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### The circadian system

*A regulatory feedback network of periphery and brain*

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## CHAPTER 3

Olanzapine-induced early cardiovascular effects are mediated by the biological clock and prevented by melatonin.

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## **Abstract**

Second generation antipsychotics (SGA) are associated with adverse cardiometabolic side-effects contributing to premature mortality in patients. While mechanisms mediating these cardiometabolic side-effects remain poorly understood, three independent studies recently demonstrated that melatonin was protective against cardiometabolic risk in SGA-treated patients. Since one of the main target areas of circulating melatonin in the brain is the suprachiasmatic nucleus (SCN), we investigated SCN involvement in SGA-induced early cardiovascular effects in Wistar rats. We evaluated the acute effects of olanzapine and melatonin in the biological clock, paraventricular nucleus and autonomic nervous system using immunohistochemistry, invasive cardiovascular measurements and Western blot. Olanzapine induced c-Fos immunoreactivity in the SCN followed by the paraventricular nucleus and dorsal motor nucleus of the vagus indicating a potent induction of parasympathetic tone. The involvement of a SCN-parasympathetic neuronal pathway after olanzapine administration was further documented using Cholera Toxin B retrograde tracing and vasoactive intestinal peptide immunohistochemistry. Olanzapine-induced decrease in blood pressure and heart rate confirmed this. Melatonin abolished olanzapine-induced SCN c-Fos immunoreactivity, including the parasympathetic pathway and cardiovascular effects while brain areas associated with olanzapine beneficial effects including the striatum, ventral tegmental area and nucleus accumbens remained activated. In the SCN, olanzapine phosphorylated the GSK-3 $\beta$ , a regulator of clock activity, which melatonin prevented. Bilateral lesions of the SCN prevented the effects of olanzapine on parasympathetic activity. Collectively, results demonstrate the SCN as a key region mediating the early effects of olanzapine on cardiovascular function, and show melatonin has opposing and potentially protective effects warranting additional investigation.

## Introduction

Second generation antipsychotics (SGA) induce severe adverse cardiometabolic effects that affect millions of patients and families by decreasing drug adherence or dramatically increasing their health burden with metabolic comorbidity (Lieberman et al. 2005). Olanzapine is a SGA effectively used to treat patients with mental disorders, but unfortunately induces adverse cardiometabolic effects including weight gain, fat mass, glucose, insulin, and lipid increases (Rummel-Kluge et al. 2010; Vidarsdottir et al. 2010; Vancampfort et al. 2013). Interestingly, in rats and humans, acute SGA treatment may induce hypotension (Markowitz 2002; Choure et al. 2014; Leung et al. 2014), while long-term SGA treatment is associated to increased blood pressure (McEvoy et al. 2007). In consequence, they have become an enormous health problem in a population in which the risk of metabolic abnormalities is 2 to 3 times greater than that of the general population (Vancampfort et al. 2013). These patients also suffer a premature death 11 to 20 years earlier, largely due to cardiovascular disease (Laursen et al. 2013; Westman et al. 2013). The SGA-induced cardiometabolic effects are also present in acute administration studies in healthy individuals, indicating that metabolism changes are independent of caloric intake and disease related factors (Vidarsdottir et al. 2010; Hahn et al. 2013). Even though intense research efforts during the past decade have focused on providing a plausible mechanism for their genesis and prevention, an integral explanation is lacking and the therapeutic options to prevent them are limited and urgently needed.

Metabolism is influenced through central hypothalamic mechanisms that involve the biological clock located in the suprachiasmatic nucleus (SCN) and the autonomic nervous system (ANS) (Buijs and Kalsbeek 2001; Buijs et al. 2013; Takeda and Maemura 2016). Disturbances of these mechanisms have been associated to metabolic problems such as the metabolic syndrome (Buijs and Kalsbeek 2001; Kreier et al. 2003), which resembles SGA-induced cardiometabolic effects.

In a recent study the pineal hormone melatonin, which signals darkness to the SCN depressing its activity (Liu et al. 1997), attenuated olanzapine-induced weight gain in rats (Raskind 2007). Hereafter in randomized controlled trial (RCT), we demonstrated that melatonin attenuates SGA induced cardiometabolic effects in patients, particularly those with bipolar disorder without affecting the psychopathological outcome (Romo-Nava et al. 2014). In two other RCT, melatonin mitigated olanzapine induced cardiometabolic effects in patients diagnosed with schizophrenia (Modabbernia et al. 2014) and bipolar disorder (Mostafavi et al. 2014).

The SCN is strongly involved in metabolic and cardiovascular control (Buijs et al. 2013). Since one of the main targets of circulating melatonin in the brain is the SCN (Reppert et al. 1988, Liu et al. 1997), these basic and clinical results could suggest the involvement of central mechanisms in SGA-induced cardiometabolic effects. Therefore, we examined in a rat model the early effects of olanzapine on hypothalamic nuclei relevant for metabolic

regulation and uncovered a hitherto unknown action of olanzapine on the biological clock resulting in a decrease in blood pressure; which was prevented by melatonin. These findings identify a novel SCN-centered mechanism for olanzapine cardiometabolic adverse effects and their prevention by melatonin without tampering the therapeutic effects.

## Materials and Methods

**Ethical statement for animal experimentation.** All experiments were carried out with the approval of the research ethics committee of the Institute of Biomedical Research at the Universidad Nacional Autónoma de México (UNAM) and were conducted in strict accordance with current legislation and technical specifications for production, care and use of laboratory animals (Norma Oficial Mexicana NOM- 062 -ZOO- 1999).

**Immunohistochemistry for c-Fos.** Male Wistar rats (200 – 250 g) were used to evaluate the effect in the brain and autonomic nervous system of acute administration of olanzapine (Zyprexa® powder for solution, Lilly USA, LLC, Indianapolis, USA) and melatonin (Sigma-Aldrich product No. M5250, Saint Louis, MO 63103, USA). Animals were in light-dark cycles of 12h: 12h (Lights on at 07:00h, lights off at 19:00h) with *ad libitum* food and water. Rats received a single subcutaneous dose of olanzapine (2.5 mg/kg), olanzapine (2.5 mg/kg) + melatonin (2.5 mg), melatonin (2.5 mg) or saline (NaCl 0.9%) at ZT11 (one hour before dark onset) and were sacrificed at ZT14. Olanzapine and melatonin doses fall within the range used for previously published studies in rats (Dawe, Huff et al. 2001, Kitagawa, Ohta et al. 2012, Weston-Green, Huang et al. 2012). Olanzapine and melatonin were dissolved in a 20% ethanol and saline solution (final volume of administration: 0.5 mL). Saline and olanzapine only groups received the same amount of ethanol (Fig. S1).

Animals were sacrificed 3 hours after drug administration (ZT14). To obtain the brains, animals were anesthetized with an overdose of intraperitoneal (i.p.) pentobarbital (210 mg/kg) and perfused with an intracardiac infusion of 250 ml saline solution (0.9% NaCl), followed by 200 ml 4% paraformaldehyde prepared in 0.1M phosphate buffer, pH 7.2. Brains and spinal cords were removed and placed in 4% paraformaldehyde for 24 hrs and subsequently cryo-preserved in 30% sucrose for 72 h. The brains were frozen and cut in 40 µm coronal plane slices and maintained in culture dishes with a 0.1M phosphate buffer, pH 7.2. The primary antibody for c-Fos (1:40,000; Calbiochem, #PC38, San Diego, CA, USA) used as a marker of neural activity, was incubated for 1 hr. at room temperature and 48 hours at 4 °C. After extensive washing, donkey anti-rabbit biotinylated secondary antibody (1:200, Jackson Immunoresearch, West Grove, PA, USA) was applied and incubated for 2 h. The avidinperoxidase complex (ABC 9:1000; Vectastain, Vector Laboratories, Burlingame, CA, USA) was applied and finally the reaction was visualized with diaminobenzidine in PBS (50 mg/100 mL, pH 7.2) combined with 0.003% peroxide and 0.01% nickel. The sections were mounted on gelatinized slides and cover slipped with Entellan® (EMD Millipore Corp., Billerica, MA, USA). Coronal sections of each side, in each nucleus or region studied

were selected using the following distance from bregma coordinates: SCN (-0.60mm); paraventricular nucleus (PVN; -1.72mm); dorsal motor nucleus of the vagus (DMV; -14.40mm); intermediolateral column (IML; T3); striatum (0.96mm); ventral tegmental area (-5.04mm); nucleus accumbens (0.96mm) (Paxinos and Watson 2007).

**Neuronal tracer injection.** In order to confirm that olanzapine's effects involve the activation of a SCN-PVN-DMV neuronal pathway, the retrograde tracer Cholera toxin-B (CtB) was injected into the DMV to analyze co-localization with c-Fos in the PVN pre-autonomic neurons after olanzapine administration and the presence of VIP projections from the SCN to them.

