# Characterization of the Monoaminergic Neurotransmission Across the Light-Dark Cycle

by

**Sergio Dominguez Lopez, BSc, MSc**

Departments of Psychiatry, Neurology and Neurosurgery

McGill University

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**Abstract**

The monoamines serotonin (5-HT) and dopamine (DA) modulate several physiological and cognitive-affective functions. Alterations in their activity have been implicated in numerous neuropsychiatric disorders. Cumulative evidence indicates that the content and release of 5-HT and DA in the brain varies across the 24 h of the light-dark cycle. In this thesis, we describe three studies. The first two were electrophysiological experiments in anesthetized rats to determine whether diurnal rhythms are present in the firing activity of 5-HT and DA neurons in the dorsal raphe nucleus (DRN) and ventral tegmental area (VTA), respectively. The influence of the hormone melatonin (MLT), a neuroendocrinal signal of photoperiod duration, on 5-HT and DA firing activity was determined also, as were the inhibitory effects of the psychostimulant amphetamine (AMPH) and a direct DA receptor agonist (apomorphine, APO) on VTA DA neuronal firing activity. In a third study, we investigated the effects of APO and AMPH on locomotor activity across the light-dark cycle. From these experiments, we were able to determine the existence of a 24 h diurnal rhythm on spontaneously active 5-HT neurons and their firing rate. The population of spontaneously active DA neurons also has a 24 h rhythm but their firing rate oscillates with a 12 h ultradian rhythm. MLT administration at lower doses (1 mg/kg, i.v.) has an inhibitory effect on DA and 5-HT cell firing rate. The effect of MLT on 5-HT neurons was only observed during daytime and likely mediated by $MT_1$ receptors. Endogenous secretion of MLT seems to exert a tonic inhibition on 5-HT neuron firing activity at night. We observed a stronger suppression of DA firing rate with the lowest dose of APO (25 μg/kg) at nighttime. In contrast, the effect of APO inhibiting locomotor activity was stronger during the light phase at doses of 50 μg/kg. No diurnal variation in DA cell response to AMPH administration was observed. However, AMPH's effect on locomotor activity was stronger

during the daytime particularly at high doses (5 mg/kg). At nighttime, the effects of AMPH were observed as a reduction in immobility. In conclusion, we were able to demonstrate the existence of diurnal rhythms of firing activity in 5-HT and DA neurons. We determined that MLT exerts an inhibitory influence on monoamine neurotransmission. We also demonstrated time of day effects in neuronal and behavioral responses with some doses of APO and AMPH.

**Résumé**

La sérotonine (5-HT) et la dopamine (DA) sont deux neurotransmetteurs monoaminergiques. Ces substances modulent plusieurs fonctions physiologiques et cognitives et l'altération de leur activité a déjà été impliquée dans plusieurs troubles neuropsychiatriques. Des données cumulatives suggèrent que la concentration et la libération de 5-HT et de DA dans le cerveau varient au cours des 24 heures du cycle lumière/obscurité. Dans cette thèse, nous avons effectué des expériences électrophysiologiques chez des rats anesthésiés pour déterminer si ces rythmes diurnes sont aussi observables dans l'activité des neurones 5-HT et DA du noyau raphé dorsal (DRN) et de l'aire tegmentale ventrale (VTA), respectivement. La mélatonine (MLT) étant un signal neuroendocrinien indiquant la durée de la photopériode, son influence sur l'activité des neurones 5-HT et DA a aussi été analysée. De plus, nous avons évalué les effets inhibiteurs d'un psychostimulant, l'amphétamine (AMPH), ainsi que d'un agoniste direct des récepteurs $D_1$-$D_2$, l'apomorphine (APO), sur l'activité des neurones DA de la VTA et sur l'activité locomotrice des rats au cours du cycle lumière/obscurité. Nous avons observé l'existence d'un rythme circadien dans l'activité de neurones 5-HT spontanément actifs. La population de neurones DA spontanément actifs suit aussi un rythme de 24h mais leur taux de décharge oscille selon un rythme ultradien de 12h. L'administration de MLT à des doses faibles (1 mg/kg, i.v.) a un effet inhibiteur sur le taux de décharge des neurones DA et 5-HT. L'effet de la MLT sur les neurones 5-HT a seulement été observé lors de la phase diurne et est probablement déclenchée par les récepteurs $MT_1$. La sécrétion endogène de MLT semble exercer une inhibition tonique sur le taux de décharge des neurones 5-HT lors de la phase nocturne. Nous avons observé une augmentation de l'inhibition du taux de décharge des neurones DA avec une dose très faible d'APO (25 µg/kg)

lors de la phase nocturne. Cependant, l'inhibition de l'activité locomotrice par l'APO est plus forte lors de la phase diurne avec des doses de 50 µg/kg. Aucune variation de la réponse des neurones DA lors de l'administration d'AMPH lors de la phase diurne n'a été observée. Par contre, l'effet de l'AMPH sur l'activité locomotrice est plus puissant lors de la phase diurne, particulièrement avec des doses élevées (5 mg/kg). Lors de la phase nocturne, nous observons une réduction de l'immobilité lors de l'administration d'AMPH. En conclusion, nous avons pu démontrer l'existence de rythmes diurnes du taux de décharge dans les neurones 5-HT et DA. Nous avons déterminé que la MLT exerce une influence inhibitrice sur la neurotransmission monoaminergique. Nous avons aussi démontré un effet du rythme circadien sur les réponses neuronales et comportementales avec plusieurs doses d'APO et d'AMPH.

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## Contributions of Authors

Sergio Dominguez Lopez designed most of the experiments, and performed the great majority of the data collection, including neuronal recordings and behavioral testing, as well as the analysis and interpretation of the data and the writing of the manuscripts. Gabriella Gobbi and Marco Leyton provided guidance, supervision and helpful feedback during the entire course of the research and they contribute in the conceptualization and writing of the manuscripts. Ian Mahar helped to develop the pinealectomy technique and to collect data included in section 2.4.6. He and Dr. Francis R Bambico helped to organize, proofread and revise the manuscript in which Chapter 2 is based. Benoit Labonte and Rafael Ochoa Sanchez helped to analyze burst activity data included in section 2.4.1. Martha Lopez Canul assisted collecting data reported in sections 3.3.1 and 3.3.3. Rebecca D. Howell assisted and helped in the behavioral experiments described in Chapter 4 and assisted collecting complementary electrophysiological reported in sections 3.3.1 and 3.3.4.

**Original Scholarship and Distinct Contributions to Knowledge**

In this thesis new evidence is provided of the existence of diurnal rhythm in monoaminergic neurotransmission. The first set of experiments described in Chapter 2, provided the first evidence that serotonin (5-HT) neuronal activity in the dorsal raphe nucleus (DRN) have a 24 h rhythm of activity associated with an inhibitory influence of melatonin (MLT) occurring at night. This supports an influence of the photoperiod in 5-HT neurotransmission. Experiments described in Chapter 3, provide the first evidence that the dopamine (DA) neuronal population in the ventral tegmental area (VTA) have a 24 rhythm of activity out of phase from a 12 h rhythm of activity of their firing frequencies. This suggested the existence of multiple factors in the modulation of diurnal DA neurotransmission. Also in Chapter 3, we have conducted an evaluation of the effects of apomorphine (APO) and amphetamine (AMPH) on DA cell firing activity at different time intervals of the light-dark cycle. To our knowledge this is the first study reported with these characteristics and it allows us to detect a stronger dose-dependent effect of APO at nighttime which may be a useful insight into diurnal changes in the brain targets of this drug. Complementary behavioral experiments also provided first evidence for time of day effects of APO inhibiting locomotor activity and add to the evidence of diurnal changes in the effects of AMPH in locomotor activity. The data in this thesis thus provided new and valuable contributions to the field.

# Chapter 1
# General Introduction

There is now compelling evidence that the monoamines, serotonin (5-hydroxytryptamine, 5-HT) and dopamine (3,4-dihydroxyphenylethylamine, DA), are involved in several neuropsychiatric disorders (Sulzer, 2011 Grace, 2012; Blier and El Mansari, 2013; Madras, 2013; Mann, 2013). Changes in brain biochemistry that lead to hypoactive and hyperactive states of monoamine neurotransmission can be associated with the development, expression and severity of disorders such as schizophrenia and depression (Laruelle *et al* 1999; Meyer, 2013). In some instances it has been observed that monoamine neurotransmission might be affected by environmental changes, such as daylight duration, in ways that could trigger well defined psychiatric entities, such as seasonal affective disorder (Rosenthal *et al*., 1984; Neumeister *et al*., 2001; Praschak-Rieder *et al*. 2008; Spindelegger *et al*, 2012). In other cases, sleep disturbances observed in a wide range of psychiatric and neurological diseases may be an indirect indicator of alterations in the diurnal physiology of monoaminergic neurotransmission (Krystal *et al*., 2008; Benedetti, 2012; Videnovic and Golombek, 2013). Therefore, it is imperative to establish how normal monoaminergic neurotransmission evolves throughout the day in order to identify disturbances that may occur in pathological states.

To begin, some evidence from research in animal models suggests that monoaminergic neurotransmission can vary during the day (Cagampang *et al*., 1993; Nagayama, 1999; Sleipness *et al.*, 2008; Webb *et al.*, 2009). However, even in animals it is difficult to define what could be a normal rhythm of activity of DA and 5-HT neurotransmission since both systems can be influenced by either behavioral activity or vigilance state (Reuter *et al*., 1997; Urbain *et al*.,

2006; Monti and Jantos, 2008; Grace, 2008; Egerton *et al*., 2009). Therefore investigations need to begin by characterizing diurnal rhythms of monoaminergic neurotransmission in isolation, with minimal interference caused by behavioral activity. In this introductory chapter, the literature will be reviewed to establish an experimental approach of assessing diurnal rhythms of firing activity in 5-HT and DA neurons located in the midbrain of the rat brain.

### 1.1 Biological rhythms

Physiological homeostasis is achieved by the coordination of multiple behaviors (*e.g.,* sleep-wake, feeding) and biological processes (*e.g.* body temperature and blood pressure). Many physiological functions occur in a non-random, and thus predictable, time locked frequency which is maintained by specific feedback mechanisms. The frequency of these biological processes varies from seconds to months and even years (e.g. heart rate, menstrual cycle). Of particular relevance for our research are those biological processes which are synchronized to an approximate 24 hour rhythm of activity, thus closely related to the day-light duration or photoperiod (Duguay and Cermakian, 2009; Golombek and Rosenstein, 2010; Tsang *et al*., 2013). In the absence of external cues, such as the photoperiod, some of these close to 24 hour biological rhythms are self-sustained by a molecular clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Morin, 2013). The term circadian rhythm is usually reserved to refer to these self-sustained biological rhythms for which external cues are not necessary. The term diurnal rhythm is used to refer either to biological rhythms for which a circadian nature has not yet been established or for those expressed on a daily basis but that are not self-sustained. There is still debate about how the SCN synchronizes and coordinates circadian functions across the body; however the secretion of the neurohormone melatonin (*N*-acetyl-5-methoxytryptamine,

MLT) by the pineal gland is considered one of the output signals of the SCN. In the following sections, the generation of the molecular mechanism in the SCN is briefly reviewed to then focus on the physiology of MLT and how it may act as a diurnal influence on monoaminergic neurotransmission.

### 1.1.1 The suprachiasmatic nucleus and clock genes

The SCN is located in the hypothalamus above the optic chiasm, ventrolateral to the third ventricle, at the base of both cerebral hemispheres (Moore *et al.*, 2002). The neurons of the SCN sustain a nearly precise 24 hour rhythm of firing activity through a mechanism of auto-regulated genes and proteins that form the so-called molecular clock (Ko and Takahashi, 2006). The endogenous oscillation of SCN neuronal activity and the secretion of its associated neurotransmitters, such as vasopressin and GABA (Moore *et al*., 2002), drive rhythms of neuronal activity and neuropeptide secretion in other areas of the brain (Lee *et al.*, 2003). In this way, the SCN is believed to synchronize circadian rhythmicity of physiological, hormonal and behavioral processes throughout the body (Golombek and Rosenstein, 2010). In the presence of light, photoreceptors located in the retina send information through the retino-hypothalamic pathway enabling the resetting of the SCN molecular clock to adapt to changes in the photoperiod (Meijer and Schwartz, 2003). The rest-activity cycle is one of the major behavioral outputs of the SCN and is strongly correlated with firing activity of SCN neurons (Inouye and Kawamura, 1979; Sato and Kawamura, 1984; Houben *et al.*, 2009) and the expression of clock genes (Bunger *et al.*, 2000; Bae *et al.*, 2001; Miyazaki *et al.*, 2007; Ramanathan *et al.*, 2010).

Rhythms in the individual neurons of the SCN are generated by complex system of feedback loops that involve several transcription factors and their final proteins, as shown in Figure 1. First, the transcription of *Period* (*Per1*, *Per2, Per3*) and *Cryptochrome 1 and 2* (*Cry1* and *Cry2*) genes is up-regulated by a protein heterodimer formed by CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like). These two proteins are members of the PAS (PER-ARNT-SIM) helix-loop-helix transcription factor family which facilitates DNA binding (Ko and Takahashi, 2006). The heterodimer formed by PER and CRY proteins down-regulates its own transcription by inhibiting the CLOCK-BMAL1 protein heterodimer, closing in this way the core loop of the molecular clock (Vitaterna *et al.*, 1994; Takumi *et al.*, 1998; Vitaterna *et al.*, 1999; Bunger *et al.*, 2000). Additional genes and proteins have been identified as contributors to these core elements, stretching this transcription-translation process over the 24 hours of the day, providing the molecular base of circadian rhythms (Reppert and Weaver, 2001; Duguay and Cermakian, 2009). In the SCN, the peak of clock genes transcription is always found during the light-phase (transcription of *Bmal1* is the only exception, in which the peak of transcription occurs during the dark phase), and this occurs independently of the active phase of the species (diurnal vs. nocturnal species) (Albrecht *et al.*, 1997; Mrosovsky *et al.*, 2001; Okano *et al.*, 2001; Reppert and Weaver, 2001; Dardente *et al.*, 2002).

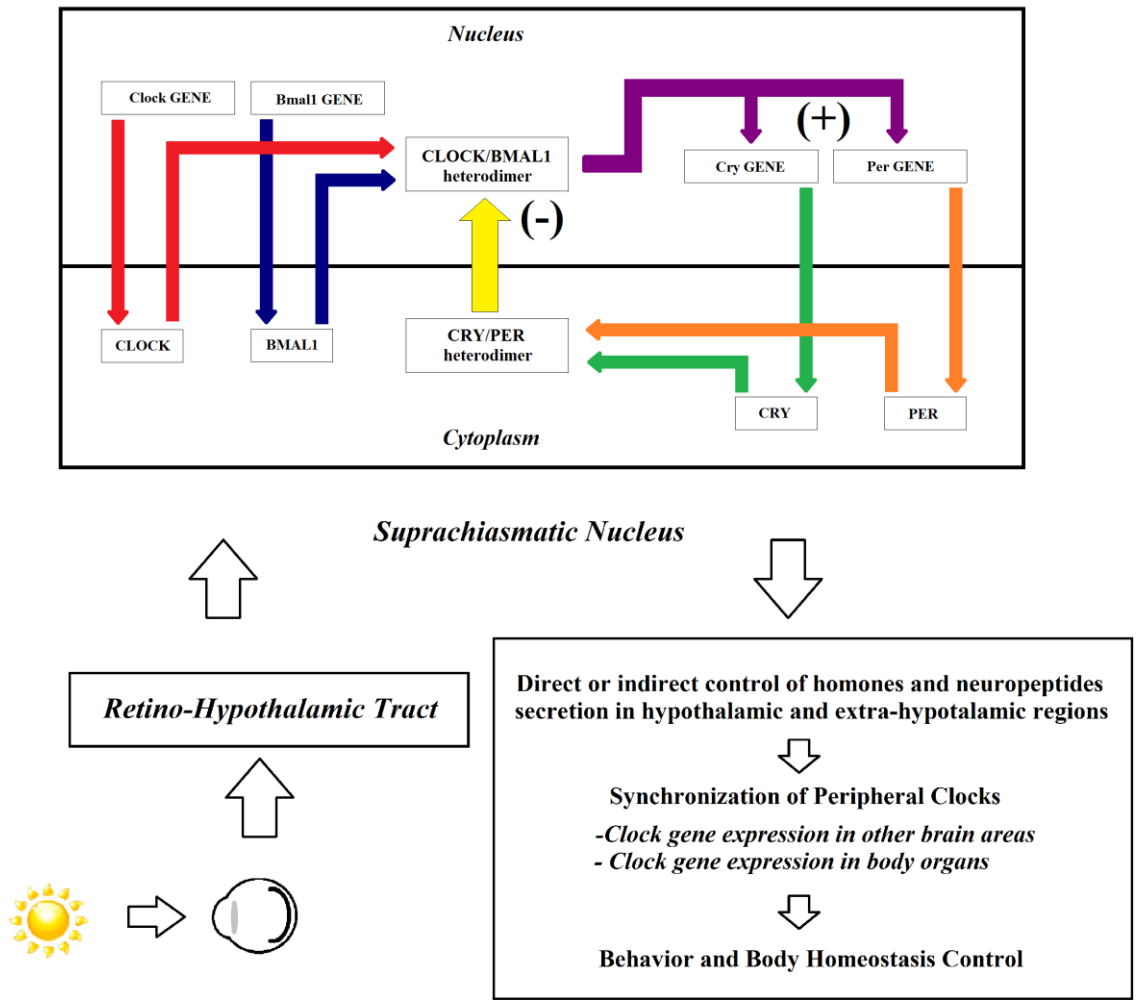


**Figure 1.** Core genes of the molecular clock in the suprachiasmatic nucleus (SCN). Transcriptional cycles of approximately 24 h form the molecular clock in the SCN. These feedback loops of autoregulated genes and their proteins are self-sustained in SCN neurons which are considered the master clock of the body. Environmental light information, sent from the retina to the SCN through the retino-hypothalamic tract, resets the molecular clock to photoperiod duration. The rhythmic activity in SCN neurons is translated to patterns of neurotransmitter release (e.g. GABA and vasopressin) in projecting areas, modulating hormones and neuropeptide release which in turn can synchronize peripheral clocks outside the SCN. Behaviors influenced by the master clock (e.g. rest-activity, feeding and sleep cycles) contribute to the homeostatic control of the body.

The SCN clock is synchronized (entrained) to the light-dark cycle and in turn is capable of phase-aligning peripheral circadian clocks, and clock-controlled output genes, distributed throughout the brain and other organs in the body (Okamura, 2007; Escobar *et al*., 2009; Morin, 2013). The expression of clock genes outside the SCN exerts an extended circadian control of homeostatic processes. An example of this can be found in the circadian regulation of molecules involved in energy metabolism such as nicotinamide adenine dinucleotide (NAD), insulin and leptin (Buijs *et al*., 2013; Challet, 2013). The synchronization of peripheral clocks to the SCN is still an area of extensive research; however it is known that the output signal that propagated the master clock circadian control is composed of several neurotransmitters, hormones, peptides and neural pathways (Cermakian *et al*, 2013; Kalsbeek and Fliers, 2013). One of its output signals is the circadian secretion of the neurohomone melatonin (MLT) which is used as a marker of SCN clock function (Cermakian and Boivin, 2009).

### 1.1.2 MLT synthesis, secretion and metabolism

MLT is synthesized and secreted by the pineal gland during the dark phase and it is considered the main endocrine signal for photoperiod duration (Simonneaux and Ribelayga, 2003; Kunz and Mahlberg, 2006). MLT is produced from 5-HT in a biosynthetic pathway that in a first step involves conversion of 5-HT to N-acetyl-serotonin by the enzyme N-acetyltransferase. N-acetyl-serotonin is then converted to MLT by the enzyme hydroxyindole-O-methyltransferase (Weissbach *et al*., 1960; Sugden 1989). MLT synthesis is under the control of the SCN, which receives photoperiod information from the retina and then sends this signal through the superior cervical ganglion, which in turn modulates MLT synthesis through noradrenaline (NA)

neurotransmission (Simonneaux and Ribelayga, 2003). The daily patterns of pineal MLT secretion reflect circadian and seasonal changes of the photoperiod that are translated to endocrine and neuronal signals in the brain via the G protein-coupled membrane receptors $MT_1$ and $MT_2$ (Dubocovich and Markowska, 2005; Pandi-Perumal *et al*., 2008; Dubocovich *et al*., 2010).

MLT is secreted by the pineal gland into the blood stream from where it can cross the blood-brain barrier and reach the brain via the choroid plexus (Tricoire *et al*., 2003; Leston *et al*., 2010). In addition, a large amount of MLT is released directly from the pineal gland into the third ventricle through the pineal recess (Tricoire *et al*., 2003; Leston *et al*., 2010). At night, plasma MLT levels are usually reported in the range of 20 to 100 pg/ml in rats, and of 30 to 200 pg/ml in humans (Pang and Ralph, 1975; Chan *et al*., 1984; Hajak *et al*., 1997). In rats, the half life of MLT in the bloodstream is short, approximately 20 minutes (Gibbs and Vriend, 1981). In humans, the half-life of endogenous produced MLT has been calculated to be approximately one hour (Fourtillan *et al*., 2000; 2001). It has been estimated that the pineal gland must release its entire contents (≈1.5 ng) every 11 seconds in order to maintain levels of MLT observed in systemic circulation at night (Huether *et al*., 1998). Indeed, MLT secretion drops to minimal values within minutes after MLT synthesis in the pineal gland stops at the onset of the light phase (Liu and Borjigin, 2006).

Metabolism of MLT occurs mainly in the liver where MLT is first transformed to 6-hydroxymelatonin and then conjugated with sulfate or glucuronic acid to finally be excreted in the urine and feces (Kopin *et al*., 1961; Semak *et al*., 2008). The amount of 6-hydroxymelatonin

sulfate excreted in urine parallels plasma MLT levels and it is sometimes used as an indirect non-invasive measure of MLT secretion (Arendt *et al*., 1985). The extent to which MLT is metabolized in the brain is still largely unknown, as well as the putative physiological actions of MLT metabolites in the CNS (Hardeland, 2010). Recently, N-acetyl-serotonin the precursor of MLT synthesis but also one of its minor metabolites has started to be investigated for its putative neuroprotective and antidepressant properties (Jang *et al*., 2010; Tosini *et al*., 2012).

### 1.1.3 MLT pharmacokinetics

In this section, some basic pharmacokinetic parameters of MLT administration will be reviewed, most of them obtained from studies in rodents. MLT is a lipophilic substance and when administered systemically it rapidly crosses the blood brain barrier reaching several brain regions including the cortex, midbrain, cerebellum, medulla-pons and hypothalamus (Kopin *et al*., 1961; Wurtman *et al*., 1964; Anton-Tay and Wurmant, 1969; Le Bars *et al*., 1991). Uptake of MLT by the brain has been observed as fast as two minutes after intravenous administration (≈100 μg, single dose), although it is estimated that only about 10-15% of systemically administered MLT reaches the brain (Pardridge and Mietus, 1980; Vitte *et al*., 1988). The half-life of exogenous MLT in rat brain tissue and plasma is similar to the half-life of endogenously produced MLT, approximately 20 to 27 minutes, and its duration in the body depends on the dose and route of administration used (Ferrerira *et al*., 1996; Venegas *et al*., 2012). For example, in rodents, following a single administration at doses ranging from the microgram range (0.8 - 2.0 μg, single dose) up to 5 mg/kg, supraphysiological levels of MLT (6-8 times higher) are observed in brain tissue and plasma for up to one hour (Gibbs and Vriend 1981; Menendez-Pelaez *et al*., 1993; Ferrerira *et al*., 1996; Yeleswaram *et al*., 1997). At doses higher than 5 mg/kg,

supraphysiological levels of MLT continue to be observed after two hours post-administration (Cheung *et al*., 2006; Venegas *et al*., 2012). In humans, a rapid and heterogeneous distribution of MLT in brain has also been reported with a peak observed about eight minutes after intravenous administration (42 μg, single dose) (Le Bars *et al*., 1991). MLT half-life in plasma after intravenous administration is 35 to 40 minutes, in men and women respectively (Fourtillan *et al*., 2000; 2001).

### 1.1.4 Extra-pineal sites of MLT synthesis

MLT is also synthesized in several extra-pineal sites such as the retina, the Harderian gland of the eye's orbit, and the gastrointestinal tract (Huether, 1993). Although it is well established that MLT of pineal origin is responsible for the nocturnal surge of MLT in brain (Pang *et al*., 1982; Venegas *et al*., 2012), the contribution of extra-pineal sites to daily circulating levels of MLT is still a matter of debate. In serum, levels of MLT decrease to daytime values after removal of the pineal gland (pinealectomy, PX) (Ozaki and Lynch, 1976; Bubenik and Brown, 1997). However, there is some evidence suggesting that a rhythm of MLT in serum can still be observable in PX rats one week after surgery (Yu *et al*., 1981). The origin of circulating MLT levels after PX has been suggested to derive from MLT synthesized in the gastrointestinal tract, associated with feeding patterns (Lynch *et al*., 1975; Huether, 1993). Interestingly, a diurnal rhythm of MLT persisted in the Harderian gland and retina three weeks after PX and the peak of MLT tissue content in these organs is significantly higher in PX rats compared with sham operated animals (Reiter *et al*., 1983). More intriguing is the report that MLT content in the membrane fraction of cerebral cortex and liver extracts from rats is increased five days after PX (Venegas *et al*., 2012).

The authors of the last study suggested that extra-pineal MLT production could be normally inhibited by circulating MLT of pineal origin (Venegas *et al*., 2012).

