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The effects of aging and chronic fluoxetine treatment on circadian rhythms and suprachiasmatic nucleus expression of neuropeptide genes and 5-HT_{1B} receptors

Marilyn J. Duncan, James M. Hester, Jason A. Hopper, and Kathleen M. Franklin

Department of Anatomy and Neurobiology, University of Kentucky Medical Center, 800 Rose Street, Lexington, KY 40536, USA

Abstract

Age-related changes in circadian rhythms, including attenuation of photic phase shifts, are associated with changes in the central pacemaker in the suprachiasmatic nucleus (SCN). Aging decreases expression of mRNA for vasoactive intestinal peptide (VIP), a key neuropeptide for rhythm generation and photic phase shifts, and increases expression of serotonin transporters and 5-HT_{1B} receptors, whose activation inhibits these phase shifts. Here we describe studies in hamsters showing that aging decreases SCN expression of mRNA for gastrin-releasing peptide, which also modulates photic phase resetting. Because serotonin innervation trophically supports SCN VIP mRNA expression, and serotonin transporters decrease extracellular serotonin, we predicted that chronic administration of the serotonin-selective reuptake inhibitor, fluoxetine, would attenuate the age-related changes in SCN VIP mRNA expression and 5-HT_{1B} receptors. *In situ* hybridization studies showed that fluoxetine treatment does not alter SCN VIP mRNA expression, in either age group, at zeitgeber time (ZT)6 or 13 (ZT12 corresponds to lights off). However, receptor autoradiographic studies showed that fluoxetine prevents the age-related increase in SCN 5-HT_{1B} receptors at ZT6, and decreases SCN 5-HT_{1B} receptors in both ages at ZT13. Therefore, aging effects on SCN VIP mRNA and SCN 5-HT_{1B} receptors are differentially regulated; the age-related increase in serotonin transporter sites mediates the latter but not the former. The studies also showed that aging and chronic fluoxetine treatment decrease total daily wheel running without altering the phase of the circadian wheel running rhythm, in contrast to previous reports of phase resetting by acute fluoxetine treatment.

Keywords

gastrin releasing peptide; hamster; serotonin selective reuptake inhibitor; SSRI; vasoactive intestinal peptide

Introduction

During aging, circadian rhythms exhibit many alterations, including reduced amplitude, attenuation of photic and nonphotic phase shifts, increased fragmentation, and dysynchrony (for review, see Duncan, 2007). These changes are associated with age-related changes in the central circadian pacemaker in the hypothalamic suprachiasmatic nucleus (SCN), including reduction in vasoactive intestinal peptide (VIP) mRNA, and augmentation of serotonin (5-hydroxytryptamine; 5-HT)_{1B} receptors and serotonin transporters (Krajnak *et al.*, 1998a;

Duncan *et al.*, 2000, 2001). These age-related SCN alterations may influence circadian rhythms in several ways. Age-attenuated SCN VIP mRNA expression may lead to decreased rhythm amplitude and photic phase resetting, because SCN VIP neurons participate in circadian rhythm generation and light entrainment (Albers *et al.*, 1991; Piggins *et al.*, 1995; Harney *et al.*, 1996; Aton *et al.*, 2005). The age-related increase in SCN 5-HT_{1B} receptors may also contribute to reduced photic resetting because activation of these receptors inhibits photic phase shifts (Pickard *et al.*, 1996). To further understand the underlying age-related changes in circadian function, the project described here tested several hypotheses using hamsters as the experimental model. The first experiment tested the hypothesis that aging decreases SCN mRNA expression for gastrin-releasing peptide (GRP). SCN GRP neurons participate in photic phase shifts, as revealed by findings that these phase shifts are mimicked by GRP microinjection into the hamster SCN or attenuated by genetic deletion of GRP receptors in mice (Albers *et al.*, 1995; Piggins *et al.*, 1995; Aida *et al.*, 2002).