After anesthesia the rats were placed in a David Kopf stereotaxic frame with the head fixed at 45°. Dissection of the dura and arachnoid to expose the dorsal surface of the bone at the level of the area postrema was performed. The head of the rat was placed so that the micropipette was aligned perpendicularly to the medulla oblongata. CtB (50 µl, 0.5%) was injected, by means of a glass micropipette with a 0.02 µl tip, unilaterally into the dorsal vagal complex by pressure (10 mbar, for 5 seconds). Following 10 days of recovery, animals received the acute subcutaneous administration of olanzapine and were sacrificed following the aforesaid procedure. Injection accuracy was confirmed by CtB immunostaining (Fig. S2).

Immunohistochemistry in PVN coronal sections was performed with c-Fos as described above. Immunohistochemistry for vasoactive intestinal peptide (VIP) to observe SCN projections to the PVN pre-autonomic neurons was then performed sequentially incubating the primary antibody for (rabbit- VIP) in a 1:2000 dilution for 1 hour at room temperature and 24 hours at 4 °C. Secondary antibody, AB and visualization with DAB procedures were followed as previously described. On the same PVN sections, CtB immunohistochemistry was performed using polyclonal rabbit anti-CtB immunoglobulin (1:1000, Sigma-Aldrich; No. C3062) to visualize PVN neurons retrogradely filled from DMV CtB injections. After incubating 24 hrs at 4°C. Secondary antibody, AB and visualization with DAB procedures were followed as described above without nickel to obtain a brown-reddish stain.

**Hemodynamic Measurements.** Blood pressure and heart rate were measured in order to evaluate the functional relevance of the effect of olanzapine and melatonin on the parasympathetic nervous system. In order to evaluate the involvement of the SCN over the cardiovascular effects of olanzapine, we included a group of bilaterally SCN lesioned (SCNxx) animals and measured the immediate effects of olanzapine on blood pressure and heart rate.

Blood pressure and heart rate measurements were made through a femoral artery catheter. Cannulation of the femoral artery was performed as described elsewhere (Jespersen, Knupp et al. 2012). In brief, rats were anesthetized with i.p. urethane (1.5 g/kg) diluted in 2 mL of saline solution (0.9% NaCl). Rats were placed in supine position and fur around the

inguinal surgical region was shaved. A small (1-2 cm) incision along the natural angle of the leg was placed and femoral vein and artery exposed through blunt dissection. The femoral artery was separated from the vein, nerve and surrounding tissue and retractors placed to fully view the artery and vein. Folded sterile 4.0 silk was placed under the femoral artery and cut to obtain proximal and distal silk pieces. The distal silk piece was pulled caudally and the proximal piece cranially to allow hemostatic control of the artery. A small incision was made on the artery section between the silk pieces; fine tip forceps were inserted into the incision and used to allow the insertion of the catheter. The catheter was pushed into the artery and proximally fixed. The functionality of the catheter was checked and two hours after surgery, connected to a blood pressure transducer.

***Bilateral lesion of the suprachiasmatic nucleus.*** The SCN lesion technique has been previously described (Buijs, Kalsbeek et al. 1993). In short, animals were anesthetized with I.P. ketamine/xylazine (90 mg and 10 mg/kg) and placed on a stereotaxic surgery frame (Model 900, David Kopf). Coordinates used to lesion the SCN were: 2.2mm posterior to bregma, 0.9mm ventral, and 0.2 mm lateral. Lesions were performed using epoxy-insulated insect pins (0.20mm) with excoriated tip. Direct electrical current for 30 seconds was applied bilaterally. Rats recovered from surgery for 2 weeks with successful lesions confirmed by actigraphic registration and SCN VIP immunostaining as described above (Fig. S3).

***Hemodynamic registration.*** Blood pressure and heart rate measurements were recorded from the artery catheter by a pressure transducer (P23 XL, Grass Instrument, Quincy, MA, USA) connected to a MP150 Research System and the data were analyzed using AcqKnowledge software (Biopac Systems Inc., Goleta, CA, USA). For the purpose of this experiment, baseline tracings, as well as saline, olanzapine and melatonin were sequentially administered i.p. at the previously described doses to ensure quantifiable responses of hemodynamic parameters. Data was obtained from three experimental groups of animals, with minutes indicating the time data was recorded after drug administration (I.P.). Group 1 (olanzapine): Baseline values were obtained (10 min) followed by 0.5 ml saline (10 min) and a single olanzapine (0.5 mg/kg) injection (60 min). Group 2 (melatonin + olanzapine): Ensuing baseline recording (10 min), a 0.5 ml saline injection (10 min) was followed by a single melatonin (2.5 mg) I.P. injection (10min) and successively olanzapine i.p. was given (60 min). Group 3 (SCNxx animals + olanzapine): Baseline tracing (10 min) preceded 0.5 ml saline I.P. (10 min), followed by a single olanzapine I.P. injection (60 min).

**Western blot.** For Nocturnal (ZT11) drug administration for saline, olanzapine, olanzapine+melatonin and melatonin, the protocol was followed as described above (n=5 animals/experimental group). Animals were sacrificed at ZT14 by decapitation. The suprachiasmatic nucleus was immediately obtained, preserved in RIPA buffer with protease and phosphatase inhibitors and frozen at -72 °C. Tissue was homogenized and total protein concentration was determined by Lowry's assay (Lowry, Rosebrough et al. 1951). Equal amounts of protein were loaded into polyacrylamide gels and phosphorylated as well as total GSK-3 $\beta$  were evaluated by Western blot. Glyceraldehyde-3-phosphate dehydrogenase GAPDH was also immunodetected as load control. In short, proteins were separated by one-dimensional SDS-PAGE in 10% polyacrylamide gels (Laemmli 1970) and transferred according to Towbin's procedure (Towbin, Staehelin et al. 1979). Phosphorylated GSK-3 $\beta$  (Ser9) was identified with an anti-phospho-GSK-3 $\beta$  antibody (1:1300, Cell Signaling Technology Inc., #9336, Danvers, MA, USA) and a peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, #711-005-152, West Grove, PO, USA) diluted 1:10,000. Detection was performed by chemiluminescence (Alegria-Schaffer 2014). After mild stripping, total GSK-3 $\beta$  and GAPDH were identified using antibodies for GSK-3 $\beta$  (1:1400, Santa Cruz Biotech, #SC-9166, Dallas, Tx, USA) and GAPDH (1:20,000, EMD Millipore Corp., MAB374, Billerica, MA, USA). Respective peroxidase-conjugated secondary antibodies were: donkey anti-rabbit IgG (1:20,000, Jackson ImmunoResearch, #711-005-152, West Grove, PO, USA) ) and goat anti-mouse IgG (1:100,000, Jackson ImmunoResearch, # 205-005-108, West Grove, PO, USA). Samples from five independent experiments were obtained and Western blots were assayed by triplicate.

Fluorogram images were obtained with a densitometer (GS-800, Bio-Rad, Hercules, CA, USA) and semi-quantitative amounts of phosphorylated GSK-3 $\beta$ , total GSK-3 $\beta$  and GAPDH were estimated by densitometry with ImageJ software (Version 1.38X, NIH, Bethesda, MD, USA). Each fluorogram was analyzed in 8-Bit type .jpg picture formats. The plot lane tool in ImageJ was used to obtain integrated optical density (OD) values from immunoreactive bands for each experimental group.

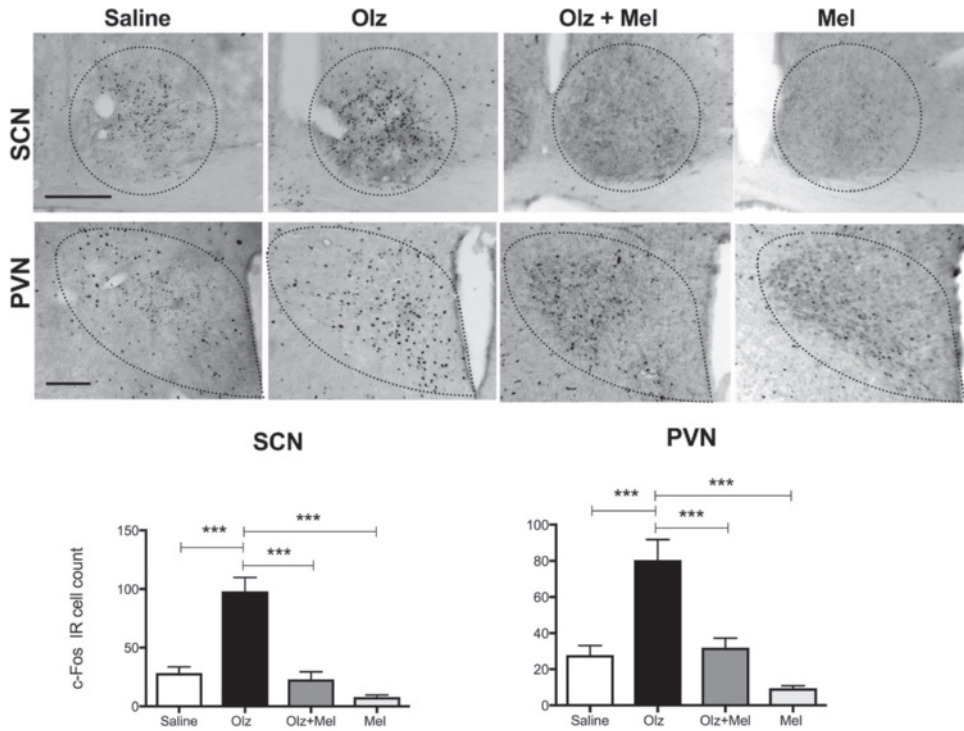
**Statistical Analysis.** The number of c-Fos immunoreactive nuclei was calculated by semi-automatic quantification of the region of interest with ImageJ software (Version 1.38X, NIH, Bethesda, MD, USA) using at least two microphotographs (jpg format) of coronal sections of each side, in each nucleus or region studied. For between group comparisons (n=4 per group), we considered the average c-Fos immunoreactivity (IR) count for each region and analysis was conducted using ANOVA with Bonferroni's post-hoc test for pairwise comparisons accordingly.