### 1.1.5 Inhibition of MLT synthesis by light

Exposure to bright light suppresses nocturnal secretion of MLT in animals and humans (Ralph *et al*., 1971; Illnerova *et al*., 1978; Lewy *et al*., 1980; Travlos *et al*., 2001). Indeed, light exposure activates the visual neuro-chemical pathway that regulates MLT synthesis, specifically activating retina-based photo-pigments and inactivating NAT and HIOMT enzymes (Zawilska, 1996; Brainard and Haninfin 2005; Foster and Hankins, 2007). The magnitude of the effect of light on MLT synthesis depends on the intensity, duration, and wavelength ($\lambda$) of the light used (Reiter, 1992; Zawilska, 1996; Brainard and Haninfin 2005). Several studies have suggested that the spectral region corresponding to wavelengths between 450 nm (blue light) and 550 nm (green light) provides the strongest stimulation on circadian and neuroendocrine responses in mammals, including suppression of MLT secretion (Brainard and Haninfin, 2005). However, ultraviolet (UV, $\lambda$ = 365 nm) and red light ($\lambda$ = 660 nm) exposures are also able to suppress MLT synthesis when applied for a long time or at high intensity (Honma *et al*., 1992; Zawilska, 1996). Dim red light (intensity < 1$\mu$ W/cm$^2$) and infrared light (IR, $\lambda$ > 800 nm) do not affect MLT synthesis and are used to observe animals in nocturnal procedures aimed at characterizing circadian rhythms (Poeggeler *et al*., 1995; Griefahn *et al*., 2002; Carazo *et al*., 2013).

### 1.1.6 MLT receptors in the brain

As previously mentioned, MLT effects are thought to be mediated by receptors which seem ubiquitously distributed in the brain. In mammals, MLT binding sites have been observed in high

density in the median eminence, pituitary, SCN, area postrema, anteroventral thalamic nucleus, and paraventricular thalamic nucleus, as well as less densely in other brain areas such as subiculum, hippocampus, cerebellum, parietal cortex, lateral habenula, amygdala, and striatum (Laudon *et al*., 1988, Weaver *et al*., 1989, Williams, 1989; Lindroos *et al*., 1993; Weaver *et al*., 1993,Williams *et al*., 1995). In addition to binding experiments, other studies have studied the presence of MLT receptors in the brain by determining mRNA expression of the two MLT receptors subtypes. In rats, $MT_1$ and $MT_2$ mRNA expression has been detected in SCN, hippocampus and vestibular nuclei (Dubocovich *et al*., 1998; Hunt *et al*., 2001; Musshoff *et al*., 2002; Ahn *et al*., 2012). Importantly, $MT_1$ receptor mRNA in mice has been detected in several brain regions which form part of the DA system such as the dorsal striatum, nucleus accumbens (NAcc), olfactory tubercle, substantia nigra (SN) and ventral tegmental area (VTA) (Uz *et al*., 2005). In humans, $MT_1$ mRNA expression has been found in areas including hypothalamus, cerebellum, cortex, NAcc, amygdala, SN, hippocampus and thalamus ( Mazzucchelli *et al*., 1996; Uz *et al*., 2005; Brunner *et al*., 2006; Adi *et al*., 2010). $MT_2$ receptor mRNA has been detected in human cerebellum, SN and amygdala (Al-Ghoul *et al*., 1998; Adi *et al*., 2010).

Despite this large body of literature on MLT receptors, just a few studies have used specific antibodies to confirm the expression of functional MLT receptors in the brain. In the rat, $MT_1$ and $MT_2$ receptors have been localized in vestibular nuclei (Ahn *et al*., 2012) and $MT_2$ receptors have been localized in the reticular thalamic nucleus, hippocampus, red nucleus, substantia nigra pars reticularis (SNpr), supraoptic nucleus and SCN (Ochoa-Sanchez *et al*., 2011). In humans, $MT_1$ and $MT_2$ receptors have been confirmed in the hippocampus, cortex and pineal gland (Savaskan *et al*., 2002, Savaskan *et al*., 2005; Brunner *et al*., 2006) and $MT_1$ receptors were

found in several other hypothalamic nuclei including the SCN (Wu *et al*., 2006). The expression of MLT receptors in the SCN is consistent with the ability of MLT to modulate SCN neuronal activity, as will be reviewed in the next section. Recently in a study done by our research group, a full mapping of the of $MT_1$ and $MT_2$ receptors localization in the rat brain was carried out. Among other structures across the brain, $MT_1$ receptors were localized in the dorsal raphe nucleus (DRN) and $MT_2$ receptors were labeled in SN and VTA (Lacoste *et al*., 2014), which could imply a direct influence of MLT on monoaminergic neurotransmission.

In addition to these high-affinity MLT receptors, another low-affinity MLT binding site, termed $MT_3$, has been characterized as a MLT-sensitive form of the human enzyme quinone reductase II (Dubocovich and Markowska, 2005; Pandi-Perumal *et al*., 2008). Finally, MLT has been suggested to bind to retinoid related orphan nuclear hormone receptors (RZR/ROR receptor family) (Becker-Andre *et al*., 1994).

### 1.1.7 Interaction between SCN and MLT

As mentioned above, MLT synthesis and secretion is under the combined control of the photoperiod and the molecular clock in the SCN. There is also evidence that MLT reciprocally modulated SCN neuronal activity and clock function due to its ability to shift circadian rhythms (Pandi-Perumal *et al*., 2008; Kunz and Mahlberg, 2006). However, the physiological mechanism by which MLT could be affecting SCN function has not been completely elucidated. It has been shown that, coincidentally with nocturnal MLT secretion, firing rates of SCN neurons are usually low at night (or the projected dark phase) and that PX abolishes these circadian fluctuations (Rusak and Yu, 1993; Brown and Piggins, 2007; Bhumbra *et al*., 2009). These findings suggest

the existence of a tonic inhibitory influence of nocturnal MLT secretion in SCN cell activity. In line with this, several *in vivo* and *in vitro* electrophysiological experiments have also reported a predominantly inhibitory effect of MLT on SCN neuronal activity, particularly at dusk (Brown and Piggins, 2007). Furthermore, MLT actions on SCN seem to be dependent on the MLT receptor subtype that is activated. For example, studies in knockout mice suggested that MLT inhibits neuronal activity of SCN neurons through $MT_1$ receptors (Liu *et al*., 1997) and induces a phase shifting response through $MT_2$ receptors (Jin *et al*., 2003). More recent evidence raises the possibility that MLT might also be able to influence clock gene expression in the SCN by acting through retinoid related orphan nuclear hormone receptors, in particular *rorβ* and *rev-erbα* (Agez *et al*., 2009).

So far, we have established that diurnal and circadian biological rhythms in the brain are generated by the interaction of SCN's molecular clock and the photoperiod, both of which influence MLT synthesis and release. The neuroanatomical distribution of MLT receptors in the brain raises the possibility that MLT can directly affect neuronal activity and potentially neurotransmitter release. This raised the possibility that MLT could have an influence on diurnal monoamine neurotransmission. In the next sections, general aspects of the 5-HT and DA systems will be reviewed as background prior to discussing evidence that they also exhibit diurnal rhythms.

### 1.2 The serotonergic system

5-HT is one of the most widely studied neurotransmitters in the central and peripheral nervous systems. In the central nervous system, most 5-HT producing neurons are located in the raphe

complex, which includes the DRN and median raphe nucleus (MRN) (Abrams *et al.*, 2004). These 5-HT cells project to all regions of the brain accounting for the transmitter's influence on diverse functions, including sleep, thermoregulation, feeding, and cognitive-affective states (Jacobs and Azmitia, 1992).

5-HT was first isolated in 1938 by the pharmacologist Vittorio Erspamer, which he called "enteramine", while he was conducting a study on the chemical composition of amphibian enterochromaffin cells in the gut (Erspamer and Asero, 1952). A decade later, Rapport *et al*. (1948) named this same molecule "serotonin" because of its actions as a vasoconstrictor. The idea that 5-HT could influence mental processes arose from two main observations: 1) 5-HT was found in the brain and since it does not cross the blood brain barrier, this suggested it must be synthesized there; 2) its molecular structure is similar to the drug, lysergic acid diethylamide (LSD), which was known for its psychotropic/hallucinogenic effects (Woolley and Shaw, 1954). Since LSD administration antagonizes the ability of 5-HT to induce muscle contractions, the drug's psychotropic effects were proposed to be a result of its antagonistic actions on the 5-HT system in the brain (Woolley and Shaw, 1954).

Subsequent work by Aghajanian and colleagues (Aghajanian *et al*. 1968) specifically associated the psychotomimetic-like effects of LSD with an inhibition of neuronal firing activity in the DRN. This nucleus had been identified a few years earlier by Dahlström and Fuxe (1964) as an area abundant in 5-HT containing neurons. The phenotype of DRN neurons responding to LSD and other 5-HT release-inducing drugs was later confirmed as serotonergic by use of a technique in which the recorded neuron was marked with a dye (ethidium bromide) that was later seen to

co-localize with the formaldehyde-induced yellow florescence of 5-HT (Aghajanian and Vandermaelen, 1982).

### 1.2.1 5-HT biosynthesis and metabolism

5-HT is synthesized from the essential amino acid, L-tryptophan, which, by definition, cannot be produced in the body and must be obtained from the diet (Grahame-Smith, 1964). L-tryptophan is then converted to 5-HT in a two-step process. First, L-tryptophan is converted to 5-hydroxy-L-tryptophan (5-HTP) by the rate-limiting enzyme, tryptophan hydroxylase (Tph) (Grahame-Smith, 1964). Second, 5-HTP is converted to 5-HT by the enzyme aromatic L-amino acid decarboxylase (Lovenberg *et al.*, 1962). Two isoforms of Tph (Tph1 and Tph2) have been identified. Only small amounts of Tph1 are found in the brain and it is predominantly expressed in the pineal gland and the periphery (Nakamura & Hasegawa 2007). Tph2 expression appears to be restricted to the brain (Walther *et al.*, 2003). 5-HT neurons in the DRN and adjacent MRN express low amounts of Tph1 mRNA, while Tph2 mRNA is expressed at higher levels (Malek *et al*., 2005).

After being released into the extracellular medium, 5-HT is reabsorbed into the axon terminal by the 5-HT transporter (5-HTT) (Rudnick and Clark, 1993). The 5-HTT is responsible for modulating the termination of 5-HT transmission by regulating the amount of 5-HT that remains in the extracellular space, available to activate 5-HT receptors (Rudnick and Clark, 1993). The human 5-HTT, encoded by a region on chromosome 17q11.2, was cloned and sequenced early in the 1990s (Ramamoorthy *et al.*, 1993). The 5-HTT gene is highly conserved in vertebrates; the rodent 5-HTT and the human 5-HTT protein have approximately 90% of structural homology in their amino acid sequence (Murphy *et al.*, 2004). Once 5-HT is back inside the neuron, its

metabolic degradation involves the enzymes monoamine oxidase (MAO) A and B, which convert 5-HT into 5-hydroxyindole acetaldehyde (Sjoerdsma *et al.*, 1955). This product is then degraded to 5-hydroxyindole acetic acid (5-HIAA) by the enzyme aldehyde dehydrogenase (Sjoerdsma *et al.*, 1955).

### 1.2.2 Neuroanatomy of the 5-HT system

As noted above, neurons that synthesize 5-HT in the brain are located along the midline of the brain stem in the raphe complex (see Figure 2). This group of cells was first described by Dahlström and Fuxe (1964) who divided the 5-HT cell clusters into nine groups (B1-B9) using histochemical fluorescence techniques. Later, the nomenclature was revised and the raphe 5-HT neurons were assigned to the superior (or ascending) and the inferior (or descending) divisions (Jacobs and Azmitia, 1992). The ascending division is composed of nuclei that send projections mainly to forebrain structures, including the caudal linear nucleus (B8), MRN (B8 and B5), DRN (B6 and B7) and the supralemniscal region (B9) (Jacobs and Azmitia, 1992). The descending division is composed of nuclei that send projections down to the spinal cord, and includes the nucleus raphe obscurus (B2), nucleus raphe pallidus (B1), nucleus raphe magnus (B3), ventral lateral medulla (B1/B3) and the area postrema (Jacobs and Azmitia, 1992). These divisions of the raphe nuclei are highly preserved across mammalian species including humans, further supporting the use of animal models to study the contribution of 5-HT transmission to brain function in health and disease (Hornung, 2003). Amongst these 5-HT raphe nuclei, the DRN has been studied the most due to its high density of 5-HT neurons and its projections to forebrain structures involved in higher cognitive processes and mood modulation (Descarries *et al*., 1982; Hornung, 2003).

The DRN is located in the midbrain just above the pons and ventral to the fourth ventricle, or Sylvian aqueduct. Abrams *et al*. (2004) further delineated a rostrocaudal topographic organization. The rostral aspect of the DRN projects to the striatum, SN and cortex and the more caudal DRN projects to the septum, hippocampus, hypothalamic areas, and entorhinal cortex (Abrams *et al*., 2004). The DRN also receives inputs from various structures, including the prefrontal cortex, hippocampus, amygdala, locus coeruleus, hypothalamus and the VTA (Jacobs and Azmitia, 1992). Recent studies using genetically modified mice expressing enhanced green fluorescence in combination with a genetic marker of 5-HT neurons (*Pet1*), which provides a fine mapping of 5-HT innervations, confirmed earlier evidence that 5-HT neurons in the DRN send axons to almost all regions of the brain (Jensen *et al*., 2008; Bang *et al*., 2012). Particularly high 5-HT DRN axonal innervation was observed in the medial septum, the nucleus of the diagonal band, shell region of the NAcc, globus pallidus, ventral pallidum, substantia innominata, basolateral amygdala, hypothalamus, VTA, substantia nigra pars compacta (SNpc) and SNpr (Bang *et al*., 2012).

### 1.2.3 5-HT receptors

5-HT receptors represent one of the most studied families of receptors described to date. They are widely expressed in the central and peripheral nervous system as well as in peripheral tissue, corresponding with the extensive physiological functions in which 5-HT is involved (Jacobs and Azmitia, 1992; Piñeyro and Blier, 1999). In the original classification of 5-HT receptors by the International Union of Pharmacology, seven classes, with a total of 14 subtypes of 5-HT receptors were described; 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$, 5-$HT_{1E}$, 5-$HT_{1F}$, 5-$HT_{2A}$, 5-$HT_{2B}$, 5-$HT_{2C}$, 5-

$HT_3$, 5-$HT_4$, 5-$HT_{5A}$, 5-$HT_{5B}$, 5-$HT_6$ and 5-$HT_7$ (Hoyer *et al.*, 1994). This classification was later extended to include the 5-$HT_{3A}$ and 5-$HT_{3B}$ and 5-$HT_{3C}$ isoforms (Nichols and Nichols, 2008). With the exception of the 5-$HT_3$ receptor class, which are ligand-gated ion channels, the rest of the 5-HT receptors are G-protein-coupled trans-membrane receptors, and can be present at both terminal and somatodendritic sites (Nichols and Nichols, 2008). A brief summary of the localization and functions of the 5-HT receptors is shown in Table 1.

The most abundant 5-HT receptor expressed in the mammalian brain is the 5-$HT_{1A}$ receptor (Nichols and Nichols, 2008). 5-$HT_{1A}$ receptors are $G_{i/o}$ protein-coupled receptors encoded by a gene on human chromosome 5q11.2-13 (Fargin *et al.*, 1988). 5-$HT_{1A}$ receptors work as autoreceptors in 5-HT DRN neurons, but they are also present as postsynaptic receptors in forebrain structures, such as the hippocampus and cortex (Pazos and Palacios, 1985; Albert *et al.*, 1990; Sotelo *et al.*, 1990). The importance of 5-$HT_{1A}$ receptors lies in their ability to auto-regulate 5-HT firing activity in DRN neurons, thereby decreasing the release of 5-HT in forebrain regions (Rogawaski and Aghajanian, 1981; Sharp and Hjorth, 1990).

### 1.2.4 Role of the 5-HT system in major depression

We are particularly interested in the possibility that disturbances to putative monoamine diurnal rhythms influence the development and expression of affective disorders. Given this, the literature on the role of 5-HT in depression and the efficacy of antidepressant drug therapy will be briefly reviewed below.

Clinical depression is characterized by low mood, anhedonia, weight changes, insomnia/hypersomnia, fatigue, worthlessness or guilty feelings, death or suicidal thoughts or suicidal ideations/attempts (American Psychiatric Association, 2000). The etiology and pathophysiology of depression remain poorly understood and are thought to reflect a complex interaction between genes and environmental factors (Olsson *et al*., 2010; Saveanu and Nemeroff, 2012). A substantive role for 5-HT is suggested by converging evidence from several lines of research. For example, early studies observed that in some patients severity of depressive symptoms correlates with lower levels of 5-HIAA in cerebrospinal fluid (CSF), thus suggesting an alteration in 5-HT metabolism (Åsberg *et al*., 1976). Subsequent studies using acute tryptophan depletion demonstrated that even a transitory decline in brain 5-HT synthesis can lead to lower mood (Young and Leyton, 2002) and promote reappearance of depressive symptoms in remitted patients (Delgado *et al.*, 1990). Further studies using positron emission tomography, provided evidence for a deficit in brain metabolic conversion of tryptophan to 5-HT in depressed patients (Rosa-Neto *et al*., 2004). In addition to a diminished synthesis of 5-HT in depression, there is also evidence that alterations in proteins involved in the modulation of 5-HT neurotransmission can also contribute to the development of a depressive state, in particular the 5-HTT and the $5\text{-HT}_{1A}$ receptor (Boldrini *et al.*, 2008; Parsey *et al.*, 2010; Murphy and Moya, 2011).

The 5-HTT appears to have an important role in susceptibility to depression and the clinical efficacy of antidepressant medications. For example, both the current generation of selective 5-HT reuptake inhibitors (SSRI) and the older tricyclic antidepressants bind to the 5-HTT, inhibiting 5-HT reuptake and increasing the extracellular availability of 5-HT (Piñeyro and Blier,

1999). Furthermore, mRNA expression of the 5-HTT in postmortem brains of depressed-suicide patients is decreased in the DRN (Arango *et al.*, 2001). Growing evidence also suggests that mutations of the 5-HTT gene (i.e. 5-HTTLPR polymorphisms in *SLC6A4,* the gene that encodes the 5-HTT) increase the risk for mood alterations in those who endured stressful childhoods (Caspi *et al.*, 2010; Karg *et al.*, 2011) and can be associated with poor antidepressant response (Murphy *et al.*, 2008).

There is compelling evidence that a common pathway of several antidepressants drugs is induction of the desensitization of the $5\text{-HT}_{1A}$ autoreceptor and increased activation of postsynaptic $5\text{-HT}_{1A}$ receptors (Piñeyro and Blier, 1999). This was first proposed based on observations that acute treatment with antidepressants induces a decrease in 5-HT firing activity that recovers after 2-3 weeks of treatment (Artigas, 1993; Blier and de Montigny, 1994). This time course corresponds to the delay for clinical efficacy in antidepressant treatment in humans, which led to the hypothesis that proper regulation of the $5\text{-HT}_{1A}$ receptor is crucial for the functioning of the 5-HT system and the regulation of mood (Blier and de Montigny, 1994). In support of this theory, alterations in $5\text{-HT}_{1A}$ receptor binding have been observed in brains from depressed suicides, specifically $5\text{-HT}_{1A}$ receptor binding was lower in the caudal and higher in the most rostral portion of the DRN (Boldrini *et al.*, 2008). This has been interpreted as the response of the system to compesate for lower levels of 5-HT (Boldrini *et al.*, 2008). In concordance, genetic alterations that produce an over-expression of the $5\text{-HT}_{1A}$ receptor in the DRN have also been associated with depression, suicidality and poor response to antidepressant treatment (Lemonde *et al.*, 2003; Parsey *et al.*, 2006).

### 1.2.5 Antidepressant treatment effect on the firing activity of 5-HT neurons

In the laboratory, changes in 5-HT neurotransmission have proven to be a helpful index of antidepressant efficacy, especially firing activity of 5-HT neurons in the DRN (Descarries *et al*., 1982; Domínguez-López *et al*., 2012a). However, the ability of antidepressant treatments to increase 5-HT neurotransmission depends on their particular mechanisms of action. For example, SSRIs increase synaptic 5-HT availability by blocking the 5-HT transporter (5-HTT) (Piñeyro and Blier, 1999). Acutely in rats, this causes a decrease in the firing activity of 5-HT neurons located in the DRN, due to the increased agonism on $5\text{-HT}_{1A}$ autoreceptors; but after chronic treatment, a desensitization of $5\text{-HT}_{1A}$ autoreceptors occurs, restoring 5-HT firing activity in the DRN to basal levels (de Montigny *et al.*, 1990; Czachura and Rasmussen, 2000). This desensitization phenomenon has been proposed to occur as a result of a decreased capacity of $5\text{-HT}_{1A}$ receptors to activate G proteins (Castro *et al.*, 2003).

Other antidepressants, such as monoamine oxidase inhibitors (MAOI) have similar acute and chronic effects on 5-HT firing activity as SSRIs (Aghajanian *et al.*, 1970; Haddjeri *et al.*, 1998). However, in addition to increasing extracellular 5-HT levels, MAOIs also increase the levels of NA and DA (Celada and Artigas, 1993; Finberg *et al.*, 1993; Wayment *et al.*, 2001; Rollema *et al.*, 2011). NA neuronal firing activity in the LC is inhibited acutely and does not recover after chronic treatment with MAOI, whereas DA neuronal firing in the VTA is slightly decreased only after chronic treatment (Blier and de Montigny, 1985; Chenu *et al.*, 2009). Antidepressants that increase NA activity are also known to increase 5-HT firing activity in DRN neurons (Haddjeri *et al.*, 1997). This is achieved by antagonism of $\alpha_2$-adrenoceptors in NA neurons of the LC that

increase 5-HT firing activity in the DRN by increasing NA activation of $\alpha_1$-adrenoceptors located on DRN 5-HT neurons (Freedman and Aghajanian, 1984; Haddjeri *et al.*, 2004). Other antidepressant strategies that do not have acute effects on 5-HT firing activity but do increase 5-HT firing activity with chronic treatment include the tricyclic antidepressants, NA reuptake inhibitors, sigma ligands, vagus nerve stimulation and electroconvulsive shocks (Hill *et al.*, 2009).

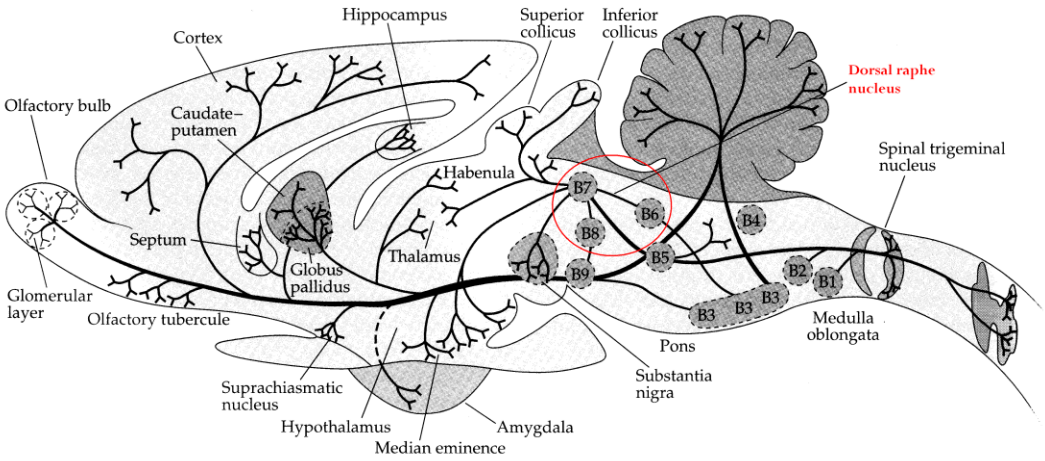


**Figure 2.** Serotonergic pathways in the rat brain. The cell clusters identified by Dahlström and Fuxe (1964) as B6 and B7 and the more dorsal part of B8 correspond to the dorsal raphe nucleus. Modified from Feldman *et al*. (1997).

**Table I.** Localization and function of 5-HT receptors

| Receptor | Localization | Associated functions |
|---|---|---|
| 5-$HT_{1A}$ | Hippocampus, septum, amygdala, DRN, cortex, hypothalamus and spinal cord. | Modulation of emotion, pre and postsynaptic receptors, 5-HT autoregulation. |
| 5-$HT_{1B}$ | Basal ganglia, SN, subiculum, DRN, cortex, striatum, cerebellum and hippocampus. | Modulation of aggression, learning and memory, presynaptic autoreceptor. |
| 5-$HT_{1D}$ | Basal ganglia, SN, hippocampus, raphe nuclei, cortex, superior colliculus, striatum, NAcc and olfactory tubercle. | Modulation of emotion, cerebral vasoconstriction, autoreceptor. |
| 5-$HT_{1E}$ | Cortex, hippocampus, striatum and amygdala. | Possible involvement in cognition and memory. |
| 5-$HT_{1F}$ | Globulus pallidus, SN, DRN, hippocampus, cortex, striatum, thalamus, hypothalamus, spinal cord, periaqueductal gray and nucleus of the tractus solitarius. | Modulation of neurogenic inflammation and migraine. |
| 5-$HT_{2A}$ | Claustrum, olfactory tubercle, cortex, basal ganglia, raphe nuclei, NAcc, spinal cord, cardiovascular tissue and gastrointestinal tract. | Modulation of hypothalamic axis, antipsychotic activity, pain and vasoconstriction. |
| 5-$HT_{2B}$ | Cochlea and inferior colliculus, fundus, gut, heart, kidney and lung. | Tissue contraction-relaxation, neurogenic pain. |
| 5-$HT_{2C}$ | Choroid plexus, globulus pallidus, SN, olfactory nucleus, cortex, amygdala, thalamus, hippocampus and lateral habenula. | Modulates DA functions, locomotor activity and feeding behavior. |
| 5-$HT_{3}$ | Dorsal vagal nerve, solitary tract nerve, trigeminal nerve, area postrema, spinal cord, limbic system, cortex, hippocampus, amygdala, habenula and gut. | Vasodilatation, respiration, gastrointestinal tone, pain, anxiety and addiction, pre and postsynaptic receptor. |
| 5-$HT_{4}$ | Colliculus, hippocampus, olfactory tubercles, NAcc, striatum, globulus pallidus, SN, cortex, vagus nerve, heart and gastrointestinal tract. | Gastrointestinal motility, memory and emotional response. |
| 5-$HT_{5A,5B}$ | Cortex, hippocampus, habenula, olfactory bulb and cerebellum. | Control of circadian rhythms, mood and cognitive functions. |
| 5-$HT_{6}$ | Striatum, olfactory tubercle, cortex, hippocampus, amygdala and NAcc. | Acetylcholine release, postsynaptic receptor, involved in learning and memory. |
| 5-$HT_{7}$ | Hypothalamus, thalamus, hippocampus, cortex, DRN, SN, lateral septum, amygdala, SCN and peripheral blood vessels. | Circadian rhythm and mood regulation, and smooth muscle relaxation. |

Information obtained from Hoyer *et al*. (1994) and Nichols and Nichols (2008).