The next two hypotheses were based on the observation of large age-related increase in SCN serotonin transporters, suggesting age-related potentiation of serotonin reuptake and decreased extracellular serotonin levels (Duncan *et al.*, 2000). In rats, serotonin fibers innervate SCN VIP neurons and trophically support VIP mRNA expression, as shown by its attenuation after administration of a serotonin-selective neurotoxin (Bosler & Beaudet, 1985; Kawakami *et al.*, 1994; Hayashi *et al.*, 2001). Therefore, the second hypothesis in this project was that the age-related increase in SCN serotonin transporter sites mediates age-related decreases in SCN VIP mRNA expression. Because serotonin denervation increases SCN 5-HT_{1B} receptor binding sites, which are not restricted to serotonin nerve terminals in this region (Manrique *et al.*, 1994, 1999; Belenky & Pickard, 2001), the third hypothesis was that the age-related increase in SCN serotonin transporter sites mediates the age-related increase in 5-HT_{1B} receptor binding sites. Chronic administration of the serotonin-selective reuptake inhibitor (SSRI) fluoxetine, which increases extracellular serotonin concentrations, was used to test these hypotheses. Finally, circadian wheel-running rhythms were monitored, because acute administration of fluoxetine influences the phase of the circadian pacemaker (Sprouse *et al.*, 2006; Gannon & Millan, 2007) and circadian rhythms are often altered in depressed patients (see Bunney & Bunney, 2000, for review). As amelioration of depression with SSRIs requires chronic treatment, it is important to determine whether chronic administration of a clinically relevant dose of fluoxetine modulates circadian rhythms.

Materials and methods

Animals and tissue preparation

These experiments used male Syrian hamsters (Harlan HSD), young (3–5 months) and old (16–19 months), exposed to a 14-h light : 10-h dark cycle (lights on from 06.00 to 20.00 h) for at least 10–14 days. Hamsters were individually housed in cages equipped with running wheels electronically interfaced with a computer for continuous monitoring of locomotor activity rhythms. Food and water were provided *ad libitum*. All animal procedures were consistent with Association for Assessment and Accreditation of Laboratory Animal Care International guidelines, and were reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

In situ hybridization

In situ hybridization was conducted as we have described previously (Duncan *et al.*, 2001, 2005). Reagents were purchased from VWR (Arlington Heights, IL, USA) unless otherwise stated. Briefly, slide-mounted tissue sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and washed with phosphate-buffered saline. The sections were acetylated, dehydrated, delipidated and air-dried. The sections were then hybridized (37°C, 21

h) with ^{35}S -end-labelled oligonucleotide probes for VIP mRNA or GRP mRNA. After hybridization, the sections were washed in $1\times$ SSC with 10 mM sodium thiosulfate at 55°C for 1 h and at room temperature for 1 h, dipped in sterile distilled water followed by 95% ethanol, and air-dried. Autoradiograms were generated by apposing the tissue sections and radioactive standards (^{14}C -microscales, Amersham, Piscataway, NJ, USA) to X-ray film (Biomax MR, VWR). Image analysis was conducted as described below (Duncan *et al.*, 2005).

Receptor autoradiography

Specific binding to $5\text{-HT}_{1\text{B}}$ receptors in the SCN was determined with quantitative autoradiography, using [^{125}I]iodocyanopindolol ([^{125}I] ICYP; Perkin Elmer/NEN, Waltham, MA, USA) with a free concentration ([Free]) of 2–12 pM as the radioligand, in the presence of isoproterenol (Tocris, Ellisville, MO, USA; 20 μM) to prevent binding to beta-adrenergic receptors and 8-OH-DPAT [Tocris; 100 nM] to prevent binding to $5\text{-HT}_{1\text{A}}$ receptors, as described in detail previously (Hoyer *et al.*, 1985; Duncan *et al.*, 2000). Nonspecific binding was determined in adjacent sections by co-incubation with serotonin (Sigma, St. Louis, MO, USA; 20 μM). Experimental slides and slides containing radioactive standards (Amersham) were apposed to X-ray film (Biomax MR; Kodak) for ~1 week. The X-ray films were developed using an automatic processor and the autoradiograms were analyzed as described below.

Specific binding to serotonin transporter sites in the SCN was determined with quantitative autoradiography using [^3H]paroxetine (Perkin Elmer/NEN; [Free] = 0.5 pM) as the radioligand, as described in detail previously (Hrdina *et al.*, 1990; Duncan *et al.*, 2000). Nonspecific binding was determined in adjacent sections by co-incubation with clomipramine (Tocris; 10 μM). Experimental slides and slides containing radioactive standards (Amersham) were apposed to X-ray film (Biomax MR, Kodak) for ~4 weeks. The resulting autoradiograms were analyzed as described below.