Blood pressure and heart rate analysis was performed using a data acquisition device with Acqknowledge software (model MP100, Biopac Systems, Goleta, CA, USA) and saved to a personal computer for offline analysis with the use of a Biopac System ECG 100C preamplifier. Ten-minute tracings of mean blood pressure values for baseline, saline,

olanzapine or melatonin injections were obtained. Changes in mean baseline values for systolic, diastolic and mean arterial blood pressure were calculated for each tracing segment of ten minutes in each experimental group. Heart rate analysis was performed using maximum values in five 2-minute segments for each ten-minute tracing to clear noise from registrations; with the mean for each ten-minute tracing calculated using these values. Mean changes in heart rate, compared to baseline values, were then calculated for saline, olanzapine and/or melatonin injections. Repeated measures ANOVA Bonferroni's post-hoc test was used for pair-wise comparisons at each time point (10-min segments). For Western blot analysis, densitometric analysis of the bands expressed as the optical density in A.U. of total GSK-3 $\beta$  pGSK-3 $\beta$ , GAPDH, and the ratio of pGSK-3 $\beta$  to total pGSK-3 $\beta$  (pGSK-3 $\beta$ / Total pGSK-3 $\beta$  ratio) were used for between-group comparisons using One-way ANOVA. All statistical tests were two-tailed and considered significant at p level <0.05.

## Results

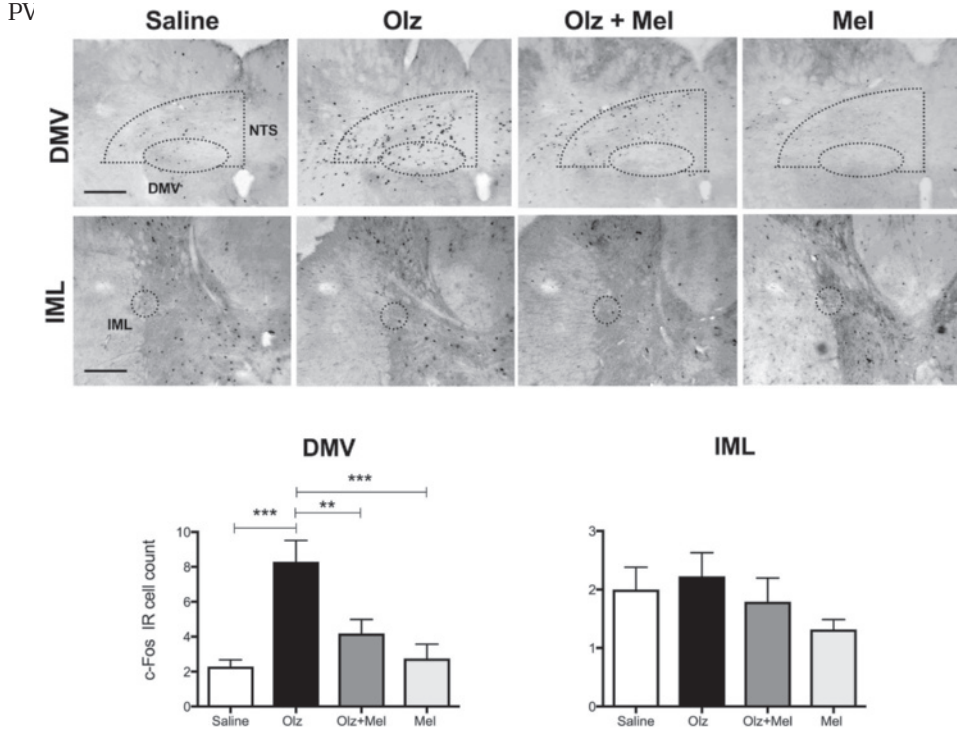
***The SCN is activated by olanzapine.*** With previous indirect indications that olanzapine may affect the biological clock (Vidarsdottir et al. 2009) and the glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), which is a regulator of the clock's activity (Iwahana et al. 2004), we examined the effect of a single subcutaneous dose of olanzapine on brain activation. Olanzapine was administered to male Wistar rats (200-250grs) at the beginning of the active phase (night) and the brain obtained 3 hrs later, whereby c-Fos detection was used as a marker of neuronal activity. The acute peripheral administration of olanzapine induced in the SCN a fourfold increase in the number of c-Fos IR nuclei as compared to saline. The co-administration of olanzapine with melatonin, a hormone able to synchronize the clock by inhibiting its activity (Liu et al. 1997), prevented this effect (Fig. 1).



**Figure 1. Olanzapine induces neuronal activation in the suprachiasmatic nucleus (SCN) and paraventricular nucleus (PVN) that melatonin prevents.** Panel with representative microphotographs of the SCN (top row) and PVN (bottom row) with c-Fos immunoreactivity as a marker for neuronal activity. The left column shows rats treated with saline, followed by a column of olanzapine (Olz), olanzapine + melatonin (Olz+Mel) and melatonin (Mel) treated rats. Bars represent the mean  $\pm$  SEM of c-Fos IR nuclei count in the SCN and the PVN. N=4 animals/group. One-way ANOVA analysis showed a significant between-group differences for SCN ( $F = 21.27$ ,  $df = 3$ ,  $P < .0001$ ) and PVN ( $F=16.80$ ,  $df = 3$ ,  $P < .0001$ ); \*\*\*  $P < .0001$ , Bonferroni's posthoc test for pair-wise comparisons. Scale bar is 100  $\mu$ m.

***Olanzapine activates the parasympathetic autonomic axis which is prevented by melatonin.***

The acute administration of olanzapine also induced a significantly increased activation in the PVN and DMV, both output pathways from the SCN to the autonomic nervous system (ANS). Similar to the SCN, the co-administration of olanzapine with melatonin prevented this effect (Fig. 2). In the spinal cord olanzapine did not induce an activation of sympathetic motor neurons in the IML (Fig. 2). These results indicate that olanzapine selectively increases the activity of the parasympathetic and not the sympathetic branch of the ANS. Since the SCN directly signals to the PVN modulating its autonomic output (Buijs et al. 2003) and melatonin receptors are densely present in the SCN and not in the PVN or DMV (Reppert et al. 1988; Lacoste et al. 2015), these results suggest that olanzapine activates directly the SCN, which consequentially activates the PV

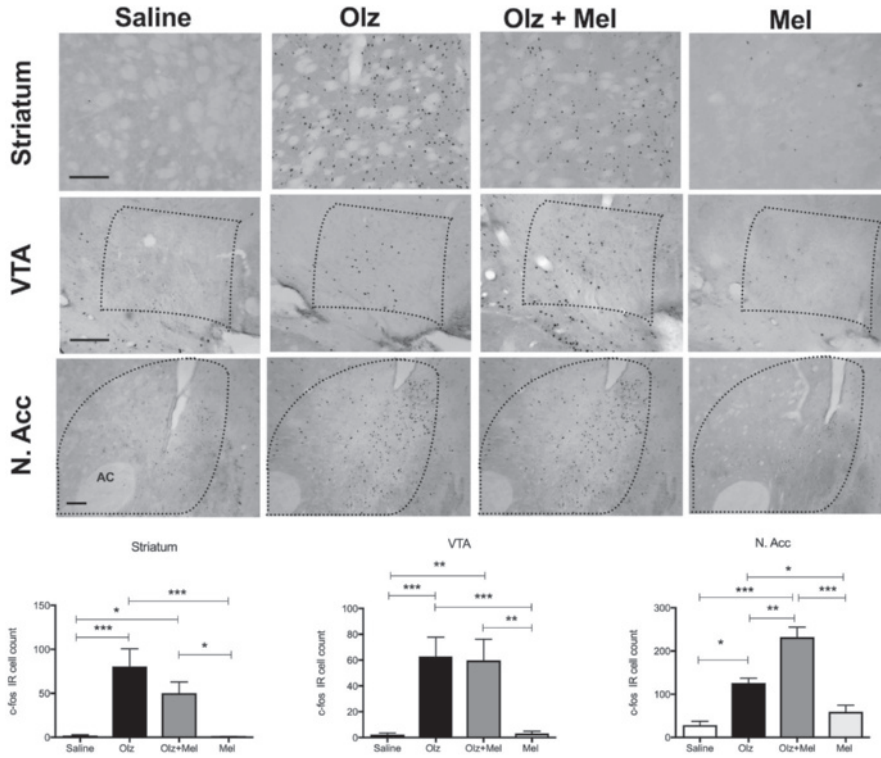


**Figure 2. Olanzapine induces activation of the dorsal motor nucleus of the vagus (DMV) which is prevented by co-administration of melatonin.** Representative microphotographs of the DMV (top) and intermediolateral column (IML; bottom row) with c-Fos immunoreactivity (IR). The left column demonstrates rats treated with saline, followed by a column of olanzapine, olanzapine + melatonin and melatonin only treated rats; N=4 animals/group. Bars represent the mean  $\pm$  SEM count of c-Fos IR nuclei in the DMV and the IML. One-way ANOVA analysis showed a significant between-group difference for DMV c-Fos ( $F = 9.028$ ,  $df = 3$ ,  $P < .0001$ ).  $** P < .001$  and  $*** P < .0001$ ; Bonferroni's post-hoc test for pair-wise comparisons. Nucleus of the tractus solitarius (NTS). Scale bar is 100  $\mu$ m