### 1.3 The DA system

DA, along with NA and adrenaline, is a monoamine belonging to the catecholamine family, which consists of neurotransmitters characterized structurally by the presence of a catechol group (Sharman, 1973). In the early 1900s, the DA molecule was proposed to be part of a biosynthetic pathway that coverts the amino acid tyrosine to adrenaline, however this proposal was received with some skepticism at the time (Halle, 1906; Ewins and Laidlaw, 1910). Shortly after, DA was synthesized under laboratory conditions by Ewing (1910) and its sympathomimetic properties were tested in peripheral tissue preparations by Barger and Dale (1910). However, it was not until the discovery of the enzymatic decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme L-aromatic amino acid decarboxylase (DOPA decarboxylase) in peripheral tissue (Holtz *et al.*, 1938), that DA was formally postulated to be part of the same biosynthetic pathway of NA and adrenaline (Blaschko, 1939; Holtz, 1939). In the 1950s, the presence of DA in peripheral mammalian tissue was established by Goodall (1950), and its presence in brain was first suggested by Montagu (1957) and then confirmed by Carlsson *et al*. (1958), who subsequently proposed that DA is a neurotransmitter involved in the control of motor functions and not just a precursor of NA (Carlsson, 1959). Since then, brain DA neurotransmission has been implicated in several neurological and psychiatric disorders such as schizophrenia and Parkinson's disease as well as in the modulation of motivated behavior for natural and drug rewards (Biju and de la Fuente-Fernandez, 2009; Taber *et al*., 2012; Salamone and Correa, 2013; Laruelle, 2014).

### 1.3.1 DA biosynthesis and metabolism

The *in vivo* synthesis of DOPA and DA from tyrosine in the brain was demonstrated in an elegant experiment performed by McGeer *et al* (1963) using radiolabeled L-tyrosine. In the first step of DA synthesis, the essential amino acid, phenylalanine, is hydroxylated in the liver by phenylalanine hydroxylase (PH). The resulting product, L-tyrosine is then carried into the brain, where it is hydroxylated to L-dihydroxy-phenylalanine within catecholamine neurons (L-DOPA) by tyrosine hydroxylase (TH), the rate-limiting enzyme in DA biosynthesis (Bagchi and McGeer, 1964; Nagatsu *et al.*, 1964, Daubner *et al.*, 2011). The final step involves the conversion of L-DOPA to DA by the action of DOPA decarboxylase (Holtz and Westermann, 1956).

The catabolism of DA is mainly due to oxidation but also O-methylation (Axelrod *et al*., 1958). After DA is released into the extracellular space, it remains there for a short period of time before diffusing to extra-synaptic regions (Cragg and Rice 2004). The DA transporter (DAT), a protein situated in the membrane of DA neurons, clears DA from the extracellular space (Cragg and Rice 2004) by reabsorbing it back inside the cell through an active transport mechanism (Eriksen *et al.*, 2010). Intracellularly, DA is catabolized by the enzyme monoamine oxidase (MAO), which was first described by Hare (1928). Two forms of MAO are known, MAO-A and MAO-B, and both are able to oxidize DA, though MAO-A is present in catecholaminergic neurons and MAO-B is found in 5-HT and histaminergic neurons and gial cells (Shih *et al*., 1999; Nagatsu, 2004). The oxidation product of DA by MAO is 3,4-dihydroxyphenylacetaldehyde (DHPA) (Nagatsu, 2004). DHPA is a short-lived metabolite of DA that is converted to DOPAC by the enzyme aldehyde dehydrogenase and is then directly excreted or further metabolized by the enzyme catechol-O-methyltransferase (COMT) to

homovanillic acid (HVA) (Marchitti *et al.*, 2007). Degradation of DA by O-methylation with COMT is considered the main metabolic pathway for extracellular degradation of DA (Männistö and Kaakkola, 1999), especially in the prefrontal cortex where the DAT is rarely expressed and DA accumulates in extrasynaptic space (Lewis *et al.*, 2001; Yavich *et al.*, 2007). This DA metabolic pathway, first described by Axelrod *et al* (1958), yields 3-methoxytyramine (3-MT) which is considered the main metabolic index of released DA (Karoum *et al.*, 1994).

### 1.3.2 Neuroanatomy of the DA system

The classification of DA brain pathways is based on the work of Dahlström and Fuxe (1964), who originally divided catecholamine containing neurons localized in the midbrain and the brainstem of rats into several clusters, of which, A8 to A12 correspond to DA producing neurons (Figure 3). Additional clusters of DA neurons (A13 to A16) were later identified in other brain regions providing a nomenclature that is still widely in use today (Bjorklund and Dunnett, 2007). In this classification, the A9 cluster is formed by densely packed neurons located in the SNpc and is traditionally considered the main origin of the nigrostriatal DA pathway with axons projecting through the medial forebrain bundle (MFB) to the caudate-putamen region in the striatum (Fuxe *et al.*, 1977). Neurons located in the VTA belong to the A10 cluster and are considered the main origin of the mesolimbic and mesocortical DA pathways that also run through the MFB but project to limbic (NAcc, olfactory tubercle, bed nucleus of the stria terminalis, lateral septum and amygdala) and cortical (prefrontal, cingulate and entorhinal) areas, respectively (Mansour *et al.*, 1995; Figure 3). The A8 cluster is a small group of cells in the mesenphalic reticular formation at the level of the red nucleus (retrorubral area), forming a bridge between the caudal parts of the A10 and A9 clusters, which projections contribute to the

three mesencephalic DA pathways (Bentivoglio and Morelli, 2005). DA neurons located in the arcuate nucleus (A12 cluster) are part of the tuberoinfudibular system with axons projecting across the median eminence to the hypothalamo-hypophyseal portal vessels (Fuxe *et al.*, 1977). A scattered group of DA neurons in the periventricular and supraoptic hypothalamic nuclei (A11, A14 and A15 clusters) and in the zona incerta of the dorso-lateral caudal hypothalamus (A13 cluster) form the incertohypothalamic DA system of the diencephalon (Mansour *et al.*, 1995). Finally, the A16 cluster is a group of DA interneurons which form part of a local circuit within the olfactory bulbs, corresponding to the cells located in the substantia innominata of the rostral forebrain in humans (Mansour *et al.*, 1995).

Although the classification of mesencephalic DA neurons in clusters (A8, A9 and A10) has remained over the years, their topographical projections and specific contributions to cortical, limbic and striatal regions has been constantly redefined and updated (Björklund and Dunnett, 2007). Many studies in the mammalian brain have observed that a single DA pathway can receive innervations from the three clusters of mesencephalic DA neurons and, in addition, the projections of these DA neurons, also referred to as the SN-VTA complex, overlap in some terminal areas (Fallon and Moore, 1978; Joel and Weiner, 2000; Haber *et al*., 2000). An example of overlapping DA innervations occurs in the cortex where a single cortical area (e.g. frontal cortex, Figure 4c) can be reached by DA neurons located all along the SN-VTA complex (Loughlin and Fallon, 1984; Björklund and Dunnett, 2007). Alternative classifications to the Dahlström and Fuxe clusters system have emerged trying to encompass these findings. For example, Fallon and More (1978), using retrograde and anterograde tracing techniques, described the organization of the SN-VTA complex, based on their connectivity, as a dorsal to

ventral gradient of neurons distributed in two layers or tiers (see Figure 4 and Figure 5). The dorsal tier included DA neurons located in the dorsal parts of the VTA and the SNpc, in addition to retroruberal area DA neurons, and it is mostly composed of calbindin-positive neurons (Joel and Weiner, 2000). This layer of neurons projects preferentially to the NAcc and the ventro-medial part of the caudate-putamen (collectively referred as the ventral striatum), as well as to limbic areas such as the olfactory tubercle and amygdala (Bentivoglio and Morelli, 2005; Björklund and Dunnett, 2007). The ventral tier included DA neurons in the ventral parts of the VTA and the SNpc and extends DA neurons in SNpr, which are calbindin-negative neurons (Joel and Weiner, 2000). The dorso-lateral part of the caudate-putamen (dorsal striatum) and dorsal forebrain structures, such as the septum, are targets of DA neurons located predominantly in the ventral tier (Bentivoglio and Morelli, 2005; Björklund and Dunnett, 2007).

### 1.3.3 Functional connectivity of mesencephalic DA neurons

Traditionally, mesolimbic and mesocortical DA pathways have been associated with motivation, reward and cognitive process and the nigrostriatal pathway with motor function and response initiation (Amalric and Koob, 1993; Joel and Weiner, 2000; Robbins *et al*., 2005). However, taking into consideration the neuro-anatomical evidence reviewed above, a strict separation of the functions in which DA neurons in the VTA and the SN are implicated based on their contribution to a single DA pathway is difficult to maintain (Bentivoglio and Morelli, 2005; Björklund and Dunnett, 2007). It has been recognized that the functions of mesencephalic DA neurons overlap and are complementary to some extent, reflecting not only their topographical projections but also the inputs received from other brain areas (Bentivoglio and Morelli, 2005; Dunnet, 2005; Wise, 2009). For example, a simple behavior such as locomotor activity, seems to

be associated with increased DA neurotransmission in both the NAcc and the caudate-putamen regions (O'Neill and Fillenz, 1985), suggesting activation of both mesolimbic and nigrostriatal DA midbrain neuron projections.

### 1.3.4 Psychostimulants and the DA system

Early evidence from behavioral studies using selective lesions of dopaminergic pathways and administration of drugs that activate or inhibit DA neurotransmission indicates that DA projections to the prefrontal cortex and the striatum, in particular the NAcc are involved in processing reward information (Schultz, 1998). More recent studies using optogenetic techniques have confirmed the important role of these DA neurons in operant reinforcement and reward-seeking behaviors (Adamantidis *et al*., 2011; Kim *et al*., 2012; Rossi *et al*., 2013). As such, mesencephalic DA neurotransmission has been central in several theories of the mechanism activated by substances of abuse including psychostimulants (Alcaro *et al*., 2007).

Originally, the mesolimbic DA pathway was proposed as the neuronal circuitry in which hyperactivity and motivational/rewarding effects induced by psychostimulants, such as amphetamine (AMPH) and cocaine, are integrated (Wise and Bozarth, 1987). Indeed, VTA lesion studies using 6-hydroxydopamine demonstrated that DA neurons originating from the VTA and projecting to the NAcc regulate spontaneous and acute AMPH-induced increases in locomotor activity (Kelly *et al*., 1975; Carey, 1983; Dunnett *et al*., 1984; Clarke *et al*., 1988; Boye *et al*., 2001). However, recent evidence suggests that an intact nigrostriatal DA pathway, especially the innervations arriving from the ventral tier of the SNpc to the dorsal striatum, is necessary for the acute locomotor activating effects of cocaine (Beeler *et al*., 2009). In addition,

the nigrostriatal pathway is involved in the occurrence of discrete, stereotypical movements (e.g. head turning, grooming) observed after acute administration of high doses of psychostimulants (Kelly *et al*., 1975; Carey 1983; Amalric and Koob, 1993; Rebec 2006). In humans, acute AMPH administration seems to affect DA neurotransmission to a higher degree in limbic and sensorimotor striatal regions while the associative striatum seem less affected (Martinez *et al*., 2003; Casey et al., 2013; see also Joel and Weiner, 2000 and Figure 5).

Moreover, repeated exposure to psychostimulants produces regional molecular and cellular changes in the striatum that have been associated with specific behavioral variations. For example, changes in the formation and strengthening of synapses modulated by glutamate and DA release (Gerdeman *et al*., 2003), as well as changes in DAT binding (Letchworth *et al*., 2001), in the NAcc are belived to be critical for acquisition in early stages of addiction. These plastic effects in the synapses spread to the caudate-putamen after long term exposure when craving and compulsive drug seeking occurs (Everitt and Wolf, 2002). In this way, the NAcc has been implicated in the motivational significance of the stimulus, while the dorsal striatum is involved in the shift from controlled drug use to compulsive drug abuse (Bentivoglio and Morelli, 2005). In a revised role of mesencephalic DA neurotransmission in reward and addiction it is now recognized that both nigrostriatal and mesolimbic pathways play complementary roles recruiting striatal regions after acute and chronic exposure to psychostimulants (Wise, 2009).

It is well established that the net effect of psychostimulants, and near all other abused drugs (e.g. ethanol) in the DA system, is an increase in extracellular DA, particularly in the NAcc and the

caudate-putamen (Di Chiara and Imperato, 1988; Moghaddam and Bunney, 1989; Kuczenski and Segal, 1999; Diana and Tepper, 2002). However, the mechanism activated by each psychostimulant is different. For example, AMPH and cocaine both increase extracellular DA levels by interacting with the DAT. However, cocaine binds to the DAT and blocks DA reuptake whereas AMPH competes with DA to bind to the DAT and, in addition, induces a release of DA via DAT reverse transport (Wall *et al.*, 1995; Sulzer *et al.*, 2005; Eriksen *et al.*, 2010). AMPH also promotes extracellular DA levels by several other mechanisms that include activation of TH, inhibition of MAO and increase of vesicular release of DA in the cytosol (Sulzer, 2011). There is also evidence that AMPH and cocaine could be activating different dopaminergic innervations originating from the SN to the striatum to exert their psychostimulant effects (Sieber *et al*., 2004).

### 1.3.5 DA receptors

Most of our knowledge about the existence of different DA receptors subtypes comes from studies in the 1970s using $^{3}$H-ligands (Seeman, 1980). DA binds to G protein-coupled receptors that are classified into the $D_1$ and $D_2$ families (Beaulieu and Gainetdinov, 2011). By the early 1990s, it was established that the $D_1$ family includes $D_1$ and $D_5$ receptors, which are somatodendritic $G\alpha_{s/olf}$ protein-coupled receptors, characterized by their ability to stimulate adenylyl cyclase activity and cAMP production (Beaulieu and Gainetdinov, 2011). The $D_2$ family includes $D_2$, $D_3$ and $D_4$ receptors which are coupled to $G\alpha_{i/o}$ proteins and, in contrast to $D_1$ family, inhibit adenylyl cyclase activity (Beaulieu and Gainetdinov, 2011). $D_2$ and $D_3$ receptors are expressed in axon terminals and at the somatodentritic level acting as autoreceptors (Shi, 2009; Ford, 2014). The $D_1$ and $D_2$ receptors are the most abundant DA receptors in the brain,

expressed predominately in the striatum, NAcc, olfactory bulb, amygdala and cortical areas (Beaulieu and Gainetdinov, 2011). Importantly, $D_2$ receptors on DA (autoreceptors) and non-DA (heteroreceptors) neurons are involved in the homeostatic control of DA synthesis and release (Anzalone *et al*., 2012). In table II, the localization and functions of DA receptors are summarized.

### 1.3.6 Autoregulation of mesolimbic DA neurotransmission

Somatodendritic release of DA in the VTA and SNpc activates $D_2$-like ($D_2$ and $D_3$) autoreceptors located in DA cell soma and dendrites (Diana and Tepper, 2002; Shi, 2009). This leads to a temporary hyperpolarization of DA cells, by potasium influx trougth GIRK2 channels, inhibiting their spontaneous firing activity and negatively modulating impulse mediated DA release (Ford, 2014). Although $D_3$ receptors are also expressed in midbrain DA neurons, inhibitory feeback is thought to be mediated by activation of $D_2$ receptors (Shi, 2009; Ford, 2014). Systemic and local iontophoretical application of $D_2$ receptor agonists such as apomorphine (APO) potently inhibits VTA DA cell firing (Bunney *et al*., 1973; Skirboll *et al*., 1979; White and Wang, 1984a; 1984b) and decreases DA release in terminal regions and in the VTA itself (Chai and Meltzer, 1992; Zhang *et al*., 1994). Comcomitant with the inhibitory effects of APO in DA neurotransmission, a decrease of locomotor activity has been observed in rodents with doses of APO that selectively activated $D_2$ autoreceptors (Strömbom, 1976; Havemann *et al.*, 1986: Depoortere *et al*., 1996). Firing rate inhibitory response to APO administration has been widely used to identify midbrain DA containing neurons during extracellular electrophysiological recordings (Diana and Tepper, 2002; Ungless and Grace 2012). It is belived that somatodendritic $D_2$ autoreceptors couple local DA release with pauses in DA cell firing shaping, in this way, patterns of firing and burst activity

in DA neurons (Ford, 2014). In addition to self-inhibition at the somatodendritic level, in the axon terminal at least three mechanisms by which $D_2$ autoreceptors regulate DA neurotransmission have been described; by decreasing voltage-dependent exocytotic DA release mediated by potassium channels (Martel *et al*., 2011), increasing DAT activity (Benoit-Marand *et al*., 2011) and inhibiting TH activity (Wolf and Roth, 1990).

Inhibition of midbrain DA cell firing by $D_2$ autoreceptor activation is also observed when local extracellular levels of DA are increased after administration of the psychostimulant AMPH (Bunney *et al.*, 1973; Kalivas *et al*., 1989; Kalivas and Duffy, 1991; Olijslagers *et al*., 2005). In addition, the overflow of extracellular DA induced by AMPH also activates a long negative feedback loop (Bunney *et al.*, 1973; Bunney and Aghajanian, 1978). This long inhibitory feedback loop is a complex mechanism that recruits cells located in terminal areas bearing DA ($D_1$-like and $D_2$-like) receptors (e.g. striatum) and it sends back direct and indirect afferents to DA midbrain neurons (Shi, 2009). However, activation of $D_2$ receptors does not stop AMPH-induced DA efflux since other mechanisms implicated in the effects of AMPH remain active besides inhibition of impulse mediated DA release (Sulzer, 2011; Daberkow *et al*., 2013).

Due to these autoregulatory mechanisms, firing activity in midbrain DA neurons is considered an index to study changes in DA neurotransmission induced by pharmacological manipulations, including psychostimulant administration (Mercuri *et al*., 1992; Diana and Tepper, 2002). Furthermore, reversal of AMPH-induced inhibition on midbrain DA neurons is used as a pharmacological model to determine a drug's potential antipsychotic activity (Stockton and Rasmussen, 1996; Ellenbroek and Cools, 2000; Diana and Tepper, 2002).

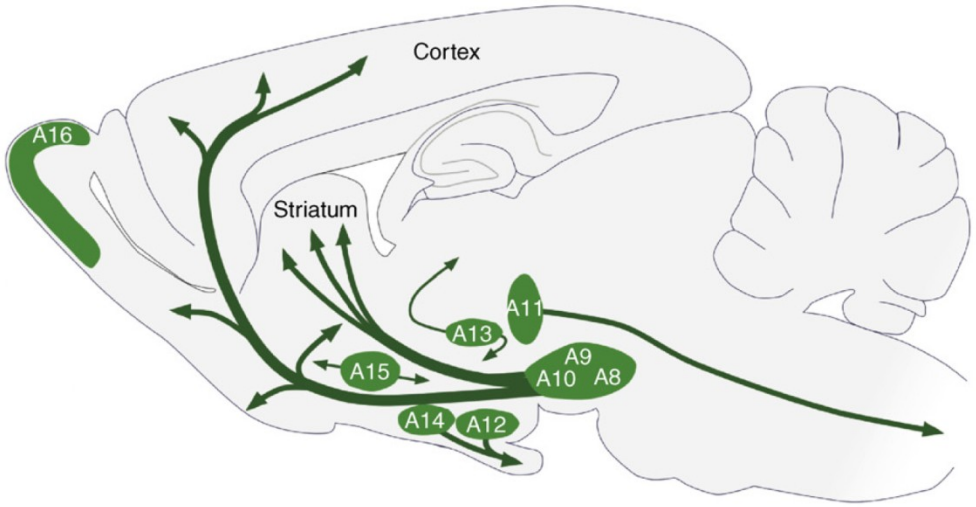


**Figure 3.** Dopamine (DA) cell clusters in the rat's brain. The mesencephalic DA neurons that localized in the A8 (retrorubral area), A9 (SN) and A10 (VTA) clusters, also known as the SN-VTA complex, are the origin of the nigrostriatal, mesolimbic and mesocortical pathways. Taken from Björklund and Dunnett, 2007.

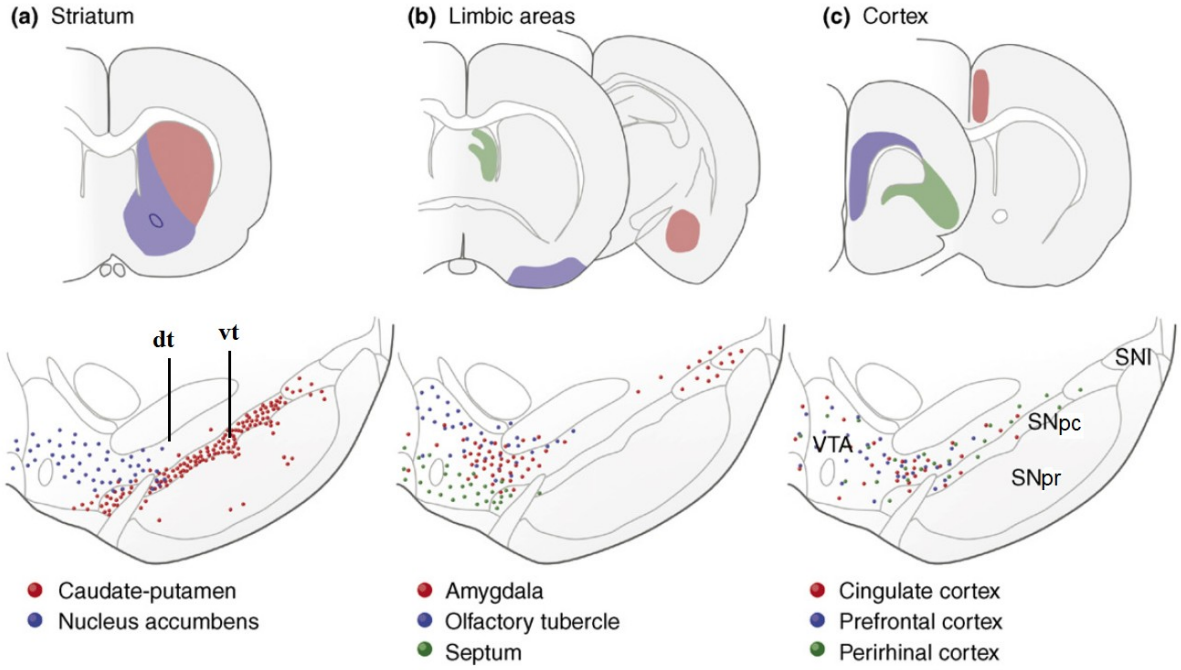


**Figure 4.** Terminal fields of mesencephalic dopamine (DA) neurons in the rat brain. Striatal (a), limbic (b) and cortical (c) pathways received mixed contributions from DA neurons located along the SN-VTA complex. dt, dorsal tier; SNL, substantia nigra pars lateralis; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; vt, ventral tier; VTA, ventral tegmental area. Taken from Björklund and Dunnett, 2007.

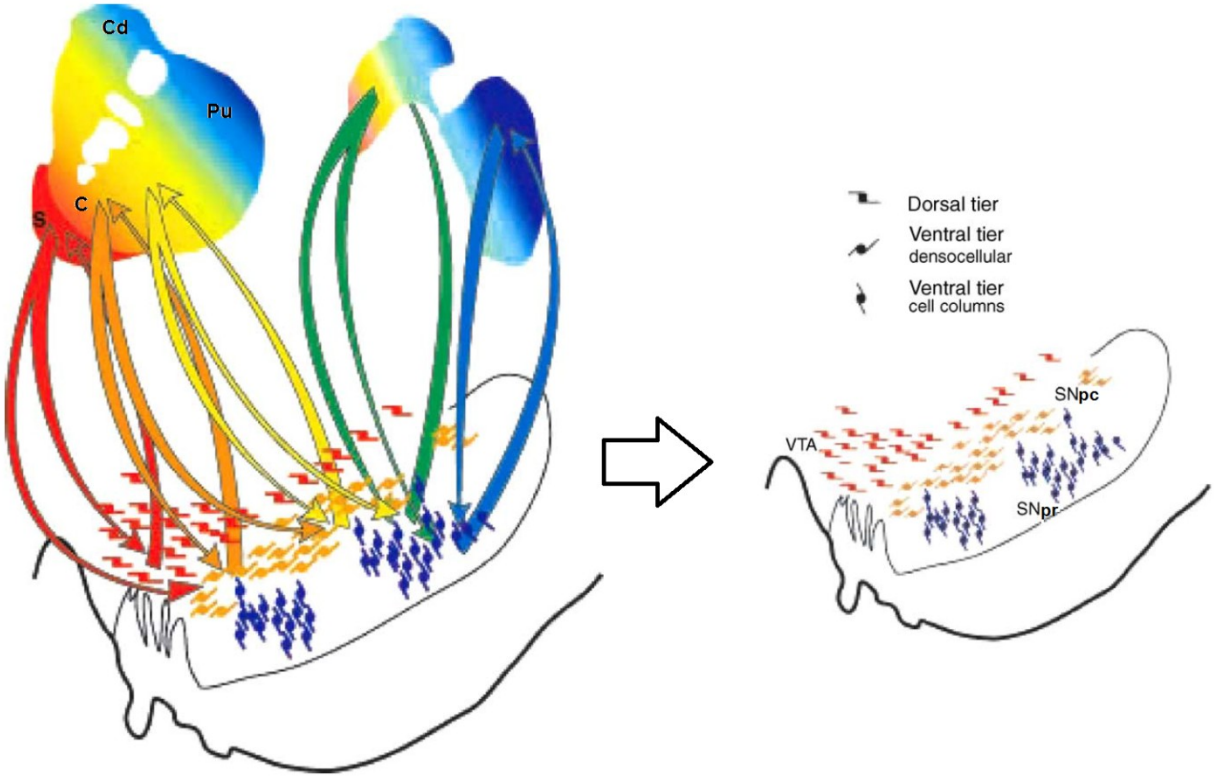


**Figure 5.** SN-VTA complex projections to the striatum in primates. Limbic (red), associative (yellow) and motor (blue) striatal regions receive and send projections to mesencephalic DA neurons that are topographically organized in a dorsal and ventral tier. C, nucleus accumbens core region; Cd, caudate; Pu, putamen; S, nucleus accumbens shell region; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; VTA, ventral tegmental area. Taken from Haber et al., 2000.