Image analysis

The SCN regions from each animal were identified on the autoradiograms by comparison with Nissl-stained tissue sections and reference to a hamster brain atlas (Morin & Wood, 2001). The autoradiographic images were captured with a CCD video camera (Diagnostic Instruments, Sterling Heights, MI, USA) interfaced with a computer running imaging software (M4; Imaging Corp., Ontario, Canada). The signal in each SCN was determined by comparing the optical density of the sampling area to a standard curve created by the imaging software from the optical densities generated by the radioactive standards. For receptor autoradiographic studies, the specific binding was calculated as the difference between total binding and nonspecific binding. Data were statistically analyzed using two-way ANOVA. When significant P values ($P < 0.05$) were obtained, *post hoc* analysis was conducted using Bonferroni's t -test.

Experiment 1. Effect of aging on GRP mRNA and VIP mRNA expressions in the SCN at multiple times of day

Young and old hamsters were anesthetized with pentobarbital (~140 mg/kg body weight; Ovation Pharmaceuticals, Deerfield, IL, USA) and decapitated at one of three times of day [zeitgeber times (ZT); by convention, ZT12 corresponds to the time of lights off] in order to assess the effect of age on GRP mRNA and VIP mRNA at times similar to those at which the circadian pacemaker exhibits pronounced phase-resetting by either nonphotic (ZT6) or photic (ZT13 and ZT19) signals. A dim red light was used when hamsters were killed during the dark phase. After dissection, brains were frozen on crushed dry ice and stored at -80°C . Coronal sections (20 μm thick) through the hypothalamus were cut with a cryostat (Microm/Thermo Scientific, Asheville, NC, USA), mounted onto negatively charged slides, and stored at -80°C , until *in situ* hybridization for GRP mRNA and VIP mRNA was conducted. The effects of age and time of day on SCN GRP mRNA and VIP mRNA were assessed by two-way ANOVA.

In the case of significant ($P < 0.05$) F -values, *post hoc* comparisons were made between groups with Bonferroni's test.

Experiment 2. Effect of aging and treatment with fluoxetine (15 mg/kg per day) on SCN expression of VIP mRNA and 5-HT_{1B} receptors at ZT6, and on circadian locomotor rhythms

Circadian wheel-running rhythms were recorded continuously for 4 weeks, using hardware and software from the Chronobiology kit (Stanford, CA, USA), from young and old hamsters ($n = 28$ each). The hamsters were untreated for the first 2 weeks, and then were given daily intraperitoneal injections (0.2 mL volume) of vehicle (distilled water; $n = 14$ per age group) or fluoxetine (Shanco, Shanghai, China; 15 mg/kg body weight, $n = 14$ per age group) at ZT6 for the second 2-week period. Preliminary studies had indicated that this dose of fluoxetine, administered to hamsters by daily i.p. injections but not by osmotic mini-pumps, induces circulating levels of fluoxetine and its active metabolite, norfluoxetine, that are within the therapeutic range in humans (M. J. Duncan and K. M. Franklin, unpublished observations). Because fluoxetine treatment has been shown to inhibit body weight in rats, body weight was monitored at the beginning of this study, at the beginning of the treatment period, and at the end of the treatment period. Also, several aspects of the circadian activity rhythms, including the times of activity onset and offset, and the amount of daily running, were determined for the pretreatment and treatment periods.

At the end of the study, the hamsters were killed by decapitation while under deep pentobarbital anesthesia at ZT6. Brains were dissected, frozen on crushed dry ice and stored at -80°C until coronal sections through the SCN were prepared. Samples of trunk blood were collected, stored at room temperature for 30 min, and centrifuged at 4°C for 20 min. Sera were aspirated, stored at -80°C , and then shipped frozen to Dr Marty Javors at the University of Texas Health Sciences Center at San Antonio for HPLC determination of fluoxetine and norfluoxetine. Tissue sections through the SCN were processed for *in situ* hybridization for VIP mRNA and receptor autoradiography for 5-HT_{1B} receptors, as described above, using [Free] = 12 pM.