***Melatonin does not impair olanzapine induced activation in the ventral striatum pathway.***

As expected the administration of olanzapine also induced an activation of the nucleus accumbens, striatum and ventral tegmental area (VTA); brain areas associated with its therapeutic effect, that do not receive direct input from the SCN (Robertson and Fibiger 1996; Sebens et al. 1998). In contrast with our observation in the SCN, the co-administration of melatonin and olanzapine did not alter the activation pattern in the striatum and VTA as observed with olanzapine alone. Moreover, in the nucleus accumbens the co-administration of melatonin with olanzapine induced a significant increase in c-Fos activation as compared to the administration of olanzapine alone. These results confirm olanzapine induced activation of brain regions involved in its therapeutic action and etiology of psychiatric disorders for which it is prescribed (Robertson and Fibiger 1996; Sebens et al. 1998). Melatonin does not counteract this therapeutic action, and, as seen in the nucleus accumbens it may even potentiate its action (Fig. 3). This concurs with clinical observations that co-administration of melatonin with an SGA does not impair its beneficial effects and may even enhance them (Modabbernia et al. 2014; Mostafavi et al. 2014; Romo-Nava et al. 2014).

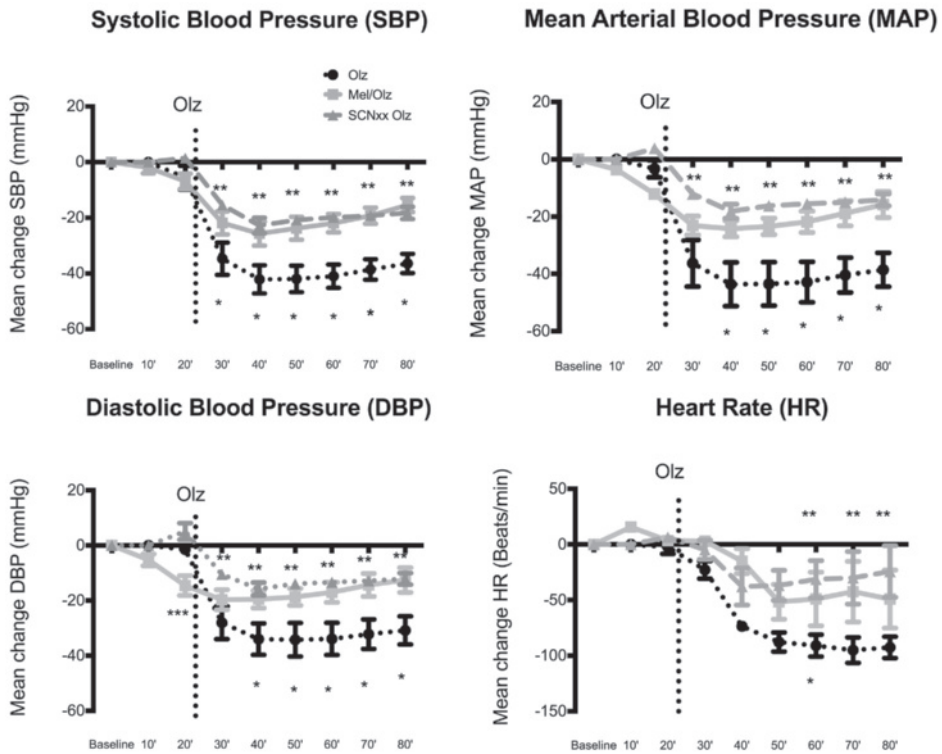
Furthermore, olanzapine induced an activation of the nucleus of the tractus solitarius (NTS), which also receives input from sensory pathways indicating that olanzapine also exerts effects on peripheral organs. Melatonin did not prevent this activation (Fig. 2 and Fig. S4), supporting a central, rather than peripheral effect of melatonin to prevent the action of olanzapine over the SCN, PVN and DMV.



**Figure 3. Olanzapine induced activation of brain regions associated with its therapeutic effect, not prevented by melatonin.** Representative microphotographs of c-Fos immunoreactivity (IR) in the striatum (top), ventral tegmental area (VTA; middle row) and nucleus accumbens (N. Acc; bottom). The left column represents rats treated with saline, followed by a column of rats treated with olanzapine (Olz), olanzapine + melatonin (Olz+Mel) and melatonin only (Mel). N=4 animals/group. Bars represent mean  $\pm$  SEM count for c-Fos IR nuclei in the striatum, VTA and N. acc. Anterior commissure (AC). One-way ANOVA analysis showed significant between-group differences for striatum ( $F=13.79$ ,  $df = 3$ ,  $P < .0001$ ), VTA ( $F = 15.78$ ,  $df = 3$ ,  $P < .0001$ ), and N.Acc.( $F = 25.19$ ,  $df = 3$ ,  $P < .0001$ ). \*  $P < .01$ ; \*\*  $P < .001$ ; \*\*\*  $P < .0001$ ; Bonferroni's post hoc test pair-wise comparisons. Scale bar is 100  $\mu\text{m}$ .

***Olanzapine activates the parasympathetic pathway via the SCN.*** The pattern of c-Fos activation in the PVN suggested that mainly autonomic neurons might be activated. In view of the activation of the NTS and DMV, we decided to place a retrograde tracer into the dorsal vagal complex and confirm activation of the retrogradely labeled neurons. CtB labeled neurons in the PVN showed a high coincidence with c-Fos when olanzapine was given and this response was higher in the autonomic and parvocellular PVN as compared to the magnocellular nuclei (Fig. S2 and Table S1). Some of these activated CtB labeled neurons showed input from SCN neurons as visualized by vasoactive intestinal peptide (VIP) immunohistochemical staining confirming a SCN-PVN-DMV neural pathway, activated by olanzapine.

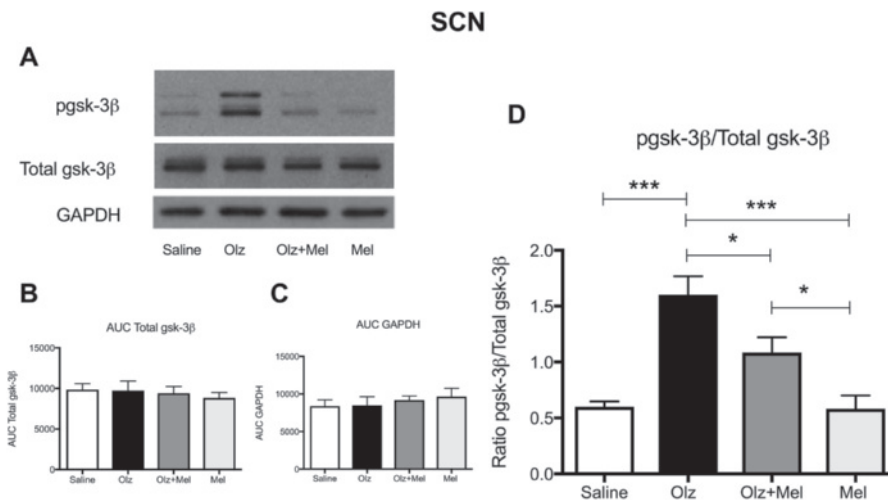
Parasympathetic activity plays a key role in blood pressure regulation, adiposity and metabolic activity (Kreier et al. 2006). Acutely, olanzapine induces a potent increase of parasympathetic tone translated into a decrease in blood pressure and heart rate in rats (Leung, Pang et al. 2014) and postural hypotension in humans (Choure et al. 2014). Parasympathetic hyperactivation may contribute to the development of metabolic syndrome, obesity (Suzuki et al. 2014) or other SGA induced adverse metabolic effects. Our anatomical analysis shows that olanzapine activates the parasympathetic branch of the ANS, with melatonin vastly mitigating this effect. To evaluate the functional significance of this finding, we measured the acute effects of olanzapine and melatonin on blood pressure and heart rate; two variables influenced by autonomic function and modified by olanzapine (Choure et al. 2014; Leung et al. 2014). In agreement with the observed increase in activity of DMV neurons, olanzapine also induced a significant decrease in systolic, diastolic and mean arterial blood pressure (MAP), as well as in heart rate. The administration of melatonin, ten minutes before the injection of olanzapine, significantly attenuated its effect on systolic and diastolic pressure, MAP and heart rate (Fig. 4). Thus, olanzapine induces activation of the parasympathetic autonomic branch, decreasing blood pressure and heart rate; this is largely avoidable by giving melatonin. Next, and considering the high concentration of melatonin receptors in the SCN, and not in PVN or DMV (Weaver et al. 1993), we hypothesized that the cardiovascular side effects of olanzapine, readily prevented by melatonin, were due to the effect of olanzapine on the SCN. To evaluate the involvement of the SCN in the hemodynamic effects of olanzapine, we performed bilateral SCN lesions (SCNxx) and after two weeks of recovery, we tested the effects of olanzapine on blood pressure and heart rate. Administration of olanzapine to SCNxx animals resulted in decreased effect on heart rate and blood pressure, similar to that observed in intact animals treated with olanzapine + melatonin (Fig. 4). This observation confirms that olanzapine acts on the SCN to induce cardiovascular adverse effects and that these effects, associated with elevated comorbidity and mortality in SGA treated patients, can be prevented by inhibiting the SCN activity with melatonin administration (Raskind 2007; Modabbernia et al. 2014; Mostafavi et al. 2014; Romo-Nava et al. 2014).