**Table II.** Localization and functions of DA receptors in the brain

| Receptor | Localization in the brain | Associated functions |
|---|---|---|
| $D_1$ | Caudate-putamen, NAcc, SN, olfactory bulb, amygdala, cortex, hippocampus, cerebellum, thalamus, hypothalamus. | Postsynaptic receptor, moderate stimulatory effect on locomotion. Critically involved in reward and reinforcement mechanisms, learning and memory. |
| $D_5$ | Cortex, SN, hypothalamus, hippocampus, caudate and NAcc. | Largely unknown, minor role in cognitive functions. It is possibly involved in the same functions than D1 receptors. |
| $D_2$ | Striatum, NAcc, olfactory tubercle, SN, VTA, hypothalamus, cortex, septum, amygdala and hippocampus. | Postsynaptic and presynaptic receptor. Presynaptic activation regulates DA release and locomotor activity. Critically involved in reward and reinforcement, learning, memory, schizophrenia and bipolar disorder. |
| $D_3$ | NAcc, olfactory tubercle, islands of Calleja, striatum, SNpc, VTA, hippocampus, septum and cortex. | Postsynaptic and presynaptic receptor. Presynaptic activation may regulate tonic DA release. Moderated inhibitory actions in locomotion. Involved in reward and reinforcement mechanisms. Minor role in cognitive functions. |
| $D_4$ | Frontal cortex, amygdala, hippocampus, hypothalamus, globus pallidus, SNpr and thalamus. | Largely unknown. Minor role in cognitive functions. |

Information obtained from Beaulieu and Gainetdinov, 2011.

### 1.4 Diurnal rhythms in the monoaminergic systems

Monoamine content in the brain is not constant across the light-dark cycle (Nagayama, 1999). Although extensive research has been done on this topic in the last 40 years, the way in which diurnal (and circadian) rhythms are generated and how they influence monoaminergic neurotransmission is still a matter of debate. Things become even more complicated due to the existence of diurnal oscillations in different components of the monoaminergic systems (e.g. synthesize enzymes, receptors, release and degradation). Due to the difficulties in assessing the human brain, most of our knowledge in this area has come from experiments in rodents.

#### 1.4.1 Diurnal rhythms in 5-HT neurotransmission

One of the first studies describing diurnal changes in content of 5-HT (and DA) in the whole brain, in rats, was performed by Scheving *et al*., (1968a; 1968b). In this paper, the authors described a clear diurnal rhythm in 5-HT content in brain tissue, with a high content during the light phase that decreased in the dark phase (Scheving *et al*., 1968a). Such a rhythm has even been observed in specific brain structures, such as the frontal cortex, hypothalamus and brain stem in rats (Quay, 1968; Héry *et al*., 1972). Later, Héry *et al*. (1977) reported that tissue content of the 5-HT metabolite 5-HIAA in frontal cortex has a diurnal rhythm but with higher levels observed in the dark phase. This led them to postulate that 5-HT synthesis is high during the light phase but 5-HT release is increased during the dark phase (Héry *et al*., 1972; 1977). Whether this same dynamic applies to other brain regions is still matter of debate. Indeed high extracellular nocturnal levels of 5-HIAA have been measured in the SCN, cortex and hippocampus (Faradji *et al*., 1983; Weiner *et al*., 1992). However, extracellular 5-HT release in the hippocampus, striatum, amygdala and frontal cortex seems to be triggered during the transition from the light to

the dark phase, which also correlates with the onset of behavioral activity (Kalén *et al*., 1989; Reuter and Jacobs, 1996). More recent studies have shown that both 5-HT and 5-HIAA content increases at night in SCN tissue (Cuesta *et al*., 2009). Manipulations that alter 5-HT neurotransmission also alter clock gene expression, which led to the hypothesis of direct communication between the 5-HT system and the molecular clock in the SCN (Cuesta *et al*., 2009).

Changes in 5-HT tissue content and extracellular release can be also modified by the duration of the light phase. Ferraro and Steger (1990) showed that the daytime increase of 5-HT tissue content in the hamster hypothalamus and olfactory bulbs is dependent on photoperiod duration. They also demonstrated that in constant dark or in constant light conditions, 5-HT tissue content in the hypothalamus remains at relatively high levels in both conditions, with no apparent rhythm (Ferraro and Steger, 1990). In the hamster SCN and intergeniculate leaflet (IGL), 5-HT release is high during the dark phase while the animal remains awake; however, locomotor activity induced by exposure to a novel environment stimulates 5-HT release in the light phase but paradoxically suppresses it in the dark phase (Dudley *et al*., 1998; Grossman *et al*., 2003). This indicates a strong influence of behavioral activity on extracellular 5-HT levels, depending on the phase of the cycle.

In addition, diurnal changes in 5-HT receptor density have also been observed. Wesemann *et al*. (1986) reported a peak of 5-HT binding sites during the dark phase in the whole rat brain (Wesemann *et al*., 1986). However, diurnal 5-HT receptor density varies between discrete brain regions and 5-HT receptor subtypes. For example, a daytime increase of $5\text{-HT}_1$ binding sites, but

not 5-$HT_2$, has been observed in the rat cerebral cortex (Koshikawa *et al*., 1988; Akiyoshi *et al*., 1989). In the hippocampus and amygdala, mRNA expression of 5-$HT_{2C}$ receptors increased at the beginning of the light phase, while no diurnal variation was observed for 5-$HT_{1A}$ or 5-$HT_{2A}$ receptor subtypes in this brain areas (Holmes *et al*., 1995). Furthermore, binding sites for 5-$HT_{1A}$ receptors in the hippocampus and 5-$HT_2$ receptor in the cortex could also be influenced by season (Weiner *et al*., 1992).

In agreement with the potential existence of diurnal patterns in 5-HT content and release, a diurnal rhythm of the enzyme Tph has been detected in 5-HT producing regions such as the DRN and the MRN. In these two nuclei, Tph activity is increased at night, especially at the end of the dark phase (Kan *et al*., 1977; Cahill and Ehret; 1981). However, expression and activity of Tph seems to be the opposite in terminal areas such as the striatum and the intergeniculate leaflet (IGL) than in the raphe region (Kan *et al*., 1977; Malek *et al*., 2005). The highest levels of 5-HT tissue content in the DRN have been reported to occur also in the light phase (Semba *et al*., 1984; Cagampang *et al*., 1993). Birkett and Fite (2005) reported that intracellular (immunolabeled) 5-HT content is high during the light phase, decreasing at the onset of the dark phase to rise sharpely at the end of the dark phase. Extracellular levels of 5-HT in the DRN are also high in awake animals compared with animals during sleep (Portas *et al*., 1998).

All the evidence reviewed above suggests that brain 5-HT neurotransmission is strongly influenced by the light-dark cycle or more generally speaking by lighting conditions. However, it is not clear yet if changes in 5-HT neurotransmission are subject to a true regulatory endogenous rhythm or if this daily changes are trigered by, for example, the active phase of the animal. The

same issue affects studies of DA.

### 1.4.2 Diurnal rhythms in DA neurotransmission

DA neurotransmission also seems to follows a diurnal rhythm of activity that can be inferred from diurnal and ultradian variations detected in DA tissue levels in the mammalian brain (Scheving *et al.*, 1968a; Owasoyo *et al.*, 1979). Evidence for a diurnal change in the rate of DA synthesis came from studies showing diurnal variations of the enzyme TH in different brain regions. In the VTA and SNpc, the peak of TH mRNA and protein expression occurs during the light phase, whereas in the NAcc and caudate the peak is observed during the dark phase (Weber *et al.*, 2004; Sleipness *et al.*, 2007; Webb *et al.*, 2009). This suggested that DA synthesis in cell bodies and axon terminals follow different diurnal rhythms of activity similar to what has been observed for 5-HT (see previous section). In the NAcc and the caudate-putamen, not only DA but also its precursor L-DOPA and its metabolites HVA and DOPAC, have higher levels during the dark phase and lower levels during the light phase (Owasoyo *et al.*, 1979; O'Neill and Fillenz, 1985; Kafka *et al.*, 1986; Paulson and Robinson, 1994; Castaneda *et al.*, 2004). This indicates that synthesis, release and metabolism of striatal DA are increased at night. However, the increase of nocturnal DA neurotransmission in the striatum (Nacc and caudate-putamen) is strongly correlated with increased locomotor activity of the rat at night (O'Neill and Fillenz, 1985).

Early studies showed that $D_2$ receptor binding in the striatum of young rats follows an ultradian rhythm with peaks in the middle of the dark and light phases (Naber *et al.*, 1980). An ultradian rhythm was also observed by quantifying $D_2$ receptor mRNA levels in the mouse striatum

(Viyoch *et al.*, 2001). However, another study using the striatum from adult rats found a single peak of $D_2$ receptor binding in the middle of the dark phase (Torner *et al*., 1998). More recent studies have reported that $D_2$, but not $D_1$, receptor mRNA expression, decreases during the light phase and increases in the dark phase in the caudate-putamen of adult rats (Weber *et al.*, 2004). In the NAcc and in the prefrontal cortex, diurnal changes in DAT have been reported; the levels of this protein seems to be higher in the NAcc during the dark phase but lower in the prefrontal cortex (Sleipness *et al.*, 2007). However in both structures (NAcc and prefrontal cortex) DA clearance peaks four hours after the beginning of the light phase (Sleipness *et al.*, 2008).

There is evidence suggesting that VTA neurons could be influenced by an indirect control from the SCN (Luo *et al*., 2008; Luo and Aston-Jones, 2009). The SCN has also been proposed to influence, to some extent, the rhythmic expression of DA transporter (DAT) and TH in the VTA and NAcc (Sleipness *et al*., 2007; Webb *et al*., 2009; Weber *et al*., 2004). A local regulation of clock genes in DA neurons is also possible. Although no rhythmic pattern of expression of the core clock genes, *Per1* and *Bmal1*, have been observed in the VTA (Webb *et al*., 2009), the transcription factor CLOCK may have a regulatory role in VTA DA neuron firing activity (McClung *et al*., 2005). For example, *Clock* mutant mice express an inactive form of this protein and have an increased DA neuron firing rate and burst activity, as well as increased levels of TH in the VTA compared with wild type animals (McClung *et al*., 2005). Still, it is possible that the increased DA neurotransmission is a result of the characteristic hyperactivity of *Clock* mutant mice.

### 1.4.3 Monoamines diurnal rhythm in human studies

Studies of potential monoamine diurnal rhythms in humans have been largely restricted to monitoring changes in monoamines, their precursors and metabolites in plasma or CSF. The available data suggest that 5-HT synthesis (tryptophan and 5-HT levels) gradually increases during the day before decreasing at night, when 5-HT metabolism (5-HIAA levels) increases (Nicoletti *et al*., 1981; Sano *et al*., 1994; Rao *et al*., 1994; Kennedy *et al*., 2002). For DA, the most consistent pattern reported is a peak of plasma HVA concentration after midnight and lower levels observed after midday (Sack *et al*., 1998; Doran *et al*., 1990; Sano *et al*., 1994; but see Poceta *et al*., 2009). Interesting, diurnal HVA and tryptophan plasma levels seem to be altered in schizophrenic patients and affected with neuroleptic treatment (Davila *et al*., 1989; Doran *et al*., 1990; (Rao *et al*., 1994); Duncan *et al*., 2006). The plasma 5-HIAA rhythm is also altered in alcoholic patients with severe acute withdrawal delirium symptoms (Sano *et al*., 1994).

Certainly, more studies are necessary to better understand these findings, but the observations listed above indicate that alterations in monoamine diurnal rhythms could be associated with symptom severity of mental disease and reflect pharmacological treatment effects. Although plasma and CSF measurements are good non-invasive indexes of central monoamine function, they are strongly influenced by diet and do not necessarily reflect accurate diurnal changes in monoamine neurotransmission occurring in the brain (Fernstrom *et al*., 1979; Kendler *et al*., 1983; Palmour *et al*., 1998; Young, 2013).

In addition, other factors such as the photoperiod may have also an influence on central monoamine level readings. For example, a higher concentration of 5-HIAA in CSF and venous

blood has been observed in samples collected in conditions of higher daylight exposure; in spring-summer vs. fall-winter seasons and bright vs. dull days (Lambert *et al*., 2002; Luykx *et al*., 2012; but see Guthrie *et al*., 1986 and Hartikainen *et al*., 1991). These patterns have also been reported for 5-HT brain tissue concentration postmortem (Carlsson *et al*. 1980) and for 5-HTT and 5-$HT_{1A}$ receptor binding in brain imaging studies (Praschak-Rieder *et al*., 2008; Spindelegger *et al*, 2012). While 5-HT neurotransmission in the brain seems to increase with more daylight exposure, DA neurotransmission seems to increase in the opposite conditions. For example, higher HVA content in CSF (Losonczy *et al*., 1984; Hartikainen *et al*., 1991) and increased DA concentration in human hypothalamic tissue has been reported during fall-winter seasons (Karson *et al*., 1984). In brain imaging studies, an increased uptake of DOPA in striatum has been observed during fall-winter (Eisenberg *et al*., 2010), while DA $D_2/D_3$ receptor availability seems to be higher during spring-summer seasons (Tsai *et al*., 2011).

The evidence reviewed above, in particular from brain imaging studies, supports the existence of diurnal rhythms in brain monoamine neurotransmission in humans. This highlights the importance of knowing how these rhythms are generated to understand the potential implications of these rhythms in normal functions and mental disorders in which monoamine neurotransmission has been implicated. It is then necessary to implement a methodology to assess changes in brain monoamine neurotransmission using a marker that represents the dynamics occurring in the system in a daily basis.

## 1.5 Methodological considerations

In the following sections the methodology used in our experiments is reviewed, with an emphasis on its advantages and potential limitations. In brief, though, single unit extracellular recording methods are well established, and have been used extensively by our research group and others to study monoamine neurotransmission. The advantage of this technique is that neuronal activity in monoaminergic nuclei (*e.g.,* DRN and VTA) reflects gross changes occurring in a given monoamine system reported as changes in cell firing frequency. This is due to existing regulatory feedback mechanisms within the monoamine systems. As such, adoption of this method to assess diurnal rhythms of activity in monoamine producing neurons opens the possibility of using this approach to gain new insights into monoaminergic neurotransmission in normal states, disease and in response to drugs across the 24 hours of the day.

### 1.5.1 Single unit extracellular recordings of monoaminergic neurons

Single-unit extracellular recording is an electrophysiological technique used to the measure voltage changes (*i.e.,* action potentials, spikes) that occur in the extracellular space surrounding an individual neuron during its discharge or firing activity. In neurons, the discharge activity of the cell is generated by the flow of ions between the inside and the outside of the cell membrane (Kress and Mennerick, 2009). This flow of ions generates an electrochemical gradient and voltage differences that increase the membrane conductivity and are responsible for the generation of the neuronal action potential (Kress and Mennerick, 2009). By introducing a conductive material near the action potential field of a neuron, it is possible to capture a signal generated by the cell's electrochemical activity (Humphrey and Schmidt, 1990). Typically an action potential is characterized by one ascending (positive) and one descending (negative)

phase, in which the amplitude and spike waveform (positive or negative first; biphasic or triphasic action potentials) can change depending on the shape of the cell and the region of the cell to which the electrode is near (Humphrey and Schmidt, 1990; Stuart *et al.*, 1997). Amplification of this signal allows the visualization of action potentials in the form of spikes that can be quantified per unit of time as firing frequency or firing rate. Single-unit extracellular recordings can be performed *in vivo* using anesthetized and awake freely moving animals. Therefore, this technique provides unique information about the electrical activity of a particular population of neurons (since many neurons can be recorded by moving the electrode through the brain region of interest) within an intact neuronal circuitry (*i.e.,* intact brain).

### 1.5.2 Functional analysis of neuronal firing activity

In the classical analysis of the firing activity, the firing rate represents the first parameter to be analyzed. This is achieved by counting the number of neuronal discharges by units of time. In this way, if the unit of time is a second (spikes/sec), the frequency of discharge can be reported in Hz, although, sometimes the firing rate can be reported in 10 sec bins to better visualize changes in firing rate across minutes or even hours. The time between the occurrence of one and another spike is also a parameter of the neuronal firing activity. In this case, the interspike interval (ISI) is used to construct histograms that allow for the analysis of the regularity of neuronal firing and the distribution of the ISI (Kostal *et al.*, 2007). Despite the fact that the ISI and the firing rate yield similar information about the neuronal activity (by definition the firing rate or frequency is the reciprocal of the ISI or period), the ISI is useful to determine other important parameters of the firing activity, such as the burst activity (Hajos *et al.*, 2007). In general, there are two basic patterns of neuronal firing activity, single and burst firing. A burst is

defined as a consecutive train of single spikes fired a higher frequency, with short ISI, followed by a relative long ISI. Burst activity is detected using ISI onsets and offsets that depend on the burst firing characteristics of the particular neuronal population under analysis (Grace and Bunney, 1983).

Finally, another consideration of neuronal activity, which is not a parameter related with the intrinsic firing properties of the neurons, is to count the number of spontaneously active neurons per electrode descent, or "neurons per track." This is achieved by sampling the brain region under analysis several times by moving, each time, the point of insertion of the electrode (in the sagittal or anterior-posterior axis). In addition to firing activity parameters, the number of neurons per track provides information about the relative population of neurons that are silent or stimulated after an experimental manipulation (Bermack and Debonnel, 2001; Bambico *et al.*, 2009).

### 1.5.3 Identification of monoaminergic neurons

To facilitate the recording and identification of monoaminegic neurons, in vivo extracellular recordings are usually restricted to the nuclei in which they are more abundant and in the regions where they represent a major percentage of the neuronal population. For example, in the DRN a higher density of 5-HT neurons is found along the brain's midline compared with most lateral parts the DRN where the population of GABA neurons increases (Descarries *et al.*, 1982; Allers and Sharp, 2003; Calizo *et al.*, 2011). In the VTA, DA neurons are intermixed with other neuronal population (e.g. glutamatergic and GABA neurons), particularly in the rostral and medial parts closer to brain's midline (Nair-Roberts *et al.*, 2008; Ungless and Grace, 2012).

However, in the central and lateral regions of the VTA the percentage of DA neurons is higher, so there are more chances of recording DA neurons in these regions (Nair-Roberts *et al.*, 2008; Ungless and Grace, 2012).

Identification of monoaminergic neurons during *in vivo* extracellular recordings is achieved primarily by using well establish electrophysiological parameters (Allers and Sharp, 2003; Ungless and Grace, 2012). Action potentials of DA and 5-HT cells can be visualized as biphasic or triphasic spikes of long duration, > 1.5 ms from start to trough, always with an initial positive rising segment (the total duration of the action potentials ranged from 2 - 9 ms). Both monoamine populations have a slow frequency of discharge, but DA neurons have a slightly higher firing rate (0.5–10 Hz) than 5-HT neurons (0.1–3.5 Hz) (Aghajanian *et al.*, 1968; Grace and Bunney, 1983; Allers and Sharp, 2003). 5-HT neurons fire with a prominently regular (clock-like) firing rate and occasionally in small bursts of two or three spikes (Hajos *et al.*, 2007). DA neurons can display regular and irregular firing patterns; the irregular is a characteristic burst-firing pattern consisting of intermittent trains of action potentials with a progressive decrease in amplitude (Ungless and Grace, 2012). Further identification of monoaminergic neurons can be accomplished by systemic administration or microiontophoretic application of pharmacological agents known to affect the firing activity of monoaminergic neurons. The most used strategy is the utilization of drugs that activate autoreceptors at the somatodendritic level which induce an inhibition of neuronal firing activity. For example, APO is used to identify DA neurons trough $D_2$ autoreceptor activation, while 8-OH-DPAT is commonly used to identify 5-HT neurons through 5-$HT_{1A}$ autoreceptor activation (Haigler and Aghajanian, 1974; Sprouse and Aghajanian, 1987; Ungless and Grace, 2012).

Neurocytochemical techniques, such as juxtacellular labeling, provide an additional way to identify monoaminergic neurons (Grace and Onn, 1989; Pinault, 1996). For this, recorded neurons are microinjected with markers such as Biocytin or Neurobiotin and then the tissue is processed by double-labeling with a cellular marker to determine monoamine neuron phenotype for example TH, DAT, Tph, 5-HTT, or DA and 5-HT themselves (Allers and Sharp, 2003; Hajos *et al.*, 2007; Ungless and Grace, 2012).

### 1.5.4 Monoaminergic activity and vigilance state

As we mentioned above, single unit extracellular recordings can be performed in anesthetized or freely moving animals. At first glance, a methodological issue that may arise using *in vivo* preparations with anesthetized animals is that changes in neuronal activity associated with the active behavioral phase of the animal (*i.e.,* dark phase in most laboratory rodents) and sensory stimulation perception (*i.e.,* phasic neuronal activation) will not be observed due to the anesthetic effect. This may also be considered a methodological constraint in assessing diurnal (or circadian) rhythms of monoaminergic neuronal activity; however several reasons supporting its use in unmasking rhythms in neuronal activity are described below.

It is quite established that the firing activity of 5-HT neurons in the DRN, and of NE neurons in the LC, changes depending on the vigilance state of the animal. In this way, NE neurons display a higher firing frequency when animals are awake, a decreased their firing rate during non-REM sleep (i.e. slow wave sleep in humans, SWS) and become practically silent during REM sleep (i.e. paradoxical sleep) (Foote *et al*., 1980; Aston-Jones and Bloom, 1981; Rasmussen *et al*., 1986). In the DRN, similar changes has been observed in 5-HT cell firing across the different

vigilance states; awake > non-REM sleep > REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1983; Gervasoni *et al*., 2000). In addition, a 5-HT neuronal population in which firing activity remains unchanged across the sleep-wake cycle and other populations that fire at higher frequencies during sleep stages (non-REM and REM) have also been reported (Sakai and Crochet, 2001; Urbain *et al*., 2006). In contrast, DA cell firing rate in the VTA and SNpc is not affected by the sleep-wake cycle but it has been observed that some DA neurons in the VTA increase their burst firing activity during REM sleep (Miller *et al*., 1983; Dahan *et al*., 2007). As the studies mentioned about have shown, transitions from one stage of sleep to another and arousal could influence the level of activity in monoamine neurons and should be considered as a potential confounding factor in the study of diurnal rhythms of activity in these neurons.

The sleep-wake cycle is not the only factor that affects monomine neurons firing activity. For example, in rodents, a phasic increase of firing rate and burst activity in NE neurons located in the LC is associated with increased behavioral activity (i.e. movement, feeding, grooming) and sensory stimulation (i.e. auditory, visual, touch stimulation or food presentation) (Foote *et al*., 1980; Aston-Jones and Bloom, 1981; Rasmussen and Jacobs, 1986; Takahashi *et al*., 2010). In the VTA and SN, DA neurons also increase their firing activity in response to sensory and natural reward stimuli and as a result of increased behavioral activity (Freeman and Bunney, 1987; Schultz, 1998; Puryear *et al*., 2010; Wang and Tsien, 2011). On the other hand, 5-HT DRN neurons display a phasic increase in firing rate in response to sensory stimulation; however this response does not seem to be associated with gross behavioral activity (McGinty and Harper, 1976; Trulson and Jacobs, 1979, Heym *et al*., 1982; Sakai and Crochet, 2001).

Therefore, any intrinsic rhythm in the activity of monoaminergic neurons will be always masked by fluctuations between awake, non-REM and REM sleep or sensory stimulation and behaviors displayed during the animal active phase. To avoid this problem the ideal setting would be to record animals in a uniform state of vigilance across the 24 hours of the light-dark cycle, stripped from all external influences. Indeed, circadian rhythms research in humans led to the development and use of protocols in which not only periodic environmental cues are removed but also periodic changes in behavior (Minors and Waterhouse, 1984). These constant routine protocols are aimed to minimize behavioral feedback that can modify endogenously generated diurnal and circadian rhythms (Duffy and Dijk, 2002). However, constant routine protocols may have a limited application in animals and probably have to involved stressful restraining procedures. To circumvent these limitations some researchers have turned to the use of anesthetized animals (Aston-Jones *et al*., 2001).

### 1.5.5 Monoaminergic activity and anesthesia

Slow wave sleep (non-REM sleep in rodents) is considered a state in which only internally generated activity takes place and the brain is disconnected from the external world (Steriade, 2000). This loss of consciousness can be induced with anesthetics, including those that modulate $GABA_A$ receptors such as chloral hydrate (Sloan, 1998; Steriade 2000). Indeed, EEG recordings in chloral hydrate anesthetized rats are characterized by a predominance of slow delta waves (0.5-4.0 Hz), as is observed in rats during natural non-REM sleep (Lu *et al*., 2008). Although cortical activity is slightly affected by anesthesia, most cortical functions remain unaffected. For this reason, anesthetized animals have been extensively used in the study of sleep physiology (Sloan, 1998; Steriade 2000). In humans, chloral hydrate is used to induce sleep in patients prior

to EEG recordings and neuroimaging procedures of diagnostic value (Low *et al*., 2008; Briton and Kosa, 2010; Sezer and Alehan, 2013).