Experiment 3. Effect of aging and treatment with fluoxetine (12 mg/kg per day) on SCN expression of VIP mRNA and 5-HT_{1B} receptors at ZT13

This experiment had a similar protocol to that of Experiment 2 with a few modifications, as described below. Fluoxetine was administered at a lower dose, 12 mg/kg per day, due to the unexpected mortality of four young hamsters treated with fluoxetine (15 mg/kg per day) in Experiment 2. A pilot study indicated that administration of 12 mg/kg fluoxetine per day to hamsters lead to circulating levels of fluoxetine and norfluoxetine that averaged 408.2 ng/mL, and were within the therapeutic range (Koran *et al.*, 1996; Kirchherr & Kuhn-Velten, 2006). Also in contrast to Experiment 2, the hamsters were killed at ZT13, and receptor autoradiography for 5-HT_{1B} receptors used [Free] = 3 pM. Finally, although the hamsters had access to running wheels, circadian activity rhythms were not recorded.

Experiment 4. Effect of aging and time of day on SCN expression of 5-HT_{1B} receptors and serotonin transporters

Because the combined results of Experiments 2 and 3 suggested that aging differentially affects 5-HT_{1B} receptors at ZT6 and 13, this possibility was examined directly in this study. Young and old hamsters were killed at ZT6 and ZT13, and coronal tissue sections through the SCN were prepared and processed for 5-HT_{1B} receptor autoradiography ([Free] = 3 pM). Furthermore, in order to investigate whether time of day as well as aging influences SCN serotonin reuptake, serotonin transporter autoradiography was conducted as well.

Results

Experiment 1. Effect of aging on GRP mRNA expression in the SCN at multiple times of day

SCN GRP mRNA expression was significantly decreased ($P < 0.02$) by aging but was not significantly affected by time of day ($P = 0.30$; Figs 1 and 2). There was a trend toward an interaction of these main effects, which did not reach significance ($P = 0.08$). The greatest difference between age groups in GRP mRNA expression was observed at ZT6, at which time GRP mRNA expression in old hamsters was on average ~50% lower than in young hamsters. Similar to GRP mRNA expression, VIP mRNA in the SCN was significantly affected only by age (age, $P < 0.005$; time of day, $P = 0.56$; interaction, $P = 0.86$; Figs 1 and 3), as reported previously (Duncan *et al.*, 2001).

Experiment 2. Effect of treatment with fluoxetine (15 mg/kg per day) on SCN VIP mRNA expression and 5-HT_{1B} receptors at ZT6 and on circadian locomotor rhythms

Chronic fluoxetine treatment did not affect SCN VIP mRNA expression in young or old hamsters, although old hamsters exhibited lower levels of VIP mRNA expression in the SCN (two-way ANOVA: age, $P < 0.05$; fluoxetine, $P = 0.108$; interaction of age and fluoxetine, $P = 0.399$; Fig. 4A). Specific [¹²⁵I]-iodocyanopindolol binding in the SCN was significantly affected by age ($P < 0.05$) and by the interaction of age and fluoxetine ($P < 0.05$) but not by fluoxetine treatment alone ($P = 0.102$). Thus, SCN 5-HT_{1B} receptor binding sites at ZT6 were robustly increased in old, vehicle-treated hamsters as compared to young hamsters (Fig. 4B), in confirmation of our previous finding (Duncan *et al.*, 2000). Treatment with fluoxetine (15 mg/kg per day) blocked this age-related increase but had no effect on SCN 5-HT_{1B} receptor binding sites at ZT6 in young hamsters (Figs 4B and 5). As well as decreasing SCN 5-HT_{1B} receptor binding sites in old hamsters, fluoxetine decreased body weight (~6–9%) in both age groups and strongly inhibited daily wheel running activity, as did aging to a lesser extent (Table 1). In contrast to the amount of daily running, the times of activity onset or offset and the duration of running were not affected by either fluoxetine treatment or aging (Table 1). The combined circulating levels of fluoxetine and nor-fluoxetine (Young, 451 ± 29 ng/mL; Old, 515 ± 47 ng/mL) were within the normal therapeutic range for fluoxetine treatment in humans (200–800 ng/mL), with no significant difference between age groups (Koran *et al.*, 1996; Kirchherr & Kuhn-Velten, 2006). Unexpectedly, four young fluoxetine-treated hamsters died before the end of the study.