**Figure 4. Blood pressure and heart rate measurements.** Olanzapine induced a decrease in systolic (SBP), diastolic (DBP) and mean arterial (MAP) blood pressure that was prevented by melatonin or a bilateral SCN lesion. Olanzapine also induced a decrease in heart rate (HR) that was counteracted by SCNxx and melatonin. Olanzapine (Olz); Melatonin/Olz (Mel/Olz); SCN lesioned animals injected with olanzapine (SCNxx). Hemodynamic parameters are reported in mean change from baseline. N=5 animals/group. Repeated measures ANOVA showed significant treatment group x time interaction for Systolic ( $F = 3.216$ ,  $df = 16$ ,  $P < .0002$ ), Diastolic ( $F = 9.561$ ,  $df = 16$ ,  $P < .0001$ ), MAP analysis ( $F = 9.164$ ,  $df = 16$ ,  $P < .0001$ ) and Heart rate ( $F = 2.089$ ,  $df = 16$ ,  $P = .014$ ). Bonferroni's post hoc test for pair-wise comparison; \*  $P < .01$  Olz vs. Mel/Olz; \*\*  $P < .01$  Olz vs. SCNxx Olz; \*\*\*  $P < .01$  Mel/Olz vs. SCNxx Olz.

**Olanzapine induces GSK-3 $\beta$  phosphorylation in the SCN which is prevented by melatonin.** Consequently we investigated the intracellular mechanisms involved in the effects of olanzapine and melatonin in the SCN. Olanzapine has a wide spectrum of action and our results suggest that it could induce SCN neuronal activity via intracellular signaling cascades that converge with those of melatonin. Olanzapine induces phosphorylation of glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) and cAMP response element binding (CREB) protein via the Akt, Wnt and PKC pathways, which are linked to its antipsychotic therapeutic and metabolic effects (Girgis et al. 2008; Aubry et al. 2009; Lee et al. 2010; Pavan et al. 2010). Notably GSK-3 $\beta$  is involved in SCN function, neurogenesis, neurotransmission and metabolic processes (Iitaka et al. 2005). Inhibition (phosphorylation) of GSK-3 $\beta$  triggers the activation (phosphorylation) of the cAMP response element binding protein (CREB),

which in turn stimulates c-Fos transcription (Ginty et al. 1994, Grimes and Jope 2001). Interestingly, the phosphorylation of GSK-3 $\beta$  shows a circadian pattern, which is lowest at night (Iitaka et al. 2005). In agreement with its action on the SCN whereby it reduces neuronal activity, melatonin decreases phosphorylation of GSK-3 $\beta$  via Akt1 (Mao et al. 2012; Ge et al. 2013). Therefore, we evaluated the effects of olanzapine and melatonin on phosphorylated GSK-3 $\beta$  levels in the SCN. Indeed, olanzapine induced an increase in phosphorylated GSK-3 $\beta$  as compared to the control group and co-administration of melatonin reduced this effect. These findings provide a possible mechanism by which melatonin prevents the activation of SCN neurons by olanzapine (Fig. 5).



**Figure 5. Olanzapine induces the phosphorylation of GSK-3 $\beta$  in the SCN and this is prevented by melatonin.**

Representative Western blots of phosphorylated GSK-3 $\beta$  (pGSK-3 $\beta$ ) and total GSK-3 $\beta$  in homogenates of the SCN. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was immunodetected as load control. (A) Neither total GSK-3 $\beta$  nor GAPDH levels were different between experimental groups (B and C). Densitometric analysis of the bands expressed as the ratio of the integrated optical density (IOD) of pGSK-3 $\beta$  and total pGSK-3 $\beta$  (pGSK-3 $\beta$ / Total pGSK-3 $\beta$ ratio) was performed for between group comparisons. Olanzapine increased pGSK-3 $\beta$ /total pGSK-3 $\beta$  ratio, which was reduced by melatonin (D). N = 5 animals/group. For: One-way ANOVA analysis showed a significant between- group difference in pGSK-3 $\beta$ /Total pGSK-3 $\beta$  ratio ( $F = 15.19$ ,  $df = 3$ ,  $P < .0001$ ). \*  $P < .01$ ; \*\*\*  $P < .0001$ ; Bonferroni's post hoc test pairwise comparisons.

## Discussion

Our animal experiments show that: 1) olanzapine activates neurons in the SCN, PVN and DMV; 2) melatonin prevents these effects; 3) pre-autonomic neurons in the PVN activated by olanzapine, project to the DMV and receive input from the SCN; 4) olanzapine induces a decrease in blood pressure, prevented by melatonin; 5) the SCN is imperative to the cardiovascular side effects of olanzapine; 6) olanzapine induces inhibition of GSK-3 $\beta$  in the SCN that is reduced by melatonin, providing a possible intracellular mechanism for the cardiovascular effects of olanzapine and melatonin.

The biological clock selectively coordinates ANS balance to influence metabolism in different parts of the body, so alteration of this output may over time precipitate problems in metabolism such as the metabolic syndrome (Kreier, Yilmaz et al. 2003) Acute parasympathetic activation induces adiposity and an increase in plasmatic adiponectin (Suzuki et al. 2014). Such effects are also observed with short-term treatment with olanzapine (Togo et al. 2004). With time, the increased parasympathetic activity induced by olanzapine favors the appearance of cardiometabolic adverse effects like obesity, as well as lipid, insulin and glucose disturbances (Lieberman et al. 2005; Leucht et al. 2013; Vancampfort et al. 2013) similar to those observed in the metabolic syndrome; whereby in the long term a compensatory increased sympathetic cardiovascular tone is reported (Kreier et al. 2003; Lieberman et al. 2005). These dynamic adjustments in autonomic activity may explain the increased sympathetic tone, increased blood pressure and decreased levels of adiponectin found after chronic use of SGAs such as olanzapine (McEvoy et al. 2007; Bartoli et al. 2015). This might be a compensatory sympathetic effort of the ANS to counterbalance the chronic parasympathetic stimulus followed by the progressive appearance of metabolic disturbances induced by olanzapine. The immediate effect of melatonin, as we have demonstrated, prevents the increased parasympathetic output induced by olanzapine. Hereby melatonin may prevent short- and long-term (Raskind 2007; Modabbernia et al. 2014; Mostafavi et al. 2014; Romo-Nava et al. 2014) hypothalamic and ANS disturbances induced by olanzapine, attenuating its adverse cardiometabolic effects. Based on this evidence, we propose a new model with a SCN-centered mechanism that could help explain SGA induced cardiometabolic effects and the beneficial role of melatonin to prevent them (Fig. S5).

## **Conclusion**

This study provides a new framework for the neuronal mechanisms involved in the cardiovascular and possibly other metabolic side effects of SGA and supports the rationale for the use of melatonin in a clinical setting to prevent them. Circulating melatonin may influence metabolic regulation through the SCN and could additionally have a direct peripheral action (Cipolla-Neto et al. 2014; Szewczyk-Golec et al. 2015). The SCN has already been shown to be linked to obesity and to metabolic syndrome, as well as hypertension. Our model could help to explain the role of the SCN and autonomic nervous system in early and chronic metabolic changes present in entities like obesity and the metabolic syndrome.

Collectively, present results and previous clinical data warrant additional basic and clinical investigation to demonstrate the SCN as a key region mediating the effects of antipsychotics on other acute and chronic metabolic changes, and show whether melatonin has opposing and potentially protective long-term effects.