Recently it has been proposed that the mechanism by which chloral hydrate and other GABAergic agents induce loss of consciousness is by activating the ventrolateral preoptic nucleus (VLPO) and inhibiting the tuberomammillary nucleus (TMN), areas involved in non-REM sleep (Lu *et al*., 2008). In addition, in electrophysiological studies it has been documented that basal firing activity of DA and 5-HT neurons are only slightly affected by chloral hydrate anesthesia (Aghajanian *et al*., 1972; Bunney *et al*., 1973; Trulson and Trulson, 1983 Kelland *et al*., 1989; McCardle and Gartside, 2012).

In any case, monoaminergic firing activity in chloral hydrate anesthetized rats reaches a homogenous level of activity similar to what is found in non-REM sleep. In these conditions, it will be possible to unmask any existent intrinsic diurnal rhythm in the firing activity of monoaminergic neurons, free of confounding factors such as the sleep cycle, sensory stimulation and behavioral activity. Using this same rationale, in studies published in Nature Neurosciences and the European Journal of Neuroscience, Aston-Jones's research group was able to unveil a diurnal rhythm in firing activity of NE neurons located in the locus coeruleus and a neuronal population in the VTA (non-DA) which selectively fired during the dark phase of rats under halothane anesthesia (Aston-Jones *et al*., 2001; Luo *et al*., 2008, Luo and Aston-Jones 2009).

## 1.6 Research hypothesis and objectives

This chapter has introduced basic concepts in biological rhythms that identify the neurohormone MLT as one of its key components influencing the generation of endogenous rhythms. The neuroanatomy and neurophysiology of the 5-HT and DA systems has been reviewed as well as their importance as targets for antidepressants and psychostimulants. A large body of literature has also been presented, generated from findings in rodents and humans, suggesting the existence of diurnal rhythms of 5-HT and DA neurotransmission. The utility of single-unit extracellular recordings as a standardized methodology to assess 5-HT and DA neuronal firing activity in anesthetized rats has been reviewed and discussed. Importantly, changes in DA and 5-HT neuronal activity during the sleep-wake cycle, sensory stimulation and behavioral activity have been identified as confounding factors that complicate attempts to elucidate diurnal rhythms.

### *General hypotheses*

We hypothesized that diurnal rhythms of monoaminergic neurotransmission can be observed in the neuronal populations synthesizing and controlling the release of monoamines in rats. These populations are represented by 5-HT neurons in the DRN and DA neurons in the VTA. We proposed that diurnal changes in the firing activity of these neurons recorded from anesthetized animals potentially represent an endogenous rhythm of activity. We also hypothesized that the hormone MLT, as a messenger of the photoperiod and circadian clock system, can potentially modulate monoamine neurotransmission. Finally, we predicted differences in monoamine neuronal response to the acute effects of pharmacological agents between discrete time intervals across the 24 h of the light-dark cycle.

***General objectives***

This research project sought primarily to identify and characterize diurnal rhythms of firing activity in 5-HT neurons located in the DRN and DA neurons located in the VTA using in vivo single-unit extracellular recordings in anesthetized rats. The basal activity of 5-HT and DA neurons was recorded in different intervals of the light-dark cycle and the data were analyzed with standard and specialized statistical methods to determine the existence of diurnal rhythms. We assessed the effects of MLT administration on both 5-HT and DA neuronal firing activity across the light-dark cycle. The effect of endogenous MLT secretion was evaluated in the firing activity of 5-HT neurons. We determined the existence of time of day differences in the response (sensitivity) of DA neurons to two drugs that affect DA: the prototypical $D_1$-$D_2$ receptor agonist APO and the psychostimulant AMPH. These two drugs were chosen for their well documented inhibitory effect in midbrain DA neurons firing activity and to compare their different mechanism of action between different times of the day. Data from the APO challenge assessed the diurnal response of DA neurons to direct $D_2$-like autoreceptors. The challenge with AMPH also assessed the response of $D_2$-like autoreceptors but in response to an overflow of DA which also involved a long-inhibitory feedback component. As an additional set of experiments, the effects of APO and AMPH on behavior across the light-dark cycle were also explored.

***Specific objectives***

The experiments described in Chapter 2 have been published in the Journal of Psychopharmacology (Domínguez-López *et al*., 2012b) and had the following objectives:

- To characterize a putative diurnal rhythm of firing activity of 5-HT neurons located in the DRN.
- To compare the acute effect of different doses of exogenous MLT administration on the firing activity of 5-HT neurons and determine an effective dose.
- To compare the effects of MLT administration on 5-HT firing activity after the blockage of MLT receptors.
- To determine the acute effect of the suppression of MLT endogenous secretion by removal of the pineal gland on 5-HT neuron firing activity.
- To compare the acute effects of MLT administration and pinealectomy in the firing activity of 5-HT neurons across the light-dark cycle.

The experiments described in Chapter 3 have been peer-reviewed and conditionally accepted for publication in Synapse (Domínguez-López *et al*., 2014) and had the following objectives:

- To characterize a putative diurnal rhythm of firing activity of DA neurons located in the VTA.
- To compare the acute effects of different doses of MLT in the firing activity of DA neurons across the light-dark cycle.
- To assess the acute effects of different doses of the direct $D_1$-$D_2$ agonist APO on DA neurons firing rate at different time intervals of the light-dark cycle.
- To assess the acute effects of different doses of the indirect DA receptor agonist AMPH on DA neurons firing rate at different time intervals of the light-dark cycle.

Finally, the experiments described in Chapter 4 are unpublished and aimed to characterize behavioral effects of APO and AMPH administration at different points of the light-dark cycle.

# Foreword to Chapter 2

The following chapter describes the first electrophysiological experiments performed in anesthetized rats to characterize the basal firing activity of 5-HT neurons located in the DRN across the light-dark cycle and to determine the influence of MLT on 5-HT firing activity. Following several lines of evidence suggesting that the 5-HT system is modulated by light environmental conditions (section 1.4.1), we first compared changes in the firing activity of 5-HT neurons in the light vs. the dark phase. In a second analysis of the data, we grouped the recordings in time intervals of four hours and determined the rhythmic nature of the changes in 5-HT neurons activity. In experiments carried out in parallel, the effects of i.v. administration of MLT on the firing activity of 5-HT neurons were tested. For this we first constructed a dose-response curve to determine the effective dose of MLT able to modify 5-HT firing activity. Further, we explored the contribution of MLT receptors to the effects of MLT on 5-HT firing activity. The converging evidence that MLT administration decreased 5-HT neurons firing activity and that 5-HT neurons recorded at night have a decreased activity, led us to speculate that endogenous MLT secretion could have an influence on 5-HT neurons activity. To determine the influence of endogenous MLT secretion on 5-HT neurons activity, we developed a technique that allowed recording of 5-HT neurons in the DRN immediately after removal of the pineal gland (pinealectomy, PX). Considering the short half-life of MLT (approximately 20 minutes in the rat, section 1.1.2), we expected to observe the effects of endogenous MLT suppression on 5-HT neurons after two or three MLT half-lives have passed (approximately 40 to 60 minutes). The effects of MLT administration and PX on 5-HT neuronal activity were also compared at different times of the light-dark cycle, since neurons in other brain regions (e.g. SCN) have

shown a time sensitive window to the effects of MLT (section 1.1.7). This chapter is based on the paper "Short-term effects of melatonin and pinealectomy on serotonergic neuronal activity across the light-dark cycle" published in the Journal of Psychopharmacology in June 2012. The final published version of the paper can be found at: http://jop.sagepub.com/content/26/6/830. Additional data have been inserted in this updated version of the manuscript including the COSINOR analysis described in sections 2.2.4 and which results are shown in section 2.4.2 and in Figure 2. This additional analysis does not change the conclusions drawn in the original published paper and was inserted to have a uniform data analysis of the diurnal activity in 5-HT and DA neurons.

# Chapter 2

# Short-term effects of melatonin and pinealectomy on serotonergic neuronal activity across the light–dark cycle

Sergio Domínguez-López, Ian Mahar, Francis Rodriguez Bambico, Benoit Labonté, Rafael Ochoa-Sánchez, Marco Leyton and Gabriella Gobbi

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**ABSTRACT**

Melatonin (MLT) and serotonin (5-HT) are two biosynthetically related compounds implicated in several common physiological functions and the etiology of mood disorders. How they interact, though, is not yet fully understood. In this study, single-unit extracellular recordings were used to monitor dorsal raphe nucleus (DR) 5-HT neuronal activity in anesthetized rats, under basal conditions (CTRL), in response to MLT administration, and after pinealectomy (PX) across the light–dark cycle. Under basal conditions, the number of spontaneously active 5-HT neurons and their firing rate were both significantly lower in the dark phase. In the light phase, administration of MLT at low doses (0.5–1 mg/kg, i.v.) decreased 5-HT firing activity. This inhibitory effect of MLT was completely blocked by the $MT_1/MT_2$ receptor antagonist luzindole, but not by the selective $MT_2$ receptor antagonist 4P-PDOT, the selective 5-$HT_{1A}$ receptor antagonist WAY100635, or by the $a_2$ adrenoceptor antagonist idazoxan. In the opposite experiment, PX increased 5-HT firing activity in the dark phase, and this was reversed by MLT administration (1 mg/kg, i.v.). Finally, in a forced swim test, MLT (1 mg/kg, i.p.) increased immobility time and decreased swimming behavior. Together, these results suggest that nocturnal MLT secretion imposes tonic inhibitory control over a sub-population of DR 5-HT neurons. This MLT-induced decrease in 5-HT neurotransmission may represent a biological mechanism underlying mood disorders characterized by increased MLT secretion, such as seasonal affective disorder.

## 2.1 Introduction

The neurohormone melatonin (*N*-acetyl-5-methoxytryptamine, MLT) is secreted by the pineal gland during the dark phase and is considered the main endocrine signal for photoperiod duration (Simonneaux and Ribelayga, 2003). MLT is synthesized from 5-HT by a mechanism that involves the sequential activity of two enzymes, N-acetyltransferase and hydroyindole-O-methyltransferase, within a pathway that includes N-acetyl-serotonin as an intermediary product (Simonneaux and Ribelayga, 2003). MLT secretion reflects circadian and seasonal changes of the photoperiod that are translated to endocrine and neuronal signals via the G protein-coupled membrane receptors $MT_1$ and $MT_2$, which are widely expressed in the mammalian brain (Dubocovich and Markowska, 2005).

MLT has been implicated in the etiology of seasonal affective disorder (SAD), a type of depression with periodic occurrence (Srinivasan *et al.*, 2006). The seasonal pattern and high incidence of SAD in populations living at latitudes where daylight length changes markedly across seasons plus the clinical efficacy of phototherapy have suggested that SAD is linked to photoperiod duration (Rosenthal *et al.*, 1984; Rosen *et al.*, 1990). One hypothesis is that increased MLT secretion occurring during the short photoperiods of the fall and winter seasons is a triggering factor for SAD (Pacchierotti *et al.*, 2001; Srinivasan *et al.*, 2006). Indeed, prolonged or increased secretion of MLT has been observed in patients with SAD (Terman *et al.*, 1987; Karadottir and Axelsson, 2001; Wehr *et al.*, 2001). Together, these observations raise the possibility that MLT rhythm could be influencing neurotransmitter systems related to mood regulation.

The best-studied neurotransmitter implicated in mood regulation and the mechanism of antidepressant efficacy is 5-HT (Belmaker and Agam, 2008). Indeed, antidepressant therapies, including monoamine oxidase inhibitors, tricyclic antidepressants and selective 5-HT reuptake inhibitors (SSRI), each act by increasing synaptic 5-HT availability and neurotransmission (Delgado *et al.*, 1990; Piñeyro and Blier, 1999; Bambico and Gobbi, 2008). For this reason, impairment of 5-HT neurotransmission is considered one of the principal causes of a depressive state (Brodie *et al.*, 1955; Owens and Nemeroff, 1994; Young and Leyton, 2002).

In the brain, the largest locus of 5-HT-producing neurons is the DRN (Dahlström and Fuxe, 1964; Descarries *et al.*, 1982). The DRN sends axons to forebrain regions such as the hypothalamus, striatum and nucleus accumbens, in which a circadian variation of 5-HT content, receptor expression and receptor binding has been reported (Kan *et al.*, 1977; Wesemann *et al.*, 1986; Akiyoshi *et al.*, 1989). Particularly in DRN, intracellular 5-HT content has been observed to reach its lowest and highest levels at the beginning and end of the dark phase, respectively (Birkett and Fite, 2005). Whether diurnal oscillations in 5-HT neurotransmission are under the influence of endogenous MLT secretion is still unknown. Furthermore, although MLT administration increases 5-HT tissue content in the midbrain and other brain areas (Anton-Tay *et al.*, 1968; Miguez *et al.*, 1997), it is unknown whether this effect is due to an accumulation of 5-HT by inhibition of its release or if MLT induce *de novo* synthesis of 5-HT.

As the influence of MLT over 5-HT neurotransmission is poorly understood, the main objective of this study was to characterize the effects of exogenous MLT administration and suppression of endogenous MLT secretion by pinealectomy (PX) on DRN 5-HT firing activity across the light-

dark cycle. In addition, we explored which MLT receptors could be involved in the hypothesized effects, and whether MLT administration induces behavioral changes in the forced swim test (FST), an animal paradigm to test antidepressant-like effects of putative drugs.

## 2.2 Materials and Methods

### 2.2.1 Animals

Adult male Sprague Dawley rats (Charles River, Saint-Constant, Quebec, Canada) weighing 300 - 330 g were housed under standard laboratory conditions with a 12/12h light-dark cycle (lights on at 0700 h) with *ad libitum* access to food and water. Experiments performed in the dark phase were conducted under dim red light provided by an infrared (IR) heating lamp (Philips, Infrared Heat, wavelength peak: 800-1200 nm). All procedures were in accordance with the guidelines set by the Canadian Institute of Health Research for animal care and scientific use. Approved protocols by the Animal Care Committee of McGill University are inserted in the Appendix.

### 2.2.2 Drugs

Desipramine (DMI) and MLT (Sigma-Aldrich, Oakville, ON, Canada) were dissolved in a vehicle (VEH) of 30% propyleneglycol (MP Biomedicals, Solon, OH, USA) in physiological saline solution (saline, NaCl 0.9%). Luzindole and 4P-PDOT (Tocris, Ellisville, MO, USA) were dissolved in propylene glycol. Chloral hydrate, WAY100635 and idazoxan (Sigma-Aldrich, Oakville, ON, Canada) were dissolved in saline. All drugs were freshly prepared the day of the experiments and just before being used. In electrophysiological experiments, intravenous (i.v.) administration was carried out using a catheter inserted into the lateral vein of the tail. The maximum volume used in electrophysiological recordings for a single i.v. injection was 0.1 ml

(infused in approximately 1 min). In the forced swim test, drugs were injected via intraperitoneal (i.p) in a volume of 0.5 ml.

### 2.2.3 Single-unit extracellular recordings

Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.) in their housing room. For experiments performed during the dark phase, anesthesia was given under IR illumination (GE, Infrared Reflector, wavelength peak: 800-1200 nm) and a black duct tape was used to cover the eyes of the rats (rats remain this way during the whole recording procedure). Animals were transported in light-tight cages to the procedural room. Surgeries and electrode placement were done with stereo microscope assistance and a fiber optic dim light source with an integrated red light filter, which was used during night time recordings. Rats were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and a hole was drilled through the skull using stereotaxic coordinates described in the rat brain atlas by Paxinos and Watson (2007). For the DRN stereotaxic coordinates are; A-P: 0.8 to 1.2 mm from the interaural line; lateral: 0 ± 0.2 mm from the midline; ventral: 5 to 6.7 mm from the brain surface. Body temperature of the animals was measured using a rectal thermometer (Yellow Springs Instrument Co., Yellow Springs, OH, USA) and was maintained at 35-36.5 °C using a IR heating lamp (Philips, Infrared Heat) which also provided dim red light illumination.

Single-barrel glass micropipettes (Harvard Apparatus, St-Laurent, QC, Canada), filled with 2% pontamine sky blue dye (Sigma-Aldrich, Oakville, ON, Canada) dissolved in sodium acetate (0.5 M, pH 7.5) and with resistances ranging from 6 to 9 MΩ, were descended into the DRN using a hydraulic micropositioner (David Kopf Instruments, Tujunga, CA, USA). Spontaneous electrical

activity of single cells was recorded using an MDA-3 amplifier system (Bak Electronics, Mount Airy, MD, USA) connected in parallel to an oscilloscope (B&K Precision, Yorba Linda, CA, USA) and a sound system for continuous monitoring. The analog signal was then converted into a digital signal via a 1401 Plus interface (CED, Cambridge, UK). Spikes frequency and waveform were collected and stored in a computer using the Spike2 software (CED, Cambridge, UK) for offline analyses.

Putative 5-HT neurons were identified according to the following criteria: a slow (0.1–3.5 Hertz, Hz) and prominently regular (clock-like) firing rate, with a broad positive action potential (0.8–3 milliseconds, ms) (Aghajanian *et al.*, 1968; Allers and Sharp, 2003). Neuronal activity was measured by calculating the mean firing rate frequency, expressed as the number of spikes per second or Hz, and the number of spontaneously active neurons per electrode descent or track (neurons/track). Additionally, the burst activity of 5-HT neurons was analyzed using a script for the Spike 2 software (available on line at www.ced.co.uk). Based on criteria previously described (Gobbi *et al.*, 2005; Hajos *et al.*, 2007), a burst was defined as a train of at least two spikes with an initial interspike interval $\leq$ 20 ms and a maximum interspike interval of 40 ms, within a regular low-frequency firing pattern. The parameters analyzed with the Spike 2 script included the percentage of spikes in burst, the number of spikes per burst, the mean burst interspike interval (ISI), and mean burst length; the latter two parameters were expressed in ms. When neurons with irregular burst pattern were detected, the coefficient of variation was calculated as the ratio of the standard deviation to the mean ISI (i.e., the lower the value, the more regular is the firing activity) (Urbain *et al*., 2006). In the final track, pontamine sky blue dye was injected iontophoretically by passing a constant positive current of 20 μA for 5 min

through the recording pipette to mark the recording site. Recordings were performed only in the medial DRN, containing substantively more 5-HT neurons than its lateral segments (Descarries *et al.*, 1982).

### 2.3 Experimental Design

#### 2.3.1 5-HT firing activity in the dorsal raphe nucleus across the light-dark cycle

In order to determine the basal 5-HT neuronal activity across the light-dark cycle, we performed a series of single-unit extracellular recordings in the DRN. We assigned several groups of rats to be recorded in one of six time intervals across the light-dark cycle, three intervals belonging to the light phase (0700-1100 h, 1100-1500 h and 1500-1900 h) and three belonging to the dark phase (1900-2300 h, 2300-0300 h and 0300-0700 h). At least one full electrode descent was performed per rat and the number of 5-HT neurons spontaneously firing and their firing rate were analyzed. The neurons were classified according with their firing pattern in single-firing neurons and burst-firing neurons. Burst activity was also analyzed. In a first analysis, recordings were grouped according with the phase of the cycle in which they were performed, to compare 5-HT neuronal firing activity between dark and light phases. To identify changes in 5-HT neuronal firing activity across time, a second analysis was performed comparing data within the six time intervals across the light-dark cycle in which recordings were performed. In addition, a COSINOR analysis (non-linear curve fitting analysis) was also performed on the data to identify rhythms in 5-HT neuronal firing activity. Based on methodology described elsewhere (Barnett and Dobson, 2010; Lentz, 1990), mean values of firing rate and neurons per track were used to fit the equation: $y = M + a \cos(2\pi x/Prd) + b \sin(2\pi x/Prd)$, where $M$ is the MESOR (Midline Estimating Statistic of Rhythm, mean of the sample), $a$ and $b$ are the coefficients to be calculate,

*Prd* is the fixed period of the cycle, *x* is a time point in the cycle, and *y* is the value of the firing activity parameter under evaluation.

### 2.3.2 Administration of exogenous MLT and 5-HT neuron firing activity

In another set of experiments, we performed a series of recordings to assess the effect of exogenous administration of MLT on DRN 5-HT firing activity. A dose-response curve was assessed by injecting distinct groups of rats, each with a different i.v. dose of MLT (0.1, 0.25, 0.5, 1, 5, 10 and 20 mg/kg) or VEH (10 rats). This range of systemic MLT doses has been reported to have a plasma half life of approximately 20 min in rats (Gibbs and Vried 1981: Yeleswaram *et al*., 1997; Venegas *et al*., 2012). In a group of rats, before MLT or VEH administration, a complete single electrode descent (track) was performed in order to record neurons under physiological conditions; these neurons were considered to be control recordings (CTRL) and were used to compare the sub-acute effects of MLT (see below). To characterize the acute effects of MLT, a 5-HT neuron with a stable firing rate of approximately 1 Hz was selected and recorded for at least three min in basal conditions. One of the doses of MLT (or VEH) was then injected, and the firing rate of this neuron was monitored until a response was observed for up to 30 min. This was based on the reported effect of MLT on other neuronal populations, such as neurons in the mesencephalic reticular formation, in which a response is observed within 10 min and last up to 1 h post administration (Pazo, 1979). Neuronal responses were classified as increased, decreased or no response, with a change of at least 40% from basal firing rate sustained over a period of at least 10 min. After the characterization of the acute effect, one or two additional tracks were performed in order to assess the post-injection (sub-acute) effects evaluating the 5-HT firing rate and the number of spontaneously active 5-HT neurons within one

hour after MLT administration.

### 2.3.3 Antagonism of MLT administration

After an effective dose of MLT was assessed, we explored, in a separate group of rats, the contribution of specific receptor subtypes in their influence of MLT over 5-HT neuronal firing in the DRN. We used the 5-$HT_{1A}$ receptor antagonist WAY100635 (WAY, 100 µg/kg) (Haddjeri and Blier, 2000), the $\alpha_2$ adrenoceptor antagonist idazoxan (IDA, 100 µg/kg) (Freedman and Aghajanian, 1984). The non-selective MLT receptor antagonist luzindole (LUZ, 1 mg/kg; $MT_1/MT_2$ affinity ratio = 16 - 26), and the $MT_2$ selective receptor antagonist 4P-PDOT (1 mg/kg; $MT_1/MT_2$ affinity ratio = 66 - 22,000) (Dubocovich *et al*., 2010). In the literature, luzindole and 4P-PDOT have been used in behavioral testing at doses of approximately 10 mg/kg and 3 mg/kg (intraperitoneal), respectively (Dubocovich *et al*., 1998). However, luzindole at doses of 10 and 30 mg/kg (intraperitoneal) is reported to induce an antidepressant effect in the forced swimming test, while a dose of 1 mg/kg have no such effects (Dubocovich *et al*., 1990). Taking in account that the molecular weight of these two compounds is close to the one of MLT (MLT = 232.28 g/mol., luzindole = 292.38 g/mol and 4P-PDOT = 279.38 g/mol), we decided to used a 1 to 1 dose to antagonized MLT intravenous administration (1 mg/kg). Each antagonist was tested separately in a single neuron per rat and was injected i.v. at approximately 5 min intervals in the following sequence: VEH→antagonist→MLT.

### 2.3.4 Suppression of endogenous MLT

To investigate the short-term effect of endogenous MLT depletion, a group of rats underwent PX and 5-HT firing activity in the DRN was recorded and compared to rats that received sham

surgery (SHAM). Removal of the pineal gland was performed based on the procedure described previously by Kuszak and Rodin (1977). Briefly, chloral hydrate-anaesthetized rats were placed in a stereotaxic apparatus. Then an incision was made on the midline of the scalp to expose the lambdoid and sagittal sutures of the skull. A hole of approximately 0.75 cm in diameter was drilled through the skull to expose the superior sagittal vein (SSV) and the lateral sinus confluence. An incision was then made at the lateral edges of the SSV and 5-0 gauge surgical threads (Ethicon, Ontario, Canada) were passed under the SSV to doubly ligate it. The SSV was then bisected and retracted to the posterior sinus confluence. The pineal gland was identified beneath the sinus confluence and removed using forceps. In the SHAM group, the surgery consisted of identical procedures as described for the PX group without the removal of the pineal gland.

Immediately after PX or SHAM surgery, single-unit extracellular recordings of neurons were performed (through the same opening used to extract the pineal gland), and 5-HT firing rate and number of spontaneously active 5-HT neurons per track were quantified for up to three hours after surgery. This procedure was chosen to avoid possible long-term compensatory mechanisms of MLT secretion by other regions (i.e., the Halderian gland or the gut) (Huether, 1993), and feedback compensatory mechanisms occurring at the level of 5-HT neurotransmission (i.e. 5-$HT_{1A}$ autoreceptor regulation) (Piñeyro and Blier, 1999), which could mask the effect of pineal MLT suppression. 5-HT neural activity of the PX and the SHAM groups was compared with CTRL recordings performed at the same period of time (1300-1900h). Only rats with histological confirmation of complete removal of the pineal gland were included in the analysis of results.

### 2.3.5 MLT and pinealectomy effects across the light-dark cycle

The effect of PX and MLT (1 mg/kg) on 5-HT firing rate and number of spontaneously active 5-HT neurons was assessed in a group of rats during the dark phase (1900h to 0700 h), and was compared with recordings obtained in a separate group of rats during the light phase (0700 h to 1900 h), using experimental manipulation (PX, MLT and CTRL) and phase (light and dark) as factors. In a further analysis, recordings were grouped into four time intervals of six hours from the beginning of the light phase (0700-1300 h; 1300-1900 h; 1900-0100 h; 0100-0700 h), and 5-HT neural activity was compared between experimental manipulations (PX, MLT and CTRL).

### 2.3.6 MLT administration in pinealectomized rats during the dark phase

In order to test whether MLT reversed the effect of PX during the dark phase, a group of rats underwent PX and 5-HT firing activity in the DRN was recorded. Two hours after the removal of the pineal gland, a single dose of MLT (1 mg/kg, i.v.) was injected and the acute and sub-acute effects of MLT on 5-HT firing rate and number of spontaneously active 5-HT neurons were assessed. Short-term effects of both manipulations were compared against CTRL recordings carried out at the same period of time.