Experiment 3. Effect of treatment with fluoxetine (12 mg/kg per day) on SCN VIP mRNA expression and 5-HT_{1B} receptors at ZT13

SCN VIP mRNA expression at ZT13 was significantly lower (~30%) in old hamsters than in young hamsters, but was not affected by fluoxetine in either age group (Fig. 6A; age, $P < 0.0001$; fluoxetine, $P = 0.309$; interaction effect, $P = 0.686$). In contrast to VIP mRNA, SCN 5-HT_{1B} receptor binding sites at ZT13 were significantly decreased (by ~30%) by fluoxetine treatment ($P < 0.01$) but were not affected by age ($P = 0.940$) or by an interaction of age and fluoxetine ($P = 0.688$; Fig. 6B). Also, fluoxetine treatment significantly reduced body weight (by ~7%, $P < 0.0001$). Body weight was not affected by age ($P = 0.146$) or by an interaction between fluoxetine and age ($P = 0.597$). (Although blood samples were collected, fluoxetine and norfluoxetine levels were not assessed because preliminary studies had indicated that these values were within the therapeutic range, and because the effect of fluoxetine on body weight was similar to that observed in Experiment 2).

Experiment 4. Effect of aging and time of day on SCN expression of 5-HT_{1B} receptors and serotonin transporters

As shown in Fig. 7A, specific 5-HT_{1B} receptor binding in the SCN was significantly affected by time of day ($P < 0.05$). The main effect of aging did not reach significance ($P = 0.106$) although the interaction between time of day and aging was significant ($P < 0.05$). As indicated by *post hoc* tests, specific 5-HT_{1B} receptor binding in the SCN was significantly greater in old than in young hamsters at ZT6 ($P < 0.05$) but not at ZT13. In contrast to 5-HT_{1B} receptor binding, specific binding to the serotonin transporters in the SCN was significantly increased by aging ($P < 0.001$) but not significantly affected by time of day ($P = 0.099$) or by an interaction of these main effects ($P = 0.722$; Figs 7B and 8).

Discussion

Aging leads to many changes in the expression of circadian rhythms as well as alterations in the neural substrates regulating them, especially the SCN. The present study extended previous reports of age-related attenuation of SCN expression of VIP mRNA (Krajnak *et al.*, 1998a; Duncan *et al.*, 2001) by demonstrating that aging also decreases SCN expression of mRNA for GRP. It should be noted that aging appears to selectively suppress GRP mRNA and VIP mRNA in the hamster SCN, because no effect of aging has been detected for several other mRNAs expressed in the hamster SCN, including those coding for vasopressin, somatostatin, calbindin-D28 and GAD67 (Duncan & Wheeler, 1999; Duncan *et al.*, 2001; Duncan & Franklin, 2007). Both VIP and GRP are expressed in the ventral SCN (although their distributions are not identical; Moore & Silver, 1998; Morin *et al.*, 2006), which is the region of the SCN that first exhibits changes in expression of the immediate-early gene *Fos* and circadian clock genes *Per1* and *Per2*, after phase-resetting light pulses (Earnest & Olschowka, 1993; Hamada *et al.*, 2001, 2004; Dardente *et al.*, 2002; Yan & Silver, 2002), and both peptides are involved in mediating light-induced circadian phase shifts. For example, exogenous administration of GRP to the SCN induces phase delays in the early subjective night and phase advances in the late subjective night, similar to light (Albers *et al.*, 1995; Piggins *et al.*, 1995; Aida *et al.*, 2002). GRP has also been implicated in conveying information from the ventral, light-responsive, SCN region to the dorsal, endogenously rhythmic, SCN region (Aida *et al.*, 2002; Antle *et al.*, 2005). Thus, decreased SCN expression of GRP mRNA during aging may contribute to age-related loss of sensitivity to photic phase shifting signals (Zhang *et al.*, 1996; Kolker *et al.*, 2003).