## References

- Alegria-Schaffer, A. (2014). "Western blotting using chemiluminescent substrates." *Methods Enzymol* 541: 251-259.
- Aubry, J. M., M. Schwald, E. Ballmann and F. Karege (2009). "Early effects of mood stabilizers on the Akt/GSK-3beta signaling pathway and on cell survival and proliferation." *Psychopharmacology (Berl)* 205(3): 419-429.
- Bartoli, F., A. Lax, C. Crocamo, M. Clerici and G. Carra (2015). "Plasma adiponectin levels in schizophrenia and role of second-generation antipsychotics: A meta-analysis." *Psychoneuroendocrinology* 56: 179-189.
- Buijs, R. M., C. Escobar and D. F. Swaab (2013). "The circadian system and the balance of the autonomic nervous system." *Handb Clin Neurol* 117: 173-191.
- Buijs, R. M. and A. Kalsbeek (2001). "Hypothalamic integration of central and peripheral clocks." *Nat Rev Neurosci* 2(7): 521-526.
- Buijs, R. M., A. Kalsbeek, T. P. van der Woude, J. J. van Heerikhuize and S. Shinn (1993). "Suprachiasmatic nucleus lesion increases corticosterone secretion." *Am J Physiol* 264(6 Pt 2): R1186-1192.
- Buijs, R. M., S. E. la Fleur, J. Wortel, C. Van Heyningen, L. Zuiddam, T. C. Mettenleiter, A. Kalsbeek, K. Nagai and A. Nijijima (2003). "The suprachiasmatic nucleus balances sympathetic and parasympathetic output to peripheral organs through separate preautonomic neurons." *J Comp Neurol* 464(1): 36-48.
- Choure, B. K., D. Gosavi and S. Nanotkar (2014). "Comparative cardiovascular safety of risperidone and olanzapine, based on electrocardiographic parameters and blood pressure: a prospective open label observational study." *Indian J Pharmacol* 46(5): 493-497.
- Cipolla-Neto, J., F. G. Amaral, S. C. Afeche, D. X. Tan and R. J. Reiter (2014). "Melatonin, energy metabolism, and obesity: a review." *J Pineal Res* 56(4): 371-381.
- Dawe, G. S., K. D. Huff, J. L. Vandergriff, T. Sharp, M. J. O'Neill and K. Rasmussen (2001). "Olanzapine activates the rat locus coeruleus: in vivo electrophysiology and c-Fos immunoreactivity." *Biol Psychiatry* 50(7): 510-520.
- Ge, D., R. T. Dauchy, S. Liu, Q. Zhang, L. Mao, E. M. Dauchy, D. E. Blask, S. M. Hill, B. G. Rowan, G. C. Brainard, J. P. Hanifin, K. S. Cecil, Z. Xiong, L. Myers and Z. You (2013). "Insulin and IGF1 enhance IL-17-induced chemokine expression through a GSK3B-dependent mechanism: a new target for melatonin's anti-inflammatory action." *J Pineal Res* 55(4): 377-387.
- Ginty, D. D., A. Bonni and M. E. Greenberg (1994). "Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB." *Cell* 77(5): 713-725.

Girgis, R. R., J. A. Javitch and J. A. Lieberman (2008). "Antipsychotic drug mechanisms: links between therapeutic effects, metabolic side effects and the insulin signaling pathway." *Molecular Psychiatry* 13(13): 918-929.

Grimes, C. A. and R. S. Jope (2001). "CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium." *J Neurochem* 78(6): 1219-1232.

Hahn, M. K., T. M. Wolever, T. Arenovich, C. Teo, A. Giacca, V. Powell, L. Clarke, P. Fletcher, T. Cohn, R. S. McIntyre, S. Gomes, A. Chintoh and G. J. Remington (2013). "Acute effects of single-dose olanzapine on metabolic, endocrine, and inflammatory markers in healthy controls." *J Clin Psychopharmacol* 33(6): 740-746.

Iitaka, C., K. Miyazaki, T. Akaike and N. Ishida (2005). "A role for glycogen synthase kinase-3beta in the mammalian circadian clock." *J Biol Chem* 280(33): 29397-29402.

Iwahana, E., M. Akiyama, K. Miyakawa, A. Uchida, J. Kasahara, K. Fukunaga, T. Hamada and S. Shibata (2004). "Effect of lithium on the circadian rhythms of locomotor activity and glycogen synthase kinase-3 protein expression in the mouse suprachiasmatic nuclei." *Eur J Neurosci* 19(8): 2281-2287.

Jespersen, B., L. Knupp and C. A. Northcott (2012). "Femoral arterial and venous catheterization for blood sampling, drug administration and conscious blood pressure and heart rate measurements." *J Vis Exp*(59).

Kitagawa, A., Y. Ohta and K. Ohashi (2012). "Melatonin improves metabolic syndrome induced by high fructose intake in rats." *J Pineal Res* 52(4): 403-413.

Kreier, F., Y. S. Kap, T. C. Mettenleiter, C. van Heijningen, J. van der Vliet, A. Kalsbeek, H. P. Sauerwein, E. Fliers, J. A. Romijn and R. M. Buijs (2006). "Tracing from fat tissue, liver, and pancreas: a neuroanatomical framework for the role of the brain in type 2 diabetes." *Endocrinology* 147(3): 1140-1147.

Kreier, F., A. Yilmaz, A. Kalsbeek, J. A. Romijn, H. P. Sauerwein, E. Fliers and R. M. Buijs (2003). "Hypothesis: shifting the equilibrium from activity to food leads to autonomic imbalance and the metabolic syndrome." *Diabetes* 52(11): 2652-2656.

Lacoste, B., D. Angeloni, S. Dominguez-Lopez, S. Calderoni, A. Mauro, F. Fraschini, L. Descarries and G. Gobbi (2015). "Anatomical and cellular localization of melatonin MT1 and MT2 receptors in the adult rat brain." *J Pineal Res* 58(4): 397-417.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature* 227(5259): 680-685.

Laursen, T. M., K. Wahlbeck, J. Hallgren, J. Westman, U. Osby, H. Alinaghizadeh, M. Gissler and M. Nordentoft (2013). "Life expectancy and death by diseases of the circulatory system in patients with bipolar disorder or schizophrenia in the Nordic countries." *PLoS One* 8(6): e67133.

Lee, J. G., H. Y. Cho, S. W. Park, M. K. Seo and Y. H. Kim (2010). "Effects of olanzapine on brain-derived neurotrophic factor gene promoter activity in SH-SY5Y neuroblastoma cells." *Prog Neuropsychopharmacol Biol Psychiatry* 34(6): 1001-1006.

Leucht, S., A. Cipriani, L. Spineli, D. Mavridis, D. Orey, F. Richter, M. Samara, C. Barbui, R. R. Engel, J. R. Geddes, W. Kissling, M. P. Stapf, B. Lassig, G. Salanti and J. M. Davis (2013). "Comparative efficacy and tolerability of 15 antipsychotic drugs in schizophrenia: a multiple-treatments meta-analysis." *Lancet* 382(9896): 951-962.

Leung, J. Y., C. C. Pang, R. M. Procyshyn and A. M. Barr (2014). "Cardiovascular effects of acute treatment with the antipsychotic drug olanzapine in rats." *Vascul Pharmacol* 62(3): 143-149.

Lieberman, J. A., T. S. Stroup, J. P. McEvoy, M. S. Swartz, R. A. Rosenheck, D. O. Perkins, R. S. E. Keefe, S. M. Davis, C. E. Davis, B. D. Lebowitz, J. Severe and J. K. Hsiao (2005). "Effectiveness of Antipsychotic Drugs in Patients with Chronic Schizophrenia." *New England Journal of Medicine* 353(12): 1209-1223.

Liu, C., D. R. Weaver, X. Jin, L. P. Shearman, R. L. Pieschl, V. K. Gribkoff and S. M. Reppert (1997). "Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock." *Neuron* 19(1): 91-102.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951). "Protein measurement with the Folin phenol reagent." *J Biol Chem* 193(1): 265-275.

Mao, L., R. T. Dauchy, D. E. Blask, L. M. Slakey, S. Xiang, L. Yuan, E. M. Dauchy, B. Shan, G. C. Brainard, J. P. Hanifin, T. Frasch, T. T. Duplessis and S. M. Hill (2012). "Circadian gating of epithelial-to-mesenchymal transition in breast cancer cells via melatonin-regulation of GSK3beta." *Mol Endocrinol* 26(11): 1808-1820.

Markowitz, J. S., C. L. DeVane, D. W. Boulton, H. L. Liston and S. C. Risch (2002). "Hypotension and bradycardia in a healthy volunteer following a single 5 mg dose of olanzapine." *J Clin Pharmacol* 42(1): 104-106.

