 2003). These findings suggest that a serotonergic pathway involving raphe and SCN plays an important role in non-photic phase resetting, although further studies are required to ask if there is a serotoninergic influence on atomoxetine's actions in the SCN.

4.5 Norepinephrine antagonist

To further examine if the administration of atomoxetine causing changes in norepinephrine levels is acting directly on the SCN, a further study was carried out. Increased levels of norepinephrine have been related to increased levels of other neurotransmitters such as DA and 5-HT (Tzavara et al, 2006), which could also play a part in this current study. Therefore to examine whether the phases shifts that occurred in this study using atomoxetine is directly related to increased levels of norepinephrine, administration of a norepinephrine antagonist prior to atomoxetine was examined. Prazosin is an alpha-2 adrenergic receptor antagonist, which can block post junctional vascular α-adrenorecptors (Menkes et al, 1981). Administration of prazosin prior to receiving atomoxetine in LL at CT6, resulted in the effect of atomoxetine being blocked, resulting in a slight phase delay. However the saline control administered prior to atomoxetine elicited a moderate phase delay in the circadian rhythm, previously seen in experiment 3.2.1, which was shown to be statistically significant. Prazosin did obstruct the pharmacological effects of atomoxetine which did not result in a large phase delay previously observed in this study.

4.6 SNRIs

A second noraderenaline reuptake inhibitor, reboxetine was also investigated in this study, to examine whether it also produced alterations in the circadian rhythms in mice. When reboxetine was administered during the subjective day CT6 in LL, a moderate phase delay was observed in the circadian rhythm of the mice compared with the saline control, which was shown to be statistically significant.

Both reboxetine and atomoxetine are SNRIs, which are non stimulant catecholaminergic drugs. Atomoxetine is the first non stimulant drug approved by the United States Food and Drug

Administration (FDA) for the treatment of ADHD (Kratochvil, 2003; Tzavara et al, 2006). It is also only drug approved by the FDA for the treatment of ADHD in adults. Reboxetine is not an approved drug for treating in ADHD patients but can be used in treating depression, and was the first SNRI used as an antidepressant agent, with specificity for the noradrenergic system (Kasper et al, 2000). In this study reboxetine showed similar results to atomoxetine when administered in the mid subjective day, resulting in a phase delay. The phase delay however was not as large as the effects atomoxetine created. In humans reboxetine has an elimination half life of 13 hours therefore making reboxetine suitable for twice daily dosing (Doster et al, 1997). Atomoxetine however only has a plasma elimination of around 5 hours (Sauer et al, 2005). Stimulants such as amphetamine and methylphenidate have only a plasma half life of 4-8 hours, therefore leading to administration of the drugs throughout the day. The differences in the pharmokinetics of atomoxetine and reboxetine may explain the different magnitude of phase-shifts elicited in the present study.

4.7 Clock gene expression

Clock gene expression of the SCN was also examined in this study with interesting results. Atomoxetine and reboxetine produces significant alteration in c-Fos and CLOCK in animals in LL, but not in DD, in agreement with the more profound phase shifts seen in LL experiments. PER2 and BMAL1expression in the SCN were not affected by administration of atomoxetine or reboxetine in either LL or DD. In LD c-Fos expression in the SCN was also not affected by the administration of atomoxetine. Therefore the photic conditions under which the animals are maintained appear to determine the effects of atomoxetine on the circadian clock. In LL c-Fos and CLOCK expression were suppressed by the administration of either atomoxetine or

reboxetine. Previous studies have shown that non-photic stimuli that produce phase-shifts also suppress c-Fos and clock gene expression in the SCN (Maywood et al, 1999; Coogan and Piggins, 2005).

4.8 Catecholamine systems

Dysfunction of catecholamine systems, mainly dopamine (DA) and norepinephrine, have being proposed to be involved in ADHD (Arnsten et al, 1996; Swanson et al, 2005). Norepinephrine plays a role in attention, and the maintenance of arousal, a cognitive function which is deficient in ADHD (Biederman et al, 1999). Studies that have been carried out have shown that impulsivity in ADHD can be reduced by increasing norepinephrine levels (Robinson et al, 2008). Studies have shown that atomoxetine (3 mg/kg i.p.) robustly increases levels of norepinephrine throughout the brain including the PFC, which has been shown to play a vital role in attention, alertness and vigilance. Atomoxetine can influence both systems in the PFC and the circadian system accordingly. The exact pharmacological mechanism by which this drug elicits positive results in ADHD patients requires further investigation. Atomoxetine unlike psycostimulant drugs does not increase DA concentrations in the nucleus accumbens that is associated with abuse potential (Bymaster et al, 2002; Swanson et al, 2006). Therefore drug abuse with the administration of atomoxetine in ADHD patients is less likely than that of administration of psycostimulant drugs.

4.9 Conclusion

Present results have shown that Atomoxetine produces a novel resetting of circadian time in mice. As there is evidence that ADHD might be associated with misalignment of the circadian clock with the environment, atomoxetine might ameliorate some of the symptoms of ADHD by re-aligning the clock. To conclude, this study has demonstrated the potential benefits SNRI drugs may have on ADHD patients. ADHD patients with irregular sleeping patterns, such as difficulty falling asleep at night and wakening in the morning are suggestive of a delayed phase in circadian organisation of sleep and wakening. Abnormalities in sleep patterns due to dysfunction of circadian timing may be the primary cause of ADHD, resulting in secondary symptoms of inattention, hyperactivity and impulsivity that certain studies have proposed (Boonstra et al, 2007). Therefore, results from this study have shown that atomoxetine produces a novel resetting of circadian time in mice. Atomoxetine might play a vital role improving some of the symptoms of ADHD by re-aligning the clock.

4.10 Future Work

Future work could probe further the roles of noradrenaline in the regulation of the SCN clock.

Another further experiment that could be carried out is the administration of atomoxetine to animals in DD with light pulses to examine the atomoxetine alters photic phase-shifting. Also a study into examining the effects of methylphenidate (Ritalin) on circadian processes is another experiment that could be carried out in the future. This experiment could examine the effects psycostimulants have on the circadian system and on clock gene expression in the SCN, compared to the SNRI's used in this study.

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