### 2.3.7 MLT administration in the rat forced swim test

Finally, to examine the effect of MLT on coping behavior, we tested MLT administration at doses of 1 and 10 mg/kg (i.p.) in the FST. Each dose was tested in a different group of rats and was compared with rats receiving DMI (10 mg/kg, i.p.; Gobbi *et al*., 2005) or VEH. The FST was performed following the original procedure described by Porsolt *et al*. (1978) with the modified drug scheduling by Page *et al*. (1999). Briefly, rats were individually placed inside

vertical Plexiglas cylinders (20 cm diameter, 50 cm height) containing water (30 cm depth) at 25 ± 1 °C. In a pretest session, rats were forced to swim for 15 min and then removed, dried and returned to their cages. In a test session 24 h later, rats were forced to swim for five min, and behavior was video-recorded and analyzed. Duration of behavior, including immobility, swimming and climbing was assessed using the Videotrack system (Viewpoint Life Science, Montreal, Quebec, Canada) via an automatic differential movement analysis. Before each FST session, locomotor activity was assessed in the open field (OF) test. For this, rats were placed in a black painted box (80 x 80 x 40 cm) for 5 min and distance traveled (m) was quantified using the Videotrack system. Drugs were injected 24 h, 5 h and 15 min before the FST test session. The first injection was given immediately after the pretest session.

### 2.3.8 Statistical analysis

Data were analyzed using the SigmaPlot software (Systat Software, Inc.). One-way ANOVA was used to analyze data from the MLT dose-response curve, and to compare the effects of MLT and PX against CTRL recordings at different time points. One-way ANOVA for repeated measures was used to analyze data from the pharmacological antagonism experiments. Two-way ANOVA was used to compare the effects of MLT and PX between phases of the light-dark cycle. Bonferroni t-test was used for post hoc comparisons. Two-tailed Student's t-tests were used to analyze effects of cycle phase on CTRL recordings, and to compare single- versus burst-firing neurons. Paired t-tests were used to analyze the acute effect of MLT at different time intervals and on PX rats. Chi-square was used to analyze differences in the proportion of burst-firing neurons and in the percentage of spikes in burst. In all cases, Bonferroni corrected t-test was used for post hoc comparisons after a significant ANOVA was detected. COSINOR analysis was done

using SigmaPlot dynamic fit wizard to perform curve fitting and regression analysis. All data are expressed as mean ± standard error of the mean (SEM). Statistical values of $p \leq 0.05$ were considered significant.

## 2.4 Results

### 2.4.1 5-HT neuronal activity in the DRN varies across the light-dark cycle

Under basal conditions (CTRL) a total of 147 neurons were recorded in the light phase and 131 neurons in the dark phase, from 32 and 25 rats respectively. In these CTRL recordings, a significant decrease in the 5-HT firing rate ($t_{(276)} = 3.056$, $p = 0.002$, Figure 1A) and number of spontaneously active neurons ($t_{(94)} = 4.486$, $p < 0.001$, Figure 1B) was found in the dark phase compared with light phase. The decrease in 5-HT firing rate was 26.41% during the dark phase compared to the light phase. Two types of 5-HT neurons were found: single- and burst-firing. The mean firing rate of burst-firing neurons was significantly greater than that of single-firing ($t_{(276)} = 4.46$, $p < 0.001$, Figure 1C). Representative waveforms of single- and burst-firing 5-HT neurons, as well as those of a non-5-HT neuron, are shown in Figure 1D. 70% of 5-HT neurons fired in single spikes, whereas 30% fired in bursts (doublets and occasionally triplets). The recording sites are show in Figure 2.

One way ANOVA's, revealed that the number of 5-HT spontaneously active neurons ($F_{(5,90)} = 4.145$, $p = 0.002$) and their firing rates ($F_{(5,272)} = 2.343$, $p = 0.042$) varies significantly between time intervals of the light-dark cycle (Figure 3). In both cases, higher values corresponded to interval of 1500-1900 h. 5-HT neuronal firing rate was only significantly higher at 1500-1900 h compared with the lower values recorded at 0300-0700 h ($p = 0.013$, Figure 3A). The number of

5-HT spontaneously active neurons was significantly higher at 1500-1900 h compared with all other intervals of the dark phase ($p = 0.004$ vs. 2300-0300 h, $p = 0.008$ vs. 1900-2300 h and $p = 0.029$ vs. 0300-0700 h, Figure 3B).

There was no significant change in the proportion of single-spiking and burst-firing neurons between phases or time intervals of the light-dark cycle and all other burst-firing parameters were also constant across the light-dark cycle and are summarized in Table 1.

### 2.4.2 Rhythms in 5-HT firing activity

The average values obtained with ANOVA's by intervals of time were used to conduct a COSINOR analysis of the firing activity data (neurons per track and firing rate), with a fixed period (*P*) of 24 h. 5-HT neuronal firing rate data significantly fit ($F_{(1,4)} = 15.017$, $p = 0.0179$) the sinusoidal equation: $y = 0.8052 – 0.0808 \cos(2\pi x/24) + 0.1438 \sin(2\pi x/24)$, with a coefficient $R^2 = 0.7897$ (adjusted $R^2 = 0.7371$), the acrophase (peak) was detected at 1454 h (14.91 radians) and the corresponding trough at 0254 h (Figure 3A). The number of 5-HT spontaneously active neurons was best represented by the equation ($F_{(1,4)} = 33.4983$, $p = 0.0044$): $y = 2.9538 – 0.1143 \cos(2\pi x/24) + 0.7783 \sin(2\pi x/24)$, with a coefficient $R^2 = 0.8933$ (adjusted $R^2 = 0.8667$), the acrophase was detected at 1333 h (13.56 radians) and the trough was observed at 0133 h (Figure 3B).

### 2.4.3 Acute inhibitory effect of MLT on 5-HT neuronal activity

A total of 74 rats were used in this experiment, and one 5-HT neuron per rat was selected to test the acute effect of one of the different doses of MLT or VEH (VEH: 10 rats; 0.1 mg/kg: 7 rats;

0.25 mg/kg: 11 rats; 0.5 mg/kg: 8 rats; 1 mg/kg: 11 rats; 5 mg/kg: 9 rats; 10 mg/kg: 7 rats; and 20 mg/kg: 11 rats). Acute administration of MLT inhibited the firing activity of DRN 5-HT neurons in a dose-dependent manner ($F_{(8,139)} = 3.159$, $p = 0.003$, Figure 4B). The decrease in 5-HT firing rate was significant at doses of 0.5 ($p = 0.027$) and 1 mg/kg ($p = 0.003$), compared to basal conditions (CTRL). No significant acute changes were found in the 5-HT firing rate at the lowest (0.1 and 0.25 mg/kg) or highest doses (5, 10, and 20 mg/kg). Figure 4B shows a representative integrated firing rate histogram of the acute effect of MLT (1 mg/kg) on a 5-HT neuron. The number of responding 5-HT neurons displayed an inverted U-shaped distribution (Figure 4C), in which MLT at the dose of 1 mg/kg had the highest rate of response (7 out of 11, or 63.63 % of tested neurons, $\chi^2_{(1)} = 4.073$, $p = 0.043$) compared to VEH (2 out of 10, or 20 % of tested neurons). The latency of 5-HT firing rate inhibition induced by MLT was 4.64 ± 0.82 min, ranging from 0.35 to 13.33 min; the distribution was bimodal, with one group of neurons responding within 2 min (average: 1.14 ± 0.81 min, n = 10) and another group after 2 min (average: 7.34 ± 0.89 min, n = 13).

### 2.4.4 $MT_1$ antagonist blocks MLT acute effect on 5-HT firing rate

Administration of the $MT_1/MT_2$ receptor antagonist LUZ potently blocked MLT-induced acute inhibition of 5-HT firing rate (6 out of 6 neurons tested; $F_{(3,15)} = 1.735$, $p = 0.203$, Figure 5A). In comparison, MLT (1 mg/kg) induced decreases in 5-HT firing rates were not prevented by pre-treatment with the $MT_2$ receptor antagonist 4P-PDOT (6 out of 6 neurons tested; $F_{(3,15)} = 8.056$, $p = 0.002$, Figure 5B), the 5-$HT_{1A}$ receptor antagonist WAY100635 (4 out of 4 neurons tested; $F_{(3,9)} = 28.431$, $p < 0.001$, Figure 5C), or the $\alpha_2$ adrenoceptor antagonist idazoxan (4 out of 4

neurons tested; $F_{(3,9)} = 233.398$, $p < 0.001$, Figure 5D). No significant changes in the 5-HT firing rate were observed after the administration of VEH or the antagonists tested.

### 2.4.5 Administration of MLT induces a sub-acute inhibitory effect on 5-HT neuronal activity

Before MLT administration, 19 tracks were complete in CTRL conditions in 19 of the 74 rats used. A total of 78 tracks were done after MLT or VEH administration. The number of tracks per dose is indicated in Figure 6B and the number of rats used per dose was described in the acute effects results. After MLT administration a dose–dependent decrease in 5-HT cell firing activity persisted for 45 min post-injection (sub-acute effect). These more enduring decreases in mean 5-HT cell firing rate were seen in rats that received MLT at doses of 0.5 ($p = 0.047$) and 1 mg/kg ($p = 0.01$), compared to CTRL recordings ($F_{(8,277)} = 2.143$, $p = 0.03$; Figure 6A). Similar results were found in the number of spontaneously active 5-HT neurons, which was decreased at doses of 0.25 ($p = 0.01$), 0.5 ($p = 0.034$) and 1 mg/kg ($p = 0.024$), compared with CTRL recordings ($F_{(8,88)} = 2.793$, $p = 0.008$; Figure 6B). In addition, the proportion of burst-firing neurons was also dose-dependently reduced by MLT administration, reaching statistical significance at the dose of 0.5 mg/kg (2 out of 22 or 9.09 % of recorded neurons, $\chi^2_{(1)} = 3.787$, $p = 0.05$), compared with CTRL recordings (21 out of 71 or 29.57 % of recorded neurons). In these burst-firing neurons, the percentage of spikes in burst was reduced at the doses of 0.25 (4.36 ± 1.03 % of spikes in burst, $\chi^2_{(1)} = 10.01$, $p<0.005$), 0.5 (3.24 ± 2.31 % of spikes in burst, $\chi^2_{(1)} = 11.971$, $p < 0.001$) and 1 mg/kg (4.52 ± 1.87 % of spikes in burst, $\chi^2_{(1)} = 8.303$, $p < 0.005$), compared with CTRL recordings (18 ± 4.34 % of spikes in burst). No differences were found in other burst activity parameters (data not shown).

### 2.4.6 Pinealectomy, but not sham surgery, increases 5-HT neuronal activity

A total of 8 rats successfully underwent PX, 8 rats received SHAM and 7 rats were included in a CTRL group. PX significantly increased the 5-HT firing rate of DRN neurons recorded 1-3 hours after the removal; in particular, 5-HT firing rate increased 1 h after the removal of the pineal gland ($p = 0.049$ vs. CTRL). No changes were observed in 5-HT firing rate after SHAM surgery (CTRL: 0.9 ± 0.1 Hz; SHAM: 0.86 ± 0.07 Hz; PX: 1.24 ± 0.13 Hz; $F_{(2,123)} = 3.991$, $p = 0.021$). No significant changes were found in the number of spontaneously active 5-HT neurons after PX or SHAM surgery (CTRL: 3.54 ± 0.24 neurons/track; SHAM: 3.26 ± 0.5 neurons/track; PX: 3.45 ± 0.5 neurons/track; $F_{(2,34)} = 0.107$, $p = 0.899$). However, PX tended to increase the percentage of burst-firing neurons (CTRL: 11 out of 39, or 28.2 % of recorded neurons; SHAM: 11 out of 49, or 22.44 % of recorded neurons; PX: 14 out of 38, or 36.8 % of recorded neurons) and the coefficient of variation of their ISI, indicating an enhanced firing irregularity (CTRL: 0.646 ± 0.09; PX: 0.745 ± 0.06).

### 2.4.7 MLT and pinealectomy have opposite effects depending on the phase of the cycle and basal 5-HT neuronal activity

Since the previous experiments with MLT administration and PX were conducted mainly during the second half of the light phase (1300-1900h), we aimed to better characterize short term effects of MLT (1 mg/kg) and PX on 5-HT neuronal activity by comparing the responses in the light phase (0700-1900h) with those in the dark phase (1900-0700h). The number of rats in each group was as follow: MLT = 19 rats, PX = 26 rats and CTRL = 28 rats. Half of the rats were recorded in the dark phase and the other half in the light phase (except in the MLT group: light phase n = 10 rats, dark phase n = 9 rats). Two-way ANOVA revealed a significant interaction

between treatment and cycle phase on 5-HT firing rate ($F_{(2,345)} = 4.003$, $p = 0.019$). Post hoc comparisons between treatments within the light phase revealed that MLT decreased 5-HT firing rate ($p = 0.04$ vs. CTRL and $p = 0.002$ vs. PX; CTRL: 0.93 ± 0.07 Hz, n = 2; MLT: 0.53 ± 0.03 Hz, n = 39; PX: 1.06 ± 0.09 Hz, n = 65), whereas PX in the dark phase increased 5-HT firing rate ($p < 0.001$ vs. CTRL and MLT; CTRL: 0.7 ± 0.06 Hz, n = 75; MLT: 0.57 ± 0.08 Hz, n = 37; PX: 1.28 ± 0.12 Hz, n = 63). Post hoc comparisons between phases of the cycle within CTRL recordings revealed that 5-HT firing rate significantly decreased in the dark phase compared with the light phase ($p = 0.04$), as indicated in Figure 7A.

Two-way ANOVA revealed a significant interaction between treatment and phase of the cycle on the number of spontaneously active 5-HT neurons ($F_{(2,120)} = 7.834$, $p < 0.001$). Post hoc comparisons between treatments within the light phase indicated that MLT administration reduced the number of active 5-HT neurons per track ($p < 0.001$ vs. CTRL and $p = 0.002$ vs. PX; CTRL: 3.78 ± 0.33 neurons/track, 19 tracks; MLT: 2.16 ± 0.2 neurons/track, 18 tracks; PX: 3.4 ± 0.32 neurons/track, 19 tracks), whereas within the dark phase PX significantly increased active neurons per track ($p < 0.001$ vs. CTRL and $p = 0.012$ vs. MLT; CTRL: 2.14 ± 0.17 neurons/track, 35 tracks; MLT: 2.31 ± 0.23 neurons/track, 16 tracks; PX: 3.5 ± 0.38 neurons/track, 18 tracks). Post hoc comparisons between phases of the cycle within CTRL recordings indicated that the number of 5-HT neurons per track was decreased during the dark phase compared with the light phase ($p < 0.001$, Figure 7B).

### 2.4.8 MLT and pinealectomy affect 5-HT neuronal activity in specific time intervals across the light-dark cycle

In order to better characterize the effects of MLT administration and PX over time, we analyzed the effects of both experimental conditions at four time intervals in the light-dark cycle. MLT administration significantly reduced 5-HT firing rate at 0700-1300 h ($p = 0.022$ vs. CTRL; $F_{(2,76)} = 3.699$, $p = 0.029$) and at 1300-1900 h ($p = 0.05$ vs. CTRL and $p < 0.001$ vs. PX; $F_{(2,94)} = 7.845$, $p < 0.001$), as shown in Figure 7A. In this time interval (1300-1900 h) PX increased 5-HT firing rate compared with CTRL ($p = 0.047$). In addition, PX increased 5-HT firing rate at 1900-0100 h ($p = 0.013$ vs. CTRL and $p = 0.001$ vs. MLT; $F_{(2,80)} = 7.258$, $p = 0.001$) and at 0100-0700h ($p<0.001$ vs. CTRL and $p = 0.009$ vs. MLT; $F_{(2,89)} = 7.782$, $p < 0.001$), as shown in Figure 7A.

Notably, the acute inhibitory effect of MLT (1 mg/kg) on 5-HT firing rate was not observed in neurons tested in the two periods of the dark phase, at 1900-0100 h (CTRL: 0.81 ± 0.2 Hz; post-MLT: 0.58 ± 0.1 Hz; $t_{(7)} = 1.166$, $p = 0.282$, $n = 8$) or at 0100-0700 h (CTRL: 0.68 ± 0.16 Hz; post-MLT: 0.65 ± 0.16 Hz; paired $t_{(5)} = 0.73$, $p = 0.496$, $n = 6$), whereas 5-HT firing inhibition was found in the two periods of the light phase, at 0700-1300 h (CTRL: 0.86 ± 0.1 Hz; post-MLT: 0.53 ± 0.1 Hz; paired $t_{(11)} = 3.23$, $p = 0.008$, $n = 12$) and at 1300-1900 h (CTRL: 1 ± 0.17 Hz; post-MLT: 0.44 ± 0.17 Hz; paired $t_{(9)} = 3.06$, $p = 0.014$, $n = 10$).

One-way ANOVA revealed that the number of spontaneously active 5-HT neurons decreased after MLT administration at 0700-1300 h ($p = 0.004$ vs. CTRL and $p = 0.006$ vs. PX; $F_{(2,25)} = 8.064$, $p = 0.002$) and at 1300-1900 h ($p = 0.025$ vs. CTRL; $F_{(2,28)} = 3.66$, $p = 0.039$), as shown in Figure 7B. In the time interval of 1900-0100 h, no differences between treatments were observed

($F_{(2,28)} = 0.568$, $p = 0.573$). In the second half of the dark phase (0100-0700 h), PX increased the number of spontaneously active 5-HT neurons ($p < 0.001$ vs. CTRL and MLT; $F_{(2,35)} = 11.239$, $p < 0.001$, Figure 7B). In general, MLT decreased 5-HT burst activity, in that the proportion of burst-firing neurons ($p < 0.001$ vs. CTRL and PX; $F_{(2,9)} = 32.608$, $p < 0.001$) and the mean percentage of spikes in burst ($p = 0.025$ vs. CTRL and $p = 0.012$ vs. PX; $F_{(2,9)} = 8.697$, $p = 0.008$) were both reduced after MLT administration, as summarized in Table 2.

### 2.4.9 MLT administration reverses the effects of pinealectomy in the dark phase

As the effects of PX were more evident during the dark phase, we tested whether these effects could be reversed by MLT administration. In this experiment, 7 rats received PX and then MLT (1 mg/kg) was injected in the dark phase; the effects were compared with a CTRL group of 7 rats recorded at the same period of time. In Figure 8A, a representative integrated firing rate histogram shows the acute effects of MLT on a 5-HT neuron recorded in a PX rat, in that MLT reduced 5-HT firing rate (PX: 1.25 ± 0.2 Hz; PX + MLT: 0.71 ± 0.2 Hz; Paired $t_6 = 2.701$, $p = 0.036$, $n = 7$). Within 45 min post-injection, MLT reversed the short-term effects of PX, decreasing the 5-HT firing rate ($F_{(2,83)} = 4.816$, $p = 0.011$, Figure 8B) and the number of spontaneously active 5-HT neurons ($F_{(2,29)} = 6.798$, $p = 0.004$, Figure 8C). In addition the proportion of burst-firing neurons was significantly reduced in PX rats after MLT administration (CTRL: 6 out of 23, or 26.08 % of recorded neurons; PX: 16 out of 42, or 38.09 % of recorded neurons; PX+MLT: 2 out of 19, or 10.52 % of recorded neurons, $\chi^2_{(1)} = 4.78$, $p = 0.028$).

### 2.4.10 MLT administration increases immobility and reduces swim behavior in rats forced to swim

In this experiment, 2 groups of rats were tested with MLT (1 or 10 mg/kg, 10 rats in each group), and 2 groups of rats were administered with DMI or VEH (9 rats in each group). In the FST, MLT administration at the dose of 1 mg/kg increased immobility duration ($p = 0.05$ vs. VEH; $F_{(3,34)} = 9.18$, $p < 0.001$, Figure 9A) and decreased swimming duration ($p = 0.043$ vs. VEH; $F_{(3,34)} = 8.15$, $p < 0.001$, Figure 9B). In comparison, the antidepressant DMI had the opposite effect on immobility ($p < 0.035$ vs. VEH) and in addition increased total climbing duration ($p = 0.002$ vs. VEH, Figure. 8A-C; $F_{(3,34)} = 7.49$, $p < 0.001$, Figure 9C). A higher dose of MLT (10 mg/kg) did not affect behavior in the FST. DMI decreased locomotor activity ($p = 0.03$ vs. VEH; $F_{(3,34)} = 4.36$, $p = 0.011$, Figure 9D), whereas no changes were observed in MLT-treated groups.

## 2.5 Discussion

These results are the first to show that acute administration of MLT at lower doses (0.5 and 1 mg/kg, i.v.) decreases the firing activity of 5-HT neurons located in the DRN, and that removal of the pineal gland has the opposite effect, increasing 5-HT firing activity. Under basal conditions, 5-HT firing rate and the number of spontaneously active 5-HT neurons in the DRN are decreased during the dark phase. Furthermore, for the first time a COSINOR analysis was performed to analyze these two parameters of 5-HT firing activity and in both cases a diurnal rhythm of 24 h was detected with acrophases in the light phase. The PX-induced increase in 5-HT firing activity was more evident in the second period of the dark phase (0100-0700 h), and was reversed by MLT administration. In contrast, the effects of MLT were only evident during the light phase (0700 h to 1900 h). Finally, the decrease in 5-HT neurotransmission induced by

MLT administration (1 mg/kg) was paralleled by a moderate but significant increase in immobility during the FST.

The effects of MLT administration in our experiments seem to be independent of 5-$HT_{1A}$ receptors or $\alpha_2$-adrenoceptors. This is in line with the negligible affinity for 5-$HT_{1A}$ receptors reported for MLT and the lack of effect of MLT on the firing rate of locus coeruleus noradrenergic neurons at the doses of 1-16 mg/kg (Millan *et al.*, 2003). Our results with IDA suggest that the decrease in 5-HT firing induced by MLT (1 mg/kg) is not due to the $\alpha_2$-adrenoceptors, which indirectly control 5-HT neurons by suppressing their excitatory $\alpha_1$-adrenergic input (Freedman and Aghajanian, 1984; Bortolozzi and Artigas, 2003; Haddjeri *et al.*, 2004). Nevertheless, the long latency of some 5-HT neurons to MLT administration may suggest an indirect action of MLT in other regions outside the DRN or the LC. As luzindole, but not 4P-PDOT, potently blocked the effect of MLT administration, it is possible that the effect of MLT on 5-HT neurotransmission is mediated by $MT_1$ receptors. To the date there are not commercially available selective $MT_1$ receptor agonists or antagonists than can be used to provide conclusive evidence of the role of $MT_1$ activation on 5-HT neurotransmission (Dubocovich *et al*., 2010). Furthermore, whether MLT is acting directly on putative $MT_1$ receptors located on DRN neurons or on $MT_1$ receptors located in other brain structures is still unknown. In humans, $MT_1$ mRNA and protein expression has been found in areas that control 5-HT activity directly or indirectly including hypothalamus (Weaver *et al.*, 1993), cerebellum (Al-Ghoul *et al.*, 1998), frontal cortex and hippocampus (Mazzucchelli *et al.*, 1996; Savaskan *et al.*, 2002). Recently, we studied the localization of MLT receptors in the rat brain using photon and electron microscopic single immunolabeling (Lacoste *et al*., 2014). In this study, we confirmed

the existence of somatodendritic $MT_1$ receptors in neurons located in the dorsal and ventral aspect of the DRN. However, it remains to be determined whether these $MT_1$-immunoreactive neurons are also serotonergic (i.e. direct modulation of 5-HT neurotransmission) or if they produce other neurotransmitters (i.e. indirect modulation).

MLT action in other brain areas and other neurotransmitters systems may explain the lack of effect of MLT at higher doses (> 5mg/kg) on 5-HT firing rate. For example, the VTA which contain MLT receptors in mice (Uz *et al.*, 2005) and rats (Lacoste *et al*., 2014), exerts a positive excitatory control on 5-HT activity (Guiard *et al.*, 2008a; 2008b) that may counterbalance the inhibition induced by MLT at doses of 5 mg/kg or more. In addition, some studies have suggested that MLT at doses above 10 mg/kg (up to 120 mg/kg) interact with the GABA and opioid systems to exert its analgesic and hypnotic effects in mice and rats (Golombek *et al.*, 1991; Yu *et al.*, 2000; Wang *et al.*, 2003). Moreover, in the rat, MLT actions in the metabolism of 5-HT (e.g., tryptophan hydroxylation ratio) are also observed at doses between 0.5 to 1 mg/kg, but not at doses of 20 mg/kg (Holmes and Sugden, 1982; Miguez *et al.*, 1994). In rat cerebral cortex and liver, the subcellular distribution of MLT reach a saturation point in the mitochondria and nucleus is still observable 2 h after systemic MLT administration at the doses of 40 mg/kg (Venegas *et al*., 2012). In membrane and cytosol, accumulation of MLT continues at doses of MLT as high as 200 mg/kg (Venegas *et al*., 2012). Considering the half life of MLT reported in by the same authors (≈27 min), it is possible that saturation of the subcellular compartments by MLT was reached at a lower dose within one hour post administration. This also can to explain the lack of effect of MLT observed at doses of 5 mg/kg and above on 5-HT neuronal firing rate.

However, a direct action within the DRN cannot be ruled out since about 40% of the 5-HT neurons tested responded within 2 min to MLT administration. In addition, the existence of $MT_1$ receptors in the DRN (Lacoste *et al*., 2014), open the possibility for a direct action of MLT on 5-HT neurons, similar to the mechanism described in SCN neurons (Dubocovich, 2007). In fact, Mason & Brooks (1988) reported that exogenous MLT administration inhibits the firing activity in 40% of SCN neurons *in vitro*, particularly in the late portion of the light phase, but not during the dark phase (Shibata *et al.*, 1989), as observed here with DRN 5-HT neurons.