McEvoy, J. P., J. A. Lieberman, D. O. Perkins, R. M. Hamer, H. Gu, A. Lazarus, D. Sweitzer, C. Olexy, P. Weiden and S. D. Strakowski (2007). "Efficacy and tolerability of olanzapine, quetiapine, and risperidone in the treatment of early psychosis: a randomized, double-blind 52-week comparison." *Am J Psychiatry* 164(7): 1050-1060.

Modabbernia, A., P. Heidari, R. Soleimani, A. Sobhani, Z. A. Roshan, S. Taslimi, M. Ashrafi and M. J. Modabbernia (2014). "Melatonin for prevention of metabolic side-effects of olanzapine in patients with first-episode schizophrenia: randomized double-blind placebo-controlled study." *J Psychiatr Res* 53: 133-140.

Mostafavi, A., M. Solhi, M. R. Mohammadi, M. Hamed, M. Keshavarzi and S. Akhondzadeh (2014). "Melatonin decreases olanzapine induced metabolic side-effects in adolescents with bipolar disorder: a randomized double-blind placebo-controlled trial." *Acta Med Iran* 52(10): 734-739.

Pavan, C., V. Vindigni, L. Michelotto, A. Rimessi, G. Abatangelo, R. Cortivo, P. Pinton and B. Zavan (2010). "Weight gain related to treatment with atypical antipsychotics is due to activation of PKC-beta." *Pharmacogenomics J* 10(5): 408-417.

Paxinos, G. and C. Watson (2007). *The rat brain in stereotaxic coordinates*. Amsterdam ; Boston ; Academic Press/Elsevier.

Raskind, M. A. (2007). "Olanzapine-induced weight gain and increased visceral adiposity is blocked by melatonin replacement therapy in rats." *Neuropsychopharmacology* 32(2): 284-288.

Reppert, S. M., D. R. Weaver, S. A. Rivkees and E. G. Stopa (1988). "Putative melatonin receptors in a human biological clock." *Science* 242(4875): 78-81.

Robertson, G. S. and H. C. Fibiger (1996). "Effects of olanzapine on regional C-Fos expression in rat forebrain." *Neuropsychopharmacology* 14(2): 105-110.

Romo-Nava, F., D. Alvarez-Icaza Gonzalez, A. Fresan-Orellana, R. Saracco Alvarez, C. Becerra-Palars, J. Moreno, M. P. Ontiveros Uribe, C. Berlanga, G. Heinze and R. M. Buijs (2014). "Melatonin attenuates antipsychotic metabolic effects: an eight-week randomized, double-blind, parallel-group, placebo-controlled clinical trial." *Bipolar Disord* 16(4): 410-421.

Rummel-Kluge, C., K. Komossa, S. Schwarz, H. Hunger, F. Schmid, C. A. Lobos, W. Kissling, J. M. Davis and S. Leucht (2010). "Head-to-head comparisons of metabolic side effects of second generation antipsychotics in the treatment of schizophrenia: a systematic review and meta-analysis." *Schizophr Res* 123(2-3): 225-233.

Sebens, J. B., T. Koch, G. J. Ter Horst and J. Korf (1998). "Olanzapine-induced Fos expression in the rat forebrain; cross-tolerance with haloperidol and clozapine." *Eur J Pharmacol* 353(1): 13-21.

Suzuki, Y., H. Shimizu, N. Ishizuka, N. Kubota, T. Kubota, A. Senoo, H. Kageyama, T. Osaka, S. Hirako, H. J. Kim, A. Matsumoto, S. Shioda, M. Mori, T. Kadowaki and S. Inoue (2014). "Vagal hyperactivity due to ventromedial hypothalamic lesions increases adiponectin production and release." *Diabetes* 63(5): 1637-1648.

Szewczyk-Golec, K., A. Wozniak and R. J. Reiter (2015). "Inter-relationships of the chronobiotic, melatonin, with leptin and adiponectin: implications for obesity." *J Pineal Res* 59(3): 277-291.

Takeda, N. and K. Maemura (2016). "Circadian clock and the onset of cardiovascular events." *Hypertens Res*.

Togo, T., K. Kojima, M. Shoji, A. Kase, H. Uchikado, O. Katsuse, E. Iseki and K. Kosaka (2004). "Serum adiponectin concentrations during treatment with olanzapine or risperidone: a pilot study." *Int Clin Psychopharmacol* 19(1): 37-40.

Towbin, H., T. Staehelin and J. Gordon (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." *Proc Natl Acad Sci U S A* 76(9): 4350-4354.

Vancampfort, D., K. Vansteelandt, C. U. Correll, A. J. Mitchell, A. De Herdt, P. Sienaert, M. Probst and M. De Hert (2013). "Metabolic syndrome and metabolic abnormalities in bipolar disorder: a meta-analysis of prevalence rates and moderators." *Am J Psychiatry* 170(3): 265-274.

Vidarsdottir, S., J. E. de Leeuw van Weenen, M. Frolich, F. Roelfsema, J. A. Romijn and H. Pijl (2010). "Effects of olanzapine and haloperidol on the metabolic status of healthy men." *J Clin Endocrinol Metab* 95(1): 118-125.

Vidarsdottir, S., F. Roelfsema, M. Frolich and H. Pijl (2009). "Olanzapine shifts the temporal relationship between the daily acrophase of serum prolactin and cortisol concentrations rhythm in healthy men." *Psychoneuroendocrinology* 34(5): 705-712.

Weaver, D. R., J. H. Stehle, E. G. Stopa and S. M. Reppert (1993). "Melatonin receptors in human hypothalamus and pituitary: implications for circadian and reproductive responses to melatonin." *J Clin Endocrinol Metab* 76(2): 295-301.

Westman, J., J. Hallgren, K. Wahlbeck, D. Erlinge, L. Alfredsson and U. Osby (2013). "Cardiovascular mortality in bipolar disorder: a population-based cohort study in Sweden." *BMJ Open* 3(4).

Weston-Green, K., X. F. Huang and C. Deng (2012). "Alterations to melanocortinerbic, GABAergic and cannabinoid neurotransmission associated with olanzapine-induced weight gain." *PLoS One* 7(3): e33548.

Olanzapine-induced effects are mediated by SCN

## Supplementary Data

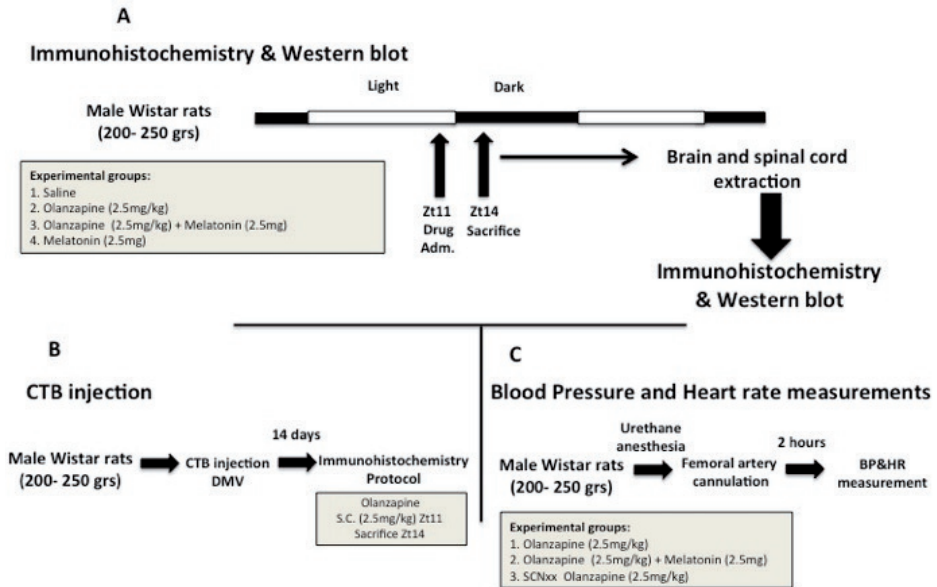
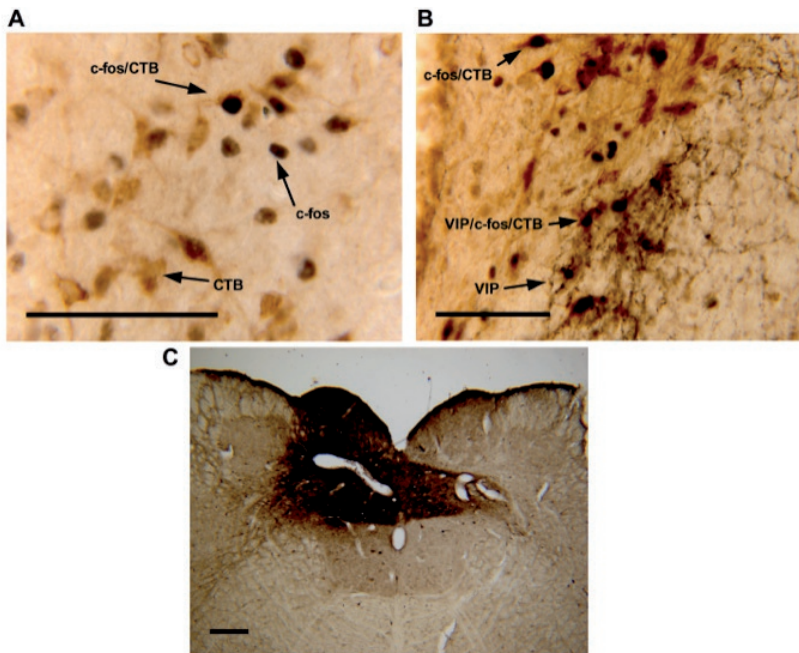
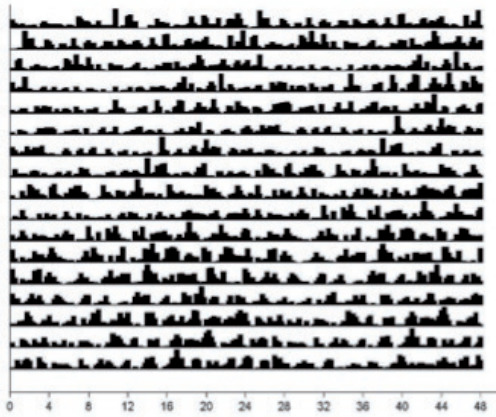