Decreased SCN expression of VIP may also contribute to this phenomenon, as well as to age-related deficits in circadian rhythm amplitude or coupling. As shown here and previously, aging decreases SCN expression of VIP mRNA in the hamster and rat SCN (Krajnak *et al.*, 1998a; Duncan *et al.*, 2001) and reduces the number of VIP-immunoreactive cells in the rat and human SCN (Chee *et al.*, 1988; Zhou *et al.*, 1995; Pereira *et al.*, 2005). In mice, genetic deletion of either VIP or the VIP receptor *Vipr2* (also called VPAC2) disrupts circadian activity rhythms during exposure to constant darkness, as well as the circadian rhythms of firing rate of SCN neurons *in vitro* (Harmar, 2003; Harmar *et al.*, 2003; Aton *et al.*, 2005). Furthermore, when circadian rhythms are observed in the SCN of a few of these mutant mice, these rhythms are characterized by lower amplitude and expression of more than one period length, suggesting desynchrony among multiple circadian oscillators (Harmar *et al.*, 2003; Aton *et al.*, 2005). These studies suggest that VIP plays an important role in oscillator coupling. Furthermore, microinjections of VIP into the hamster SCN induce photic-like phase shifts (Huhman & Albers, 1994; Piggins *et al.*, 1995). Thus, VIP acting through *Vipr2* receptors participates in light-induced phase shifts and is necessary for consolidated high-amplitude circadian rhythms. VIP is also an important SCN output signal regulating circadian endocrine rhythms, such as the glucocorticoid rhythm and the preovulatory luteinizing hormone surge, which exhibit

attenuation with aging (Krajnak *et al.*, 1998b; Gerhold *et al.*, 2005; Loh *et al.*, 2008). Therefore, age-related decreases in VIP mRNA expression probably contribute to age-related deficits in photic phase shifts as well as age-related attenuation of rhythm amplitude and perhaps fragmentation or loss of synchrony of rhythms (Satinoff *et al.*, 1993; Cai *et al.*, 1997; Kolker *et al.*, 2003).

Because VIP neurotransmission is critical for SCN function, the current study investigated a possible mechanism mediating age-related inhibition of SCN VIP mRNA by testing the hypothesis that the age-related increase in SCN serotonin transporter sites (Duncan *et al.*, 2000) mediates the age-related decrease in VIP mRNA expression (Duncan *et al.*, 2001). The results of both Experiments 2 and 3 revealed no effect of fluoxetine on SCN VIP mRNA expression; thus, there was no support for this hypothesis. The lack of effect of fluoxetine treatment on SCN VIP mRNA expression contrasts with previous studies indicating that this expression is modulated by serotonergic innervation. For example, serotonergic depletion of the rat SCN by treatment with selective toxins [e.g., parachlorophenylalanine or 5,6-dihydroxytryptamine (5,6-DHT)] induced a decrease of ~25% in SCN VIP mRNA expression at either ZT5 or ZT8 (Kawakami *et al.*, 1994; Hayashi *et al.*, 2001). Although this previous study indicates that virtual total loss of serotonin innervation has an inhibitory effect on SCN VIP mRNA expression in rats, the present results suggest that the converse is not the case, i.e., that the enhanced extracellular serotonin levels presumably produced by fluoxetine treatment do not have a stimulatory effect on SCN VIP mRNA levels, at least in hamsters. There may also be important species differences in the regulation of VIP mRNA expression in the SCN, in view of the day–night differences observed in rats but not hamsters (Albers *et al.*, 1990; Duncan *et al.*, 2001). Furthermore, while 5,6-DHT treatment decreased VIP mRNA expression in the rat SCN at ZT8, this treatment increased expression of VIP mRNA at ZT20 (Hayashi *et al.*, 2001).

In contrast to the lack of effect of fluoxetine on SCN VIP mRNA in the current study, the results did support the third hypothesis that the age-related increase in SCN serotonin transporter sites mediates the age-related increase in SCN 5-HT_{1B} receptor binding sites observed at ZT6 (Duncan *et al.*, 2000). Chronic fluoxetine administration selectively decreased SCN 5-HT_{1B} receptor binding sites at ZT6 in old hamsters, restoring the expression of these binding sites to the level seen in young hamsters at this timepoint. This finding is consistent with our previous prediction that if the age-related increase in [³H]paroxetine binding sites represents increased serotonin reuptake, then decreased serotonin levels in the synaptic space may cause the age-related increase in the 5-HT_{1B} receptor binding sites (Duncan *et al.*, 2000). The lack of effect of fluoxetine on 5-HT_{1B} binding sites in the SCN of young hamsters during the mid-day is similar to other findings that chronic fluoxetine treatment in young adult mice did not alter 5-HT_{1B} binding sites in the globus pallidus or substantia nigra of mice (Shanahan *et al.*, 2009) or hamsters (M. J. Duncan and K. M. Franklin, unpublished observations).