Our results suggest that MLT administration inhibit impulse activity of 5-HT neurons and likely inducing a decrease of 5-HT release in terminal regions. Consistent with this hypothesis, in neurochemical studies exogenous administration of MLT (0.5-1 mg/kg) induces a short-term (1 h) decrease in 5-HT content in the cerebral cortex, accompanied by an increase in the midbrain and hypothalamic regions (Anton-Tay *et al.*, 1968; Miguez *et al.*, 1994). An accumulation of 5-HT in the hypothalamus is compatible with the reports decrease in 5-HT release from synaptosomes of the preoptic and anterior hypothalamic areas after a single dose of MLT in the same range (0.5 mg/kg) (Miguez *et al*., 1995). However, no effect on 5-HT content after MLT administration at high doses (ranging from 10 to 50 mg/kg) was found in the whole mouse brain or in rat cortex, brain stem, pineal gland, or serum (Sugden, 1983; Manzana *et al.*, 2001), although following administration of an even higher dose (60 mg/kg) a decrease in 5-HT release in the hypothalamus, corpus striatum and nucleus accumbens has been reported (Chuang and Lin, 1994). Based on the above-mentioned studies, a lower dose of MLT induces an accumulation of 5-HT in discrete brain areas, but decreased release in synaptic terminals. Since the electrical stimulation of the DRN in short burst sequences elicits increased 5-HT release and

neuronal activity in the prefrontal cortex (Gartside *et al.*, 2000; Puig *et al.*, 2005), one may hypothesize that the MLT (0.25-1 mg/kg) induced decrease in 5-HT burst activity leads to a decrease in release at the terminal areas.

Our experiments have explored for the first time the acute effects of PX on DRN 5-HT neuronal activity. In previous neurochemical studies, 5-HT content in the DRN and median raphe was found to be unaltered one month after PX (Miguez *et al.*, 1997). In electrophysiological experiments, Castillo-Romero *et al*. (1993) found no change in the firing rate of dorsal striatum neurons recorded in rats one week after PX. However, circadian rhythms were not considered in these studies and experimental procedures were performed only during the light phase of the cycle. As MLT concentration in the brain depends on the integrity of the pineal gland and its circadian secretion pattern (Pang *et al.*, 1982), the increased 5-HT neuronal activity observed in our experiments in PX rats suggests a tonic inhibition of 5-HT by MLT during the dark phase. In hamsters, a similar mechanism involving MT1 receptors has been proposed to explain the loss of nocturnal inhibition in SCN neurons after PX (Rusak and Yu, 1993; Dubocovich, 2007). Together, these data suggest that the circadian effects of MLT over different neuronal populations disappear after removal of the pineal gland in the short-term. However, further studies are needed to clarify if the effects of pineal MLT secretion on 5-HT neurons occur directly in the DRN, or whether an indirect mechanism is involved.

We were able to determine the occurrence of a diurnal rhythm of 5-HT neuronal firing activity using standard (ANOVA) and specific (COSINOR) statistical testing. Our results indicated that both the population of active 5-HT neurons and their firing rate are at highest levels of activity

during the middle of the light phase and at the lowest in the dark phase. This occurs under a well defined 24 h diurnal rhythm of activity. Therefore, it is likely that tonic 5-HT release reaches its maximal levels in the middle of the light phase.

In agreement with our results, under basal conditions, the highest levels of 5-HT in the DRN have been reported to occur in the light phase, and the lowest levels at the onset of the dark phase (Semba *et al.*, 1984; Birkett and Fite, 2005), although not all reports reach consensus. Indeed, Poncet *et al*. (1993) reported an increase of 5-HT content in the raphe centralis during the light phase, but in the DRN they reported higher 5-HT content during the dark phase. However, using electrophysiological techniques in anesthetized rats, an early study by Mosko and Jacobs (1974) showed that approximately 60% of the 5-HT neurons recorded in the DRN discharged at a higher rate when illumination conditions were changed from dark to light. This observation is in line with the increased 5-HT firing activity recorded in the light phase in our experiments.

A limitation in the present study is that our recordings were performed in a constant slow-wave sleep (SWS)-like state induced by anesthesia (Alkire *et al.*, 2008). In non-anesthetized rats, most 5-HT neurons discharge at a higher rate when rats are awake, decreasing their activity during SWS and becoming silent during paradoxical sleep (Urbain *et al.*, 2006). However, this may explain the lack of effects of MLT at high doses on 5-HT firing activity, as 5-HT neurons responding to anesthetic SWS-induced effects may already have decreased firing activity. Therefore, it is possible that the decrease in basal 5-HT firing activity observed during the dark phase was due only to a sub-population of 5-HT neurons tonically inhibited by nocturnal MLT secretion. This idea is supported by the increased 5-HT firing activity observed after PX in the

dark phase, which was restored by MLT administration to basal dark phase levels. Interestingly, a circadian rhythm in spontaneous and KCl-evoked release of 5-HT was reported in hippocampal slices (Monnet, 2002); this may be evidence supporting the existence of a subpopulation of 5-HT neurons exhibiting a circadian rhythm, within a circuit involved in mood regulation (Piñeyro and Blier, 1999; Bambico *et al.*, 2010).

Different pharmacological properties have been attributed to MLT, including analgesic, hypnotic, anti-convulsant, anxiolytic, and antidepressant effects (Sugden, 1983; Golombek *et al.*, 1993; Chase and Gidal, 1997; Shaji and Kulkarni, 1998; Ambriz-Tututi *et al.*, 2009). Although the analgesic and sleep-inducing properties of MLT seem to be consistent across studies, its antidepressant properties are still controversial. For instance, some studies have reported that MLT have an antidepressant-like effect reducing immobility in the FST and in the tail suspension test (Shaji and Kulkarni, 1998; Micale *et al.*, 2006; Binfare *et al.*, 2010). However, reports are inconsistent when considering dose, schedule and antidepressant-like efficacy of MLT (Brotto *et al.*, 2000; Bourin *et al.*, 2004). Indeed, MLT agonists such as agomelatine and antagonists such as luzindole have also been reported to have antidepressant-like properties (Bourin *et al.*, 2004; Sumaya *et al.*, 2005). However, the consistent antidepressant activity of agomelatine seems to be independent of its MLT agonistic properties and rather mediated by its $5HT_{2C}$ antagonist properties (Millan *et al.*, 2003; Bourin *et al.*, 2004; Zupancic and Guilleminault, 2006).

From our results, low (1 mg/kg) but not high (10 mg/kg) doses of MLT increase immobility duration and decrease swimming duration in the FST. This may represent, at best, switching

from an active to a passive mode of coping. However, these results should be interpreted cautiously, because although an increase in immobility has been used as an indication for depressive-like behavior triggered by pro-depressant manipulations, e.g. chronic stress (Bielajew *et al.*, 2003) and drug withdrawal (Cryan *et al.*, 2003), the FST was used primarily to assess antidepressant efficacy (Porsolt *et al.*, 1978), not pro-depressant liabilities. Nevertheless, our results corroborate those of others who reported a decrease in active coping (swimming duration) in the FST after 14 days of MLT treatment (Brotto *et al.*, 2000). Others have also reported a lack of effect of acute MLT administration at higher doses (4, 16, and 64 mg/kg) (Bourin *et al.*, 2004) in the same test. Furthermore, recent evidence has shown that both short photoperiod conditions and MLT administration, with a schedule that mimics short photoperiods, can induce depressed or anxiety-modeling behaviors in diurnal animals (Ashkenazy *et al.*, 2009), supporting a direct role of endogenous MLT secretion in mood regulation. This increase in passive coping behavior after MLT administration could be related to the MLT-induced decrease in 5-HT neurotransmission that we found in DRN neurons. Consistent with this interpretation, changes in MLT activity and impairment in 5-HT neurotransmission are both seen in stress-associated conditions such as sleep and pain disorder, as well as in alterations of feeding behavior (Blundell, 1992; Jouvet, 1999; la Fleur *et al.*, 2001; Sounvoravong *et al.*, 2004; Dubocovich, 2007; Ambriz-Tututi *et al.*, 2009).

Finally, some reports indicate that MLT administration induces a negative effect on mood, increasing fatigue and decreasing alertness in both healthy and depressed individuals (Carman *et al.*, 1976; Lieberman *et al.*, 1984; Dollins *et al.*, 1993). In a recent long-term trial with evening MLT administration (2.5 mg/daily) in older patients, a negative impact on mood was found,

although MLT was effective in improving sleep (Riemersma-van der Lek *et al.*, 2008). On the other hand, in 2007 a prolonged release MLT formulation was approved by the European Medicines Agency (EMEA) for the treatment of insomnia in elderly people, acting mostly as a sleep inducer and reversing blunted MLT levels in these patients (Wade *et al.*, 2011). However, other reports affirm that low doses of MLT (0.3 - 0.5 mg), at different times of the day, may be considered as a valid treatment for phase shift disorders, although this theory requires validation by large double-blind studies (Lewy, 2009). Nevertheless, the increased or prolonged secretion of MLT observed in SAD patients during the fall and winter seasons (Karadottir and Axelsson, 2001; Pacchierotti *et al.*, 2001; Wehr *et al.*, 2001; Srinivasan *et al.*, 2006), as well as the effectiveness of light therapy on these patients (Rosenthal *et al.*, 1984; Martiny *et al.*, 2004; Lam *et al.*, 2006), support the idea of tonic inhibition of 5-HT neurotransmission by MLT in response to photoperiod.

### 2.6 Conclusion

In conclusion, the present study is the first to demonstrate a periodical 24 h rhythm of firing activity in 5-HT neurons of the DRN with a decrease associated with the dark phase and a modulation of 5-HT firing activity by exogenous and endogenous MLT. This MLT modulation over 5-HT neurotransmission is inhibitory in nature, and can be elicited by MLT administration at low doses during the light phase and appears to be mediated by $MT_1$ receptors. During the dark phase, endogenous MLT inhibition of 5-HT activity appears to occur; PX leads to increased 5-HT cell firing and the lower firing rates usually seen during the dark phase can be restored by exogenous MLT administration. This down-regulation of 5-HT neurotransmission by MLT could represent a general mechanism by which circadian and seasonally related functions are

influenced by 5-HT neurotransmission, MLT secretion and photoperiod duration. Further studies are needed to clarify whether this modulation occurs indirectly or directly on DRN neurons, and to better characterize the distinct role of $MT_1$ and $MT_2$ receptors in 5-HT neurotransmission.

# Figures and Tables

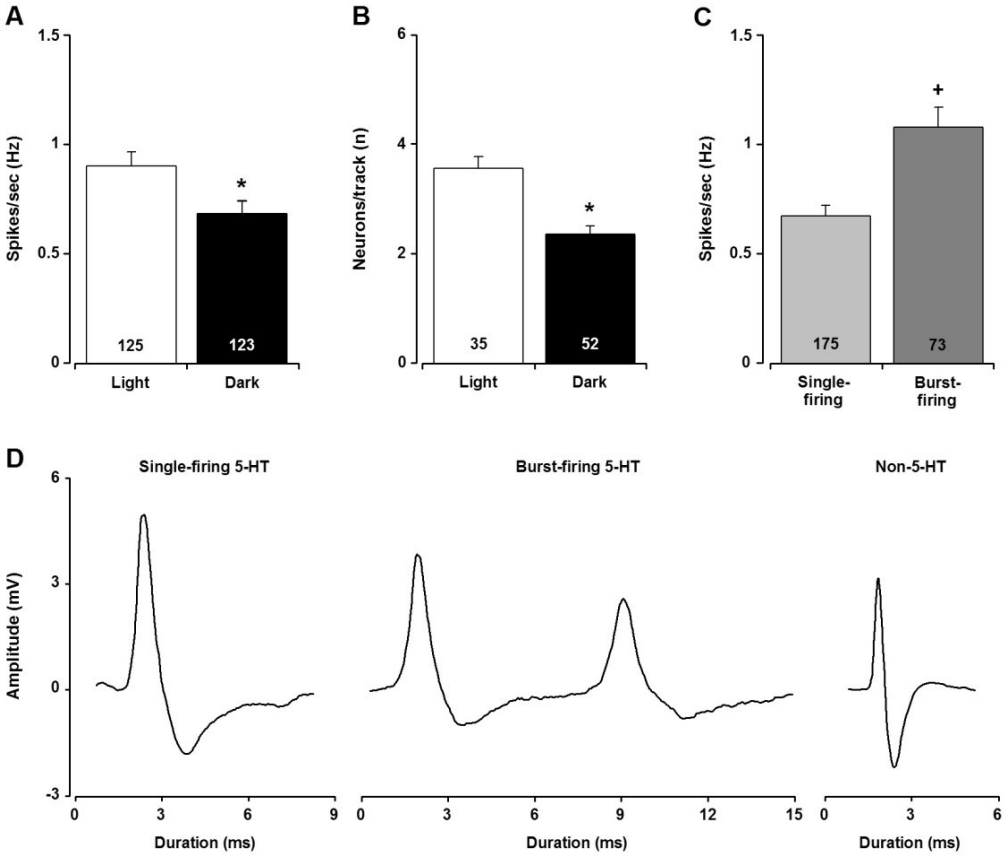



**Figure 1.** The basal firing activity of serotonin (5-HT) neurons located in the dorsal raphe decreases during the dark phase. (A) The mean firing rate of 5-HT neurons recorded during the dark phase (1900-0700 h) was significantly lower (-24.11%) than the firing rate of 5-HT neurons recorded during the light phase (0700-1900 h). (B) In addition, a decrease in the number of spontaneously active 5-HT neurons was also observed during the dark phase. (C) As depicted above, two types of firing were observed in 5-HT neurons: burst-firing accompanied by a significantly higher firing rate, and single-firing representing 70% of recorded neurons. (D) From left to right, representative waveforms are shown of single, doublet burst-firing, and non-5HT neurons. Bars represent mean ± SEM, numbers within bars in A and C indicate number of recorded neurons and in B indicate number of tracks. Student's t-test: * $p < 0.05$ vs. light phase; + $p < 0.001$ vs. single firing.

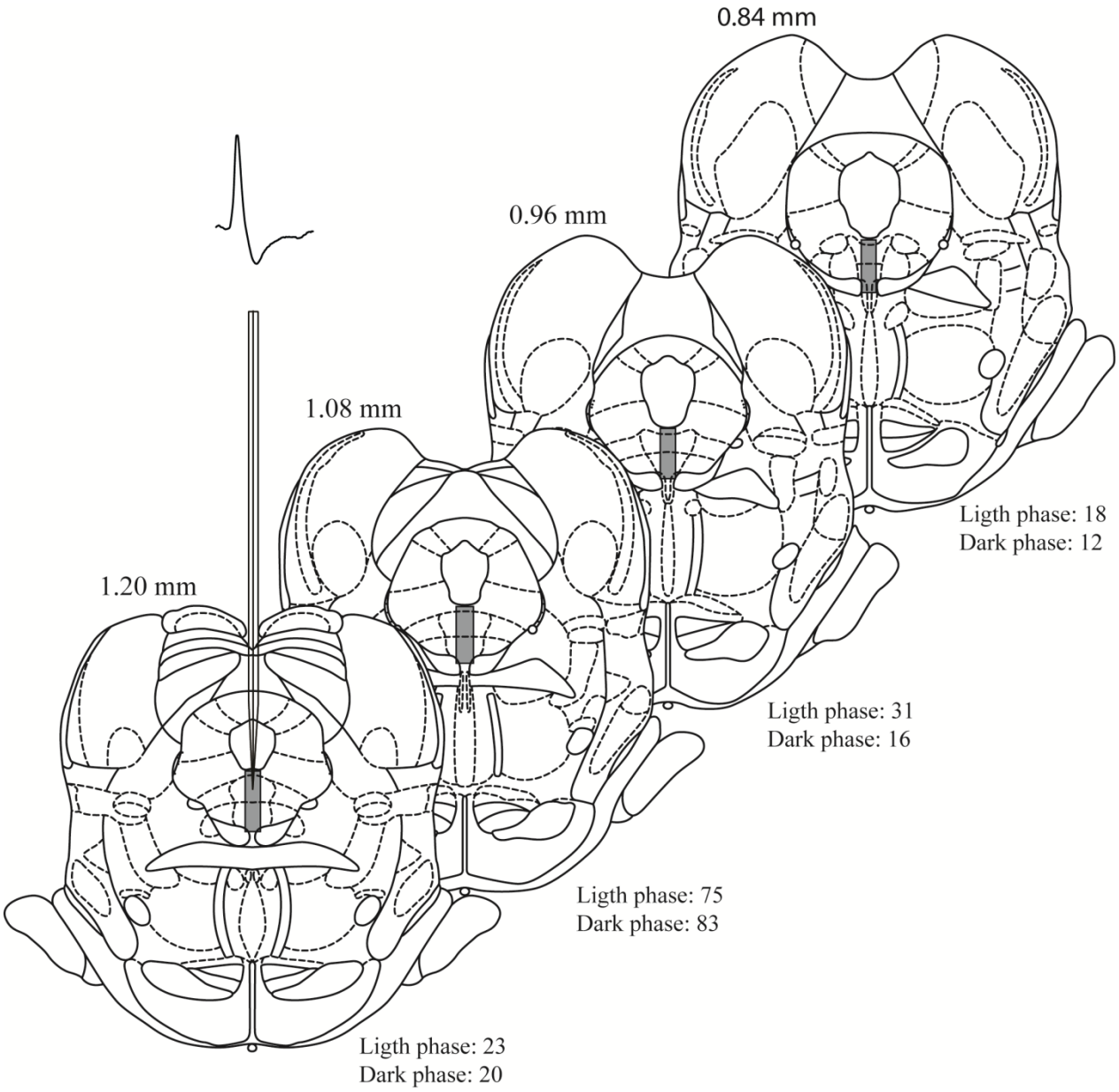



**Figure 2.** Recording sites for 5-HT neurons in the DRN. The shaded area indicates an illustrative depiction of the sites in which glass electrodes were descended into the DRN to record 5-HT neurons. The number on the top of each diagram indicates the anterior-posterior coordinates with reference to the interaural line, based on the stereotaxical atlas of Paxinos and Watson (2007). The number of neurons recorded in each phase of the cycle is indicated in the bottom of each diagram.

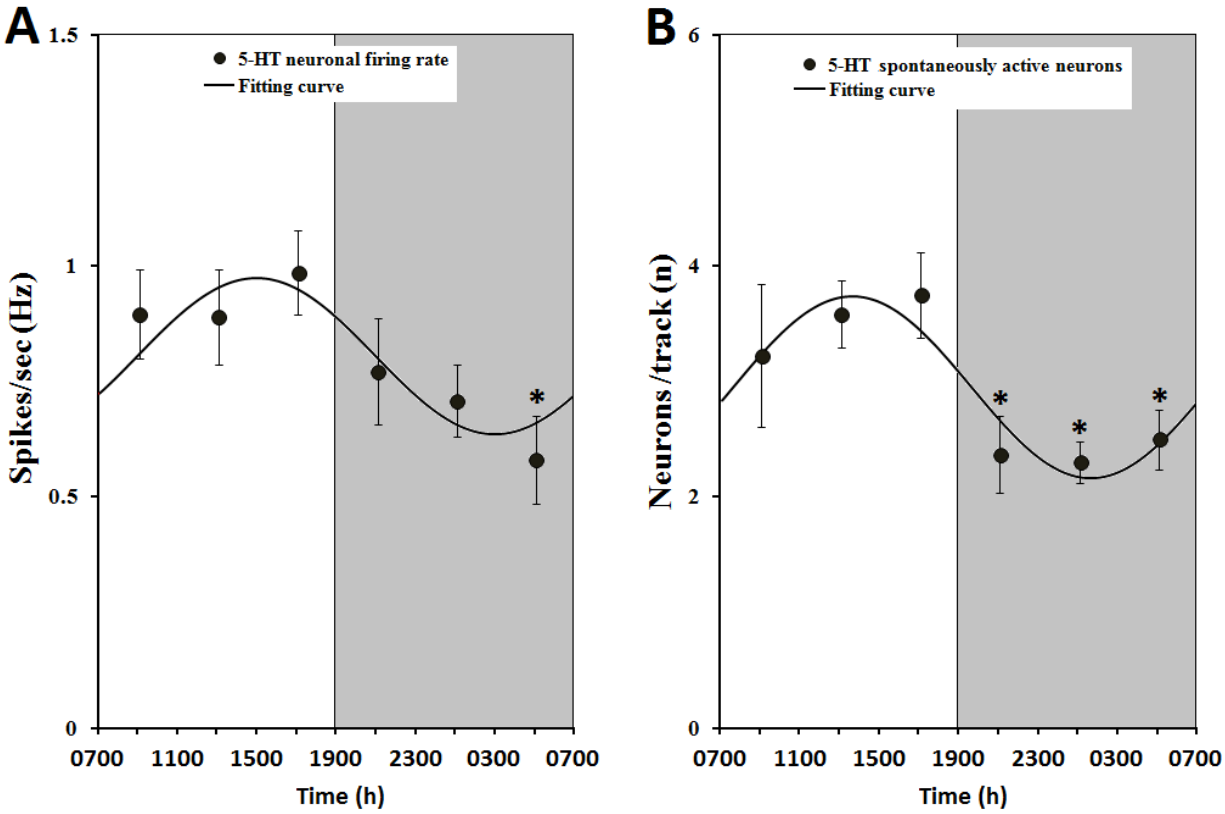



**Figure 3.** Basal firing activity of serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN) across the light-dark cycle. A) The mean firing rate of 5-HT neurons recorded in the DRN varies across the different intervals of the light-dark cycle with a period of 24 h detected with COSINOR analysis ($F_{(1,4)}$ = 15.017, p = 0.0179; adjusted $R^2$ = 0.7371). The peak 5-HT firing rate was detected at 1454 h. B) The number of spontaneously active 5-HT neurons found per electrode track showed a significant decrease at all intervals of the dark phase. A diurnal rhythm of 24 h was also detected for this parameter ($F_{(1,4)}$ = 33.4983, p = 0.0044; adjusted $R^2$ = 0.8667) with a peak detected at 1333 h. The total number of neurons recorded and the tracks performed by time interval are as follow (neurons/track): 0700-1100 h = 29/9, 1100-1500 h = 43/12, 1500-1900 h = 75/20, 1900-2300 h = 45/19, 2300-0300 h = 46/20 and 0300-0700 h = 40/16. Points represent mean ± SEM within the time interval that is indicated. In both graphs, the shadowed background indicates the dark phase. ANOVA was also performed on the data with Bonferroni t-test post hoc comparison: * $p \leq 0.05$ vs. 1900-2300 h.