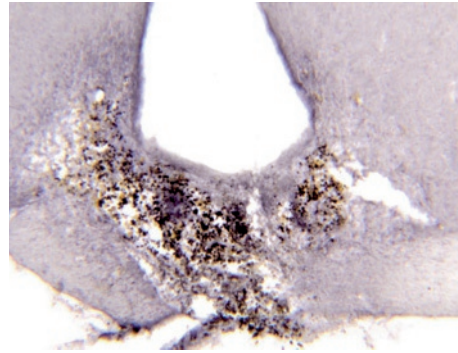
Figure S1. Experimental settings.



**Figure S2. Olanzapine induces activation of pre-autonomic neurons in the PVN that receive input from the SCN and project to the DMV.** A representative microphotograph shows co-localization of c-Fos (black nuclei)/CTB (red-brown cytoplasm) in the ventral parvocellular PVN (A). Representative microphotograph shows pre-autonomic neurons IR to c-Fos (black nuclei) in the ventral parvocellular PVN after olanzapine injection and retrogradely filled with CtB (red-brown cytoplasm) that receive VIP input (dark projections) from the SCN (B). Immunohistochemical confirmation of CtB injection in the DMV complex 14 days after injection (C). Scale bar is 100µm.

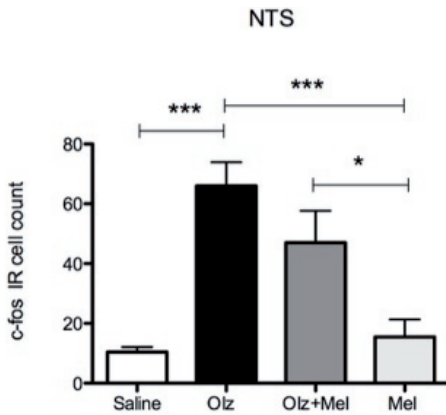


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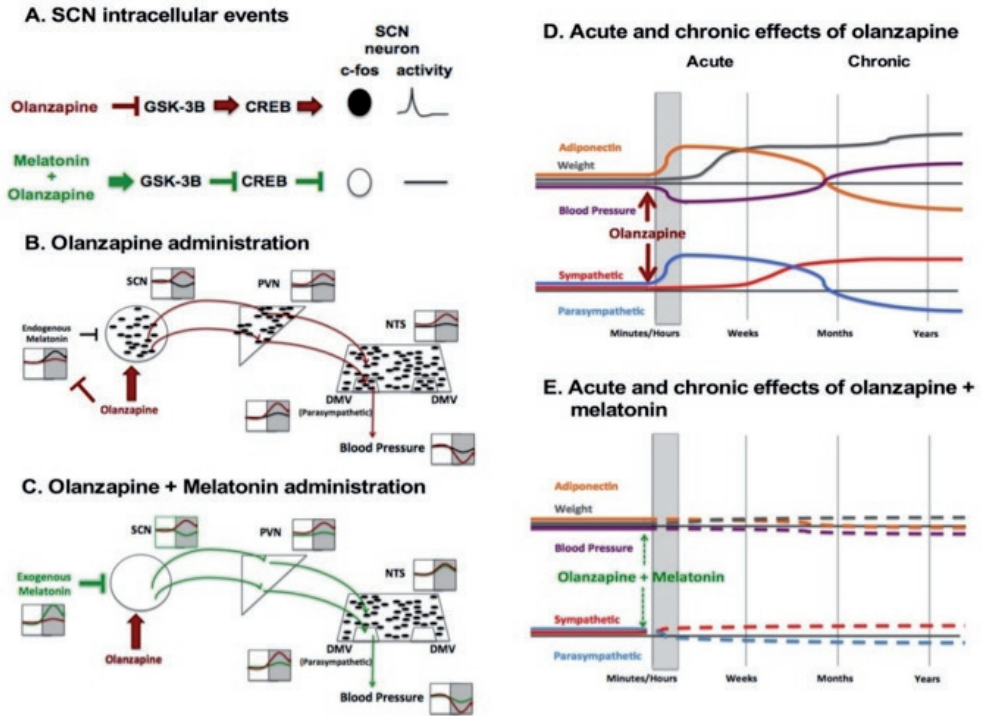
B

**Figure S3. Functional and histological confirmation of bilateral SCN lesion.** Actigraphic confirmation for arrhythmicity after SCN lesion (A), VIP immunohistochemical confirmation of bilateral SCN lesion (B).



**Figure S4. Melatonin does not modify olanzapine induction of c-Fos in the nucleus of the tractus solitarius (NTS).** Bars represent the mean ± SEM count for c-Fos IR nuclei in the NTS. For representative microphotographs refer to **Fig. 2**. One-way ANOVA ( $F=10.75$ ,  $df=3$ ,  $p<0.0001$ ). Bonferroni's post hoc test pairwise comparisons; \* $p<0.01$ ; \*\*\*  $p<0.0001$ .

3



**Figure S5. A hypothesis for SGA induced cardiometabolic effects and their prevention by melatonin.** The SCN prepares the body to function according to activity/rest periods by signaling via the PVN to the autonomic nervous system (ANS). The ANS functions using two antagonistic branches; the sympathetic nervous system (fight, fright and flight), with motor neurons in the intermediolateral column (IML); and the parasympathetic nervous system (PNS) (rest & digest), with outputs in the dorsal motor nucleus of the vagus (DMV) 12,14,15. Olanzapine disrupts this balance by SCN activation at a time point in which it is normally inactive and thus decreases the production of endogenous melatonin 17. Olanzapine could induce an increase in c-Fos by inactivating the GSK-3 $\beta$  and thus activating CREB; which facilitates c-Fos transcription 41-44. Melatonin counteracts this by GSK-3 $\beta$  activation and consequent CREB inhibition, which prevents c-Fos transcription 48-49.(A) An activated SCN by olanzapine signals to the pre-autonomic neurons in the PVN and increases the parasympathetic activity via the DMV; inducing a decrease in blood pressure.(B) The administration of melatonin prevents these effects by inhibiting the activation of the SCN (C). The autonomic effects of olanzapine are time-dependent; acutely (gray vertical background rectangle) it induces parasympathetic activity, increasing adiponectin levels and decreasing blood pressure 5,6,50. Chronically, olanzapine then favors weight increase and generates an autonomic countermeasure resulting in the observed increased sympathetic activity, that results in blood pressure increase and decreased adiponectin levels 4,8,52.(D) The co-administration of melatonin would prevent this change acutely (gray vertical rectangle) and chronically mitigating olanzapine-induced adverse cardiometabolic effects 18-20. (E) Arrows indicate activation; capped lines indicate inhibition; width and font size indicate magnitude. In (A), (B) and (C) small squares illustrate circadian activity divided in day (white) and night (gray); Black lines (endogenous melatonin); Green lines (exogenous melatonin + olanzapine); Blue lines (olanzapine). Black dots inside the schematic nuclei illustrate neuronal activity according to c-Fos IR.

**Table S1.** Co-localization of c-Fos and CtB in the PVN.

PVN Region	CtB	c-Fos	CtB & cFos	(CtB & c-Fos)/CtB
	Mean(SD)	Mean(SD)	Mean(SD)	%
Ventral parvocellular	25.8(17.8)	23.0(5.1)	10.5 (6.4)	43.2
Medial parvocellular	13.5 (10.2)	12.5 (1.9)	4.0 (2.7)	37.0
Dorsal parvocellular	4.8 (4.1)	4.5 (3.5)	1.0 (0.8)	31.5
Mean parvocellular	14.6 (9.8)	13.3 (0.4)	5.1 (3.0)	37.2 *
Magnocellular	6.8 (7.8)	17.8 (17.4)	1.3 (2.8)	14.7

\*  $p < 0.0001$ ; Mean Parvocellular vs. Magnocellular Chi-square test.