However, the current study showed that when SCN 5-HT_{1B} receptor binding sites were measured at ZT13 instead of ZT6, lower levels were exhibited in both young and old fluoxetine-treated hamsters, as compared to vehicle-treated controls. Furthermore, there was no age difference in SCN 5-HT_{1B} receptors at ZT13, as indicated by the results of both Experiments 3 and 4. The finding that SCN 5-HT_{1B} receptor binding sites are elevated in old hamsters vs. young at ZT6 but not ZT13 cannot be explained by differences in serotonin transporter binding sites at these times, as shown in Experiment 4. The finding that fluoxetine suppressed SCN 5-HT_{1B} binding sites in young as well as old hamsters at ZT13 but not ZT6 may be explained by the previous finding that serotonin release in the adult hamster SCN is maximal at ZT13 and basal at ZT6 (Dudley *et al.*, 1998). Therefore, the potentiating effect of fluoxetine on SCN extracellular serotonin levels is likely to be greatest at ZT13; this effect may lead to enhanced

receptor down-regulation and thus account for the decreased 5-HT_{1B} receptor binding sites observed at ZT13 after chronic fluoxetine treatment in the current study.

The current project also investigated the effect of fluoxetine treatment and aging on circadian wheel-running rhythms. In view of a recent report that acute fluoxetine administration to the SCN *in vitro* acts synergistically with tryptophan to robustly advance the circadian pacemaker (Sprouse *et al.*, 2006), we sought to determine whether chronic fluoxetine treatment, as used to ameliorate depression, also advances the circadian pacemaker. No effects of fluoxetine treatment were observed on the times of activity onset or offset, which are used as markers for phase angle. This finding is reminiscent of a previous study in which fluoxetine administered in the drinking water did not alter the phase angle of entrainment of wheel running rhythms of hamsters exposed to a short day photoperiod (10 h light : 14 h dark; 10L : 14D) for 2 weeks (Klemfuss & Kripke, 1994). In contrast to this previous study, the present study investigated hamsters exposed to a long day photoperiod, and confirmed that the dose and route of administration of fluoxetine produced clinically relevant circulating levels of this drug and its biologically active metabolite in young and old animals. Furthermore, the present study tested the effect of administration of fluoxetine during the mid-day because this is the time when the circadian system is most sensitive to serotonergic induction of phase shifts (Tominaga *et al.*, 1992; Edgar *et al.*, 1993; Prosser, 2003) and when the therapeutic effect of the another antidepressant, chlomipramine, has been shown to be maximal (Nagayama *et al.*, 1991). Taken together, the current and previous studies indicate that although acute administration of fluoxetine can advance the circadian pacemaker *in vitro* (Sprouse *et al.*, 2006) or attenuate photic phase shifts *in vivo* (Gannon & Millan, 2007), chronic fluoxetine treatment does not appear to alter photic entrainment.

Similar to chronic fluoxetine, aging also did not alter entrainment in the current study. This finding was consistent with a previous report that the time of activity onset was not affected by age when hamsters were exposed to a long day photoperiod (14L : 10 D), although it was significantly altered by age, by >2 h, in hamsters exposed to a short day photoperiod (6L : 18D; Scarbrough *et al.*, 1997). As well as entrainment, the current study investigated the effect of aging and fluoxetine on the amount of daily wheel running. Both aging and fluoxetine significantly reduced the average number of wheel revolutions per day. As hamsters run almost exclusively at night, this effect represents a reduction in the amplitude of the circadian locomotor activity rhythm. Changes in circadian rhythm amplitude, as well as changes in activity, have previously been associated with aging in rodents and humans (Golombek *et al.*, 1997; Wei *et al.*, 1999).

In conclusion, the present studies demonstrated that SCN expression of neuropeptide genes (GRP and VIP) and receptors (5-HT_{1B} receptors) that modulate photic resetting of circadian rhythms are affected by aging, and that the latter is also affected by chronic treatment with a therapeutically relevant dose of fluoxetine. Furthermore, age-related changes in SCN expression of VIP mRNA and 5-HT_{1B} receptors are differentially regulated because only the latter are mediated by age-related increases in serotonin reuptake sites. Also, while aging and fluoxetine administration decrease the amount of daily wheel running, reflecting attenuation of the amplitude of this circadian rhythm, these factors do not influence the rhythm phase during exposure to a long day photoperiod (14L : 10D).

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Abbreviations

[¹²⁵I]ICYP	[¹²⁵ I]iodocyanopindolol
[Free]	free concentration
5-HT	5-hydroxytryptamine or serotonin
GRP	gastrin-releasing peptide
SCN	suprachiasmatic nucleus
VIP	vasoactive intestinal peptide
ZT	zeitgeber time

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Fig. 1.

The effects of aging and time of day on SCN expression of GRP and VIP mRNAs. (A) Aging but not time of day decreased SCN expression of GRP mRNA. (B) SCN VIP mRNA was lower in old hamsters and was not affected by time of day. Bars are the mean + SEM ($n = 5-8$ per group).

Fig. 2. Photomicrographs depicting *in situ* hybridization of GRP mRNA in the SCN. Scale bar, 1 mm (refers to all panels).

Fig. 3. Photomicrographs depicting *in situ* hybridization of VIP mRNA in the SCN. Scale bar, which represents 1 mm, refers to all panels.

Fig. 4.

The effects of aging and fluoxetine administration (15 mg/kg per day) on SCN expression of VIP mRNA and specific [¹²⁵I]iodocyanopindolol (ICYP) binding to 5-HT_{1B} receptors at ZT6. (A) Aging significantly decreased SCN VIP mRNA expression but fluoxetine treatment had no effect. (B) Aging increased specific [¹²⁵I]ICYP binding in the SCN; fluoxetine administration blocked this age-related increase but did not alter specific [¹²⁵I]ICYP binding in young hamsters. Bars represent the mean + SEM (n = 7–12 per group). *P < 0.05 vs. young vehicle-treated group.

Fig. 5.
Photomicrographs depicting specific [^{125}I]ICYP binding in the SCN. Scale bar, 1 mm (refers to all panels).

Fig. 6.

The effects of aging and fluoxetine administration (12 mg/kg per day) on SCN expression of VIP mRNA and specific [¹²⁵I]ICYP binding to 5-HT_{1B} receptors at ZT13. (A) Aging decreased SCN VIP mRNA expression at ZT13; this effect was not attenuated by fluoxetine. (B) Fluoxetine but not aging decreased specific [¹²⁵I]ICYP binding in the SCN at ZT13. Bars are the mean + SEM ($n = 8-15$ per group).

Fig. 7.

The effects of aging and time of day on specific [¹²⁵I]ICYP binding in the SCN and specific [³H]paroxetine (PRX) binding in the SCN. (A) Specific [¹²⁵I]ICYP binding in the SCN was greater in old hamsters than in young hamsters at ZT6 but not at ZT13. * $P < 0.05$ vs. young at same timepoint. (B) Aging increased specific [³H]paroxetine binding sites in the SCN while time of day had no effect.

Fig. 8. Photomicrographs depicting specific [³H]paroxetine binding in the SCN. Scale bar, 1 mm (refers to all panels).

Effect of aging and fluoxetine administration (15 mg/kg per day) on body weight and wheel running activity

Table 1

	Young		Old		P-values for effect of		
	Vehicle	FLX	Vehicle	FLX	Age	FLX	Interaction
Body weight change (% initial)	3.32 ± 0.66	-6.82 ± 0.02	-1.06 ± 0.92	-8.75 ± 2.08	0.0383	<0.0001	0.4133
Daily wheel revolutions (×1000)	7.46 ± 0.46	4.30 ± 0.62	6.80 ± 0.55	2.50 ± 0.54	0.0286	<0.0001	0.3060
Activity							
Onset (h)	21.2 ± 0.07	21.5 ± 0.17	21.2 ± 0.07	20.8 ± 0.90	0.4611	0.8658	0.4239
Offset (h)	03.62 ± 0.31	03.39 ± 0.53	03.10 ± 0.033	03.98 ± 0.59	0.9368	0.4598	0.2194
Duration (h)	6.01 ± 0.28	4.86 ± 0.53	5.53 ± 0.25	5.93 ± 0.83	0.5707	0.4706	0.1412

FLX, fluoxetine. Values are the mean ± SEM for each group (n = 11–14 each).