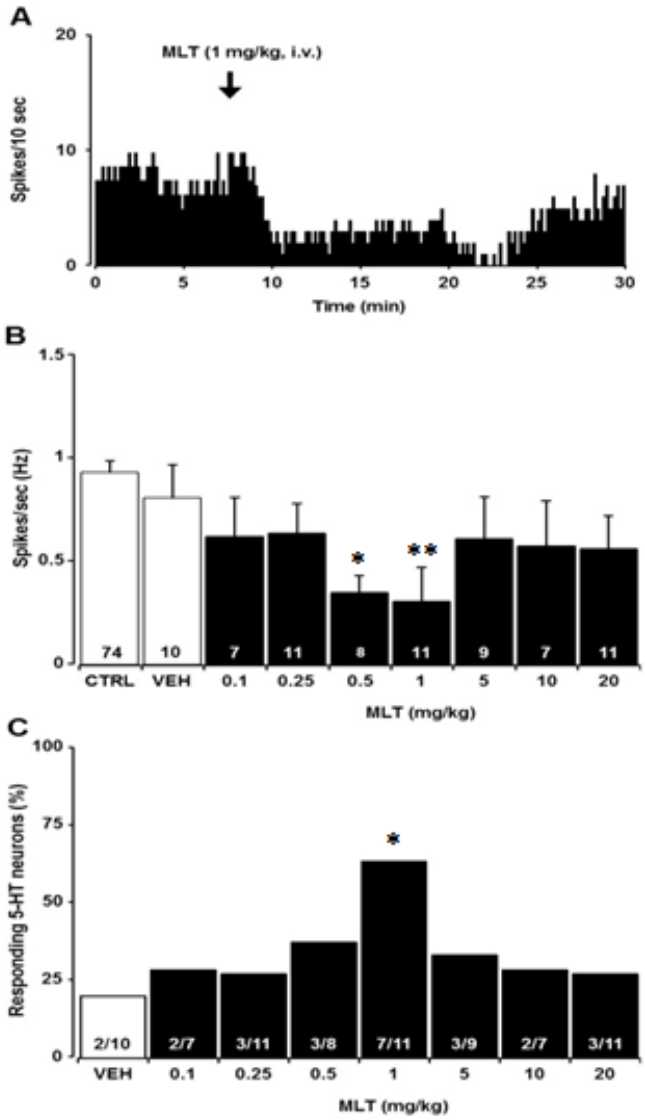
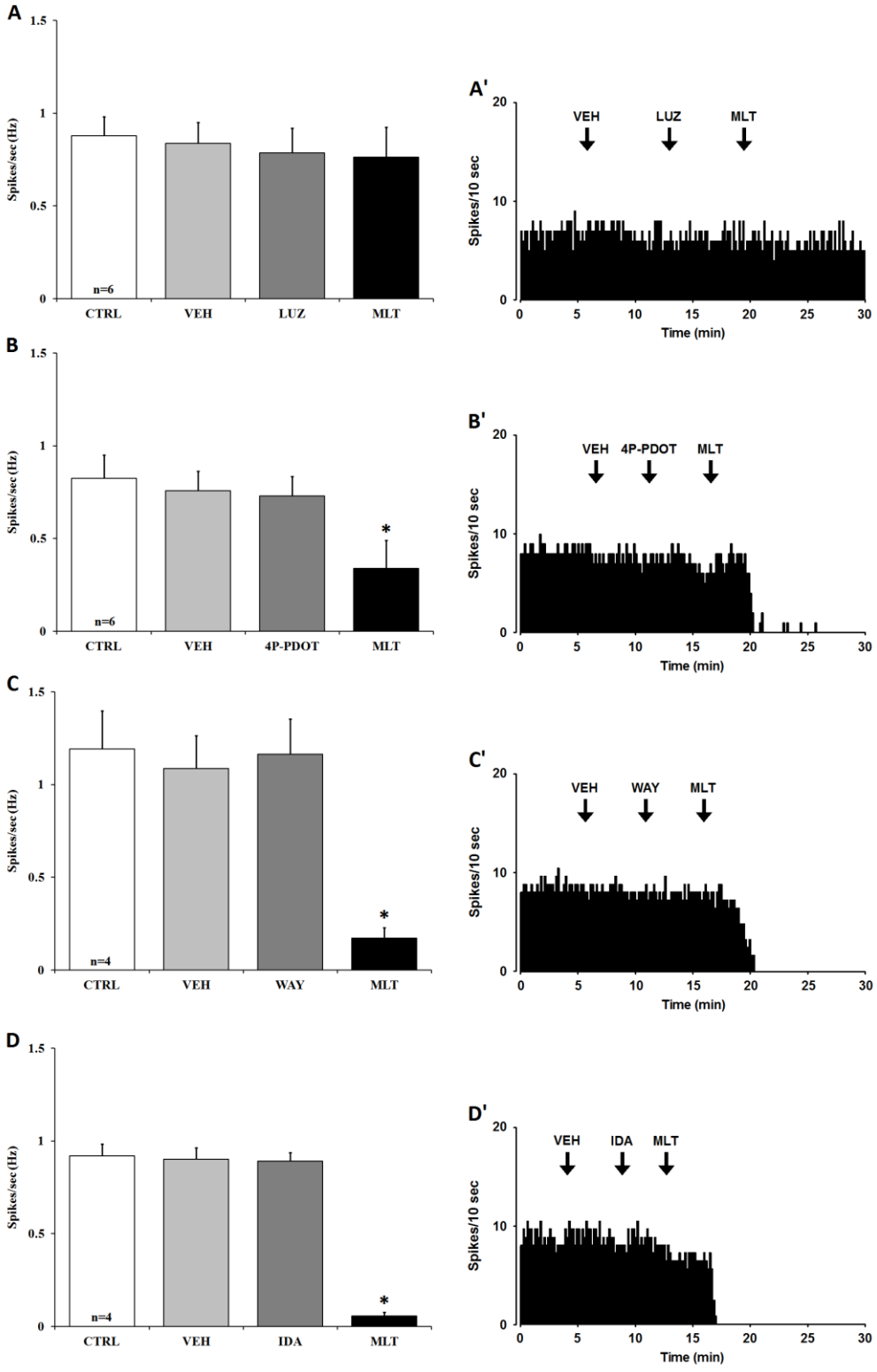



**Figure 4.** Acute dose-dependent response of dorsal raphe nucleus (DR) serotonin (5-HT) neurons to melatonin (MLT) administration. (A) Integrated firing rate histogram (10 sec bins) of a single 5-HT neuron, recorded in the DR, responding to intravenous (i.v.) injection of MLT (1 mg/kg). The arrow indicates time of injection. (B) Summary of the acute effects of the different doses of MLT from all the 5-HT neurons tested. The group of 5-HT neurons tested with the doses of 0.5 and 1 mg/kg of MLT, showed a significant inhibition from the mean firing rate (CTRL). No significant changes were observed in the 5-HT neurons tested with vehicle (VEH). Bars represent mean ± SEM; numbers within bars indicate number of neurons tested. Bonferroni t-test post-hoc comparisons: * $p < 0.05$, ** $p = 0.003$ vs. CTRL. (C) The dose of 1 mg/kg had the highest percentage of responding 5-HT neurons (i.e., firing rate inhibition of 40% from basal). Numbers within bars indicate responding neurons out of total number of neurons tested. Chi square: * $p < 0.05$ vs. CTRL.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Spikes/sec (Hz)
n=6
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**Figure 5.** Antagonism of the $MT_1$ receptor blocks melatonin (MLT)-induced acute inhibition of serotonin (5-HT) firing rate. (A) Administration of the $MT_1/MT_2$ receptor antagonist luzindole (LUZ, 1 mg/kg, i.v.) prevented the 5-HT firing-rate inhibition induced by MLT (1 mg/kg). (B) Administration of the selective $MT_2$ receptor antagonist 4P-PDOT (1 mg/kg, i.v.) did not prevent the acute inhibitory effect of MLT. (C-D) Neither pretreatment with the 5-HT1A receptor antagonist WAY100635 (WAY, 100 μg/kg, i.v.) nor with the $\alpha_2$-adrenoceptor antagonist idazoxan (IDA, 100 μg/kg, i.v.) prevented the 5-HT firing rate inhibition induced by MLT. No changes were observed after injection of vehicle (VEH). (A' to D') Representative integrated firing rate histograms of DRN 5-HT neurons showing the effect of different receptor antagonists before intravenous (i.v.) administration of MLT. Arrows indicate time of injection. Bars represent mean ± SEM; numbers within bars indicate number of neurons tested. Bonferroni t-test post-hoc comparisons: * $p < 0.05$ vs. CTRL.

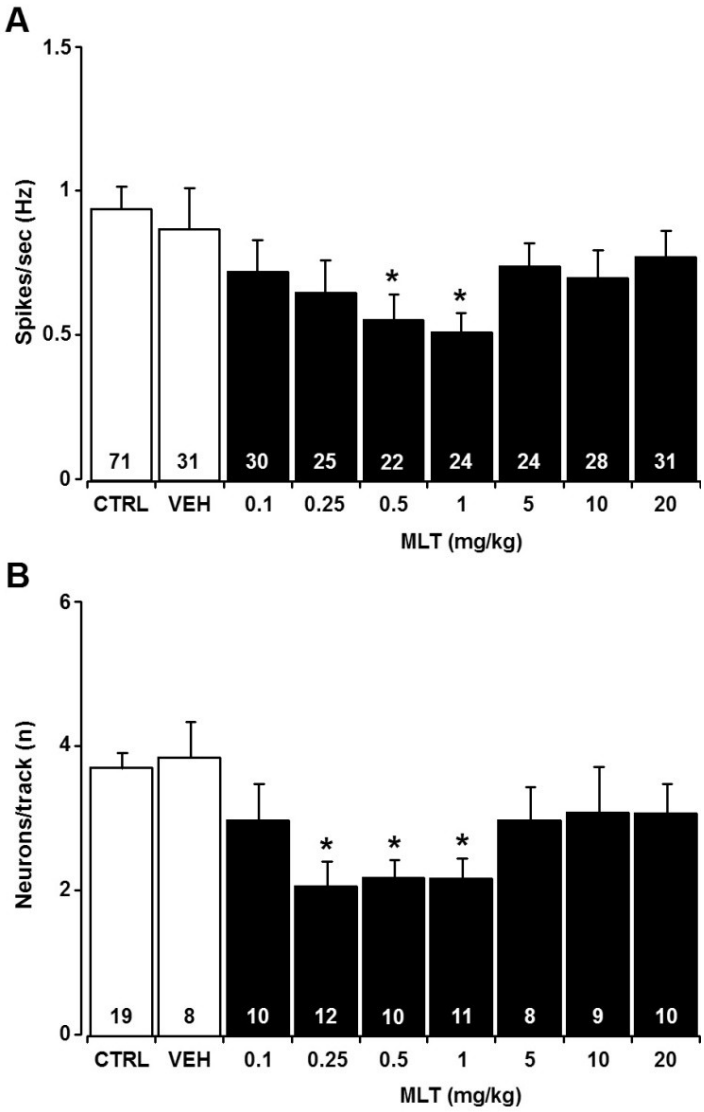


**Figure 6.** Sub-acute response of dorsal raphe (DR) nucleus serotonin (5-HT) neurons after melatonin (MLT) intravenous administration. Before MLT administration, 19 complete tracks were done in basal conditions, these recordings were considered as control (CTRL) recordings. (A) Within 45 min post-injection, MLT dose-dependently decreased 5-HT firing rate, achieving significance in the group of rats that received the doses of 0.5 and 1 mg/kg, compared with (CTRL). (B) MLT administration also reduced the number of spontaneously active 5-HT neurons per track in the rats that received the doses of 0.25, 0.5 and 1 mg/kg. No significant changes were observed after vehicle (VEH) administration. Bars represent mean ± SEM, numbers within bars in A indicate number of recorded neurons, and those in B indicate number of tracks. Bonferroni t-test post-hoc comparisons: * $p < 0.05$ vs. CTRL.

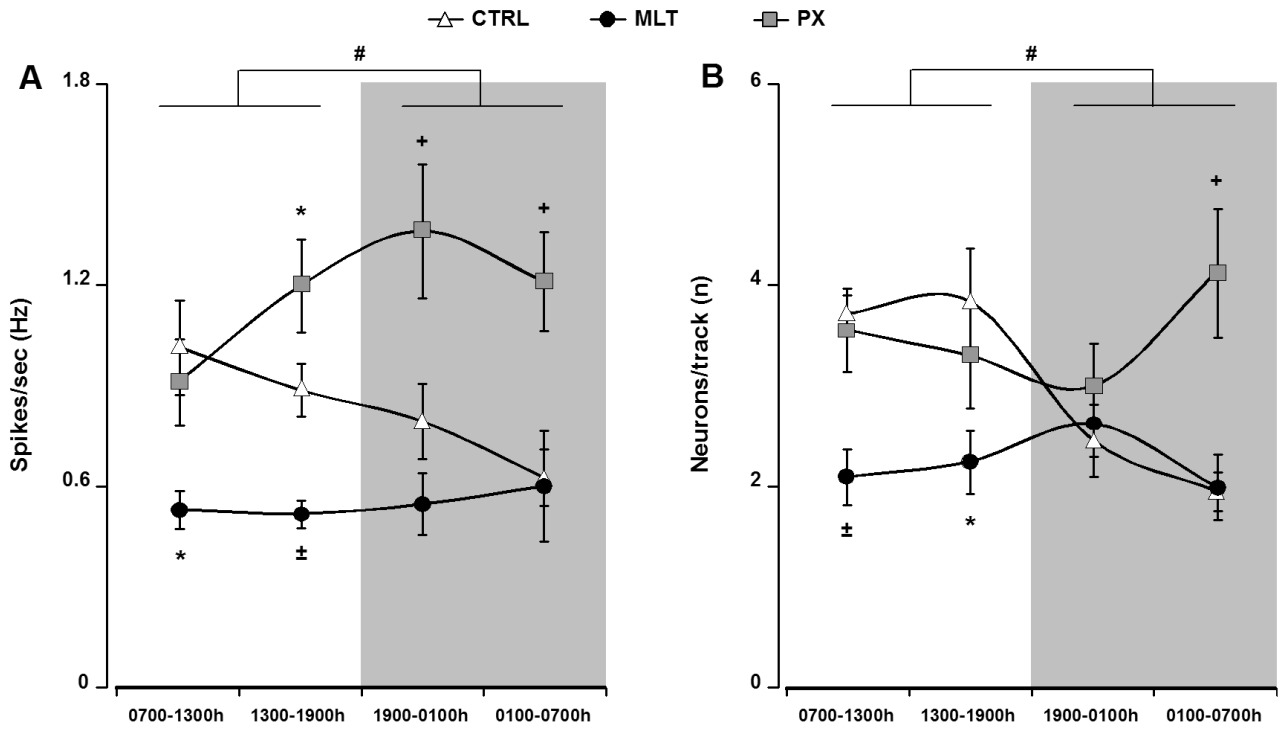


**Figure 7.** Effects of melatonin (MLT) and pinealectomy (PX) on dorsal raphe (DR) nucleus serotonin (5-HT) activity across the light-dark cycle. (A) Under basal conditions (CTRL), the mean 5-HT firing rate from the two time intervals of the light phase was greater than that of the two time intervals of the dark phase. (B) An identical pattern was observed with the number of spontaneously active 5-HT neurons. MLT (1 mg/kg) decreased 5-HT firing rate and the number of 5-HT neurons per track in the light phase but not in the dark phase (A and B, respectively, pooled data from the two time points of each phase). Conversely, PX increased 5-HT firing rate and the number of 5-HT neurons per track in the dark phase but not in the light phase (A and B, respectively, pooled data from the two time intervals of each phase). Across different time points of the light-dark cycle, MLT administration at 0700-1300 h and at 1300-1900 h reduced 5-HT firing rate (A) and the number of 5-HT neurons per track (B) in comparison to CTRL. PX increased 5-HT firing rate at 1300-1900 h, at 1900-0100h and at 0100-0700 h (A), and also increased the number 5-HT neurons per track at 0100-0700 h (B). Neurons/track per time interval and treatments are as follows: 0700-1300 h (CTRL = 26/7, MLT = 21/10, PX = 32/9); 1300-1900 h (CTRL = 46/12, MLT = 18/9, PX = 33/10); 1900-0100 h (CTRL = 32/13, MLT = 2 1/8, PX = 30/10); 0100-0700 h (CTRL = 43/22, MLT = 16/8, PX = 33/8). Points represent mean ± SEM. In both graphs, the shadowed background represents the dark phase. Bonferroni t-test post-hoc comparisons: # $p<0.05$ CTRL light phase vs. CTRL dark phase; * $p < 0.05$ vs. CTRL; + $p < 0.05$ vs. CTRL and MLT; ± $p < 0.05$ vs. CTRL and PX.

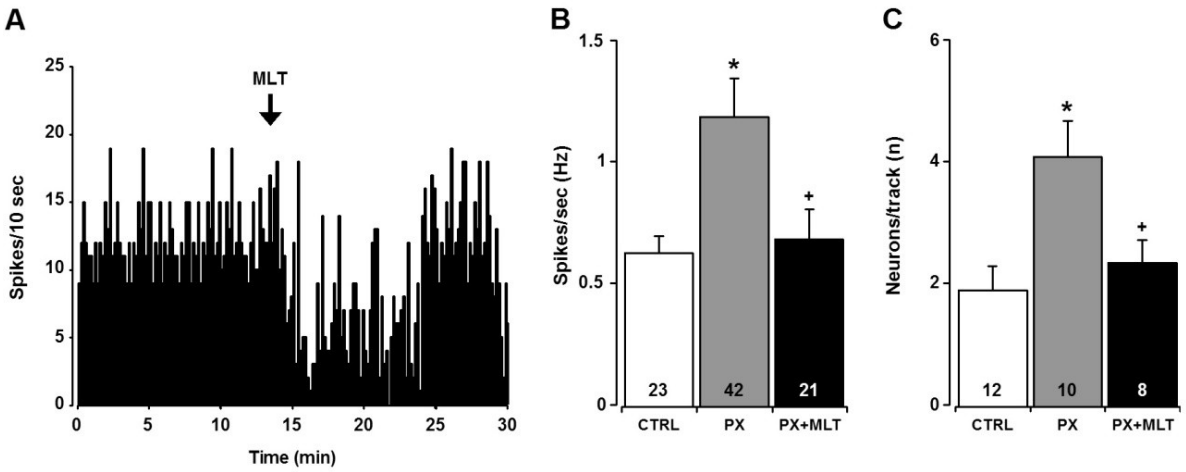


**Figure 8.** Melatonin (MLT) reversed pinealectomy (PX) effects on dorsal raphe (DR) nucleus serotonin (5-HT) firing activity during the dark phase. (A) Representative integrated firing rate histogram showing the acute inhibition induced by intravenous MLT administration (1 mg/kg) in a burst-firing DR 5-HT neuron recorded in a PX rat during the dark phase. Note the high and more irregular firing rate pattern in this particular neuron. (B-C) Sub-acutely, MLT administration reversed the increase in 5-HT firing rate and in the number of spontaneously active 5-HT neurons induced by PX during the dark phase (B and C, respectively). Bars represent mean ± SEM, numbers within bars in B indicate number of recorded neurons, and in C indicate number of tracks. Bonferroni t-test post-hoc comparisons: * $p < 0.05$ vs. CTRL; + $p < 0.05$ vs. PX.

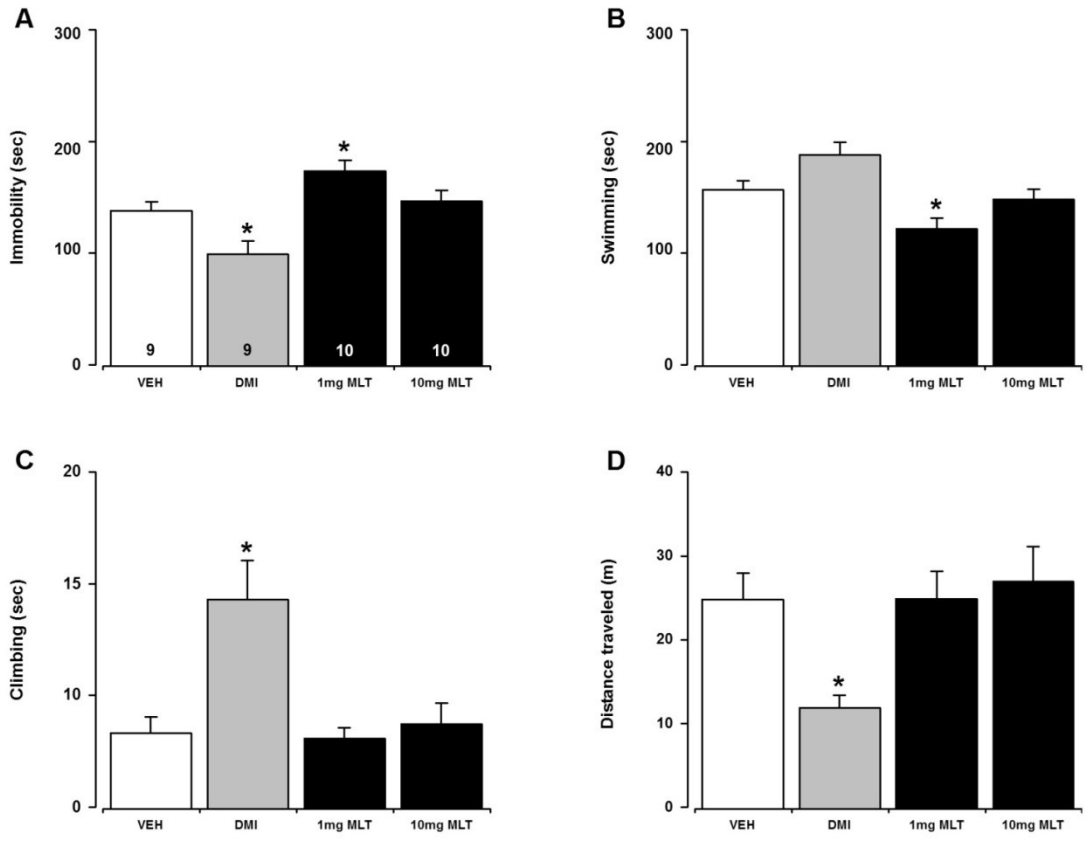


**Figure 9.** Behavioral effects of melatonin (MLT) in the forced swim test (FST). (A-B) Intraperitoneal administration of 1 mg/kg but not 10 mg/kg of MLT induced a significant increase in immobility duration (A), and a decrease in total swimming duration (B) in rats forced to swim. Desipramine (DMI) showed an antidepressant profile, reducing immobility duration and increasing swimming duration (A and B, respectively). (C) In addition, DMS induced an increase in struggling behavior. (D) DMI but not MLT (1 or 10 mg/kg) significantly decreased distance traveled during the locomotor activity test. Bars represent mean ± SEM, numbers within bars in A indicate number of rats tested. Bonferroni t-test post-hoc comparisons: * $p < 0.05$ vs. VEH.

**Table 1**. Burst activity of 5-HT neurons recorded in basal conditions in the DRN across the light-dark cycle.

| Time interval | Burst-firing neurons (%) | Firing rate (Hz) | Spikes in burst (%) | Spikes per burst (n) | Burst interspike interval (ms) | Burst length (ms) |
|---|---|---|---|---|---|---|
| 0700-1100 h | 34.48 | 1.19 ± 0.18 | 22.1 ± 6.0 | 2.2 ± 0.1 | 6.2 ± 0.6 | 13.9 ± 1.8 |
| 1100-1500 h | 27.90 | 1.12 ± 0.19 | 13.4 ± 3.0 | 2.0 ± 0.0 | 6.7 ± 0.4 | 14.5 ± 1.0 |
| 1500-1900 h | 29.33 | 1.26 ± 0.18 | 12.8 ± 3.3 | 2.0 ± 0.0 | 5.3 ± 0.5 | 11.1 ± 1.1 |
| *Light Phase* | *29.93* | *1.17 ± 0.11* | *15.6 ± 2.3* | *2.0 ± 0.0* | *6.2 ± 0.3* | *13.6 ± 08* |
| 1900-2300 h | 33.33 | 1.11 ± 0.23 | 26.0 ± 7.5 | 2.0 ± 0.0 | 5.9 ± 0.3 | 12.4 ± 0.8 |
| 2300-0300 h | 28.26 | 1.07 ± 0.18 | 16.4 ± 3.0 | 2.1 ± 0.1 | 5.6 ± 0.3 | 12.6 ± 1.1 |
| 0300-0700 h | 27.5 | 0.75 ± 0.20 | 23.1 ± 8.2 | 2.0 ± 0.0 | 5.6 ± 0.7 | 11.9 ± 1.4 |
| *Dark Phase* | *29.77* | *0.99 ± 0.12* | *22.0 ± 3.8* | *2.0 ± 0.0* | *5.7 ± 0.2* | *12.3 ± 0.6* |
| Total | 29.85 | 1.09 ± 0.08 | 18.6 ± 2.2 | 2.0 ± 0.0 | 6.0 ± 0.2 | 13.0 ± 0.2 |

Values represent mean ± SEM.

**Table 2.** Burst activity of serotonergic neurons recorded in the DRN across the light-dark cycle after pinealectomy (PX) or melatonin (MLT) administration

| Group | Time interval | Burst-firing neurons (%) | Spikes in burst (%) | Spikes per burst (n) | Burst interspike interval (ms) | Burst length (ms) |
|---|---|---|---|---|---|---|
| CTRL | 0700-1300 h | 38.5 | 18.0 ± 5.0 | 2.0 ± 0.0 | 5.3 ± 0.5 | 10.8 ± 1.0 |
| (control | 1300-1900 h | 26.1 | 16.3 ± 6.0 | 2.1 ± 0.0 | 6.1 ± 0.4 | 13.2 ± 1.0 |
| recordings) | 1900-0100 h | 25.0 | 21.8 ± 8.8 | 2.0 ± 0.0 | 6.0 ± 0.4 | 12.7 ± 0.9 |
| | 0100-0700 h | 30.2 | 10.5 ± 2.7 | 2.1 ± 0.0 | 5.9 ± 0.5 | 12.8 ± 1.1 |
| | Total | 29.9 ± 3.0 | 16.7 ± 2.3 | 2.05 ± 0.02 | 5.8 ± 0.2 | 12.3 ± 0.5 |
| MLT | 0700-1300 h | 9.5 | 9.1 ± 5.7 | 2 ± 0 | 6.4 ± 1.0 | 12.9 ± 2.1 |
| (1 mg/kg, i.v.) | 1300-1900 h | 11.1 | 8.9 ± 7.7 | 2 ± 0 | 5.8 ± 0.4 | 11.6 ± 0.8 |
| | 1900-0100 h | 9.5 | 3.1 ± 2.4 | 2.5 ± 0.5 | 11.1 ± 2.5 | 25.6 ± 8.5 |
| | 0100-0700 h | 12.5 | 11.9 ± 7.2 | 2 ± 0 | 5.3 ± 0.1 | 11.0 ± 0.5 |
| | Total | 10.6 ± 0.7* | 8.25 ± 1.8* | 2.12 ± 0.12 | 7.15 ± 1.3 | 15.3 ± 3.5 |
| PX | 0700-1300 h | 31.2 | 18.7 ± 9.2 | 2 ± 0 | 4.6 ± 0.4 | 9.5 ± 1.0 |
| | 1300-1900 h | 42.4 | 18.2 ± 5.1 | 2.14 ± 0.09 | 4.8 ± 0.4 | 10.7 ± 0.4 |
| | 1900-0100 h | 33.3 | 15.8 ± 4.6 | 2.5 ± 0.17 | 7.6 ± 0.8 | 19.6 ± 2.9 |
| | 0100-0700 h | 39.4 | 18.6 ± 6.0 | 2.23 ± 0.12 | 6.9 ± 0.6 | 15.5 ± 1.6 |
| | Total | 36.5 ± 2.6 | 17.8 ± 0.7 | 2.21 ± 0.1 | 5.9 ± 0.7 | 13.8 ± 2.3 |

Values represent mean ± SEM. Bonferroni t-test post-hoc comparisons: * $P < 0.05$ vs. CTRL and PX.

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# Foreword to Chapter 3

This chapter describes electrophysiological experiments performed to characterize the firing activity of DA neurons located in the VTA across the 24 hours of the light-dark cycle. Compared to 5-HT, diurnal changes in DA neurotransmission have been less studied. This noted, there is more evidence linking DA neurotransmission with clock genes activity than directly with the photoperiod (section 1.4.2). This is in contrast with a clear influence of photoperiod in 5-HT firing activity, an effect probably mediated by MLT endogenous secretion, as discussed in Chapter 2. The research approach was similar to that described in Chapter 2 using anesthetized rats. First basal DA firing activity was measured throughout the light and dark phases. In order to detect the existence of a diurnal rhythm of DA firing activity a COSINOR analysis was applied to the data. The COSINOR analysis is a regression and curve fitting analysis developed for the study of rhythmic changes across time. The use of this methodology helped us to detect periodical changes in firing frequency. Second, we tested whether pharmacological responses of DA neurons are influenced by time of day. For this, we used two pharmacological agents known to inhibit DA neurons firing activity; the psychostimulant AMPH and the direct DA receptor agonist APO (section 1.3.6). Using these two drugs we were able to compare the effect of selective $D_2$ autoreceptor activation by APO with an increase of DA outflow induced by AMPH. The results are discussed in the context of the mechanism of action of both drugs and the interaction between time of the day and potency. Finally, we tested the effect of acute MLT administration. We used a range of doses including the dose of 1 mg/kg, previously demonstrated to inhibit 5-HT cell firing (see Chapter 2). A manuscript based in this chapter has been accepted for publication in Synapse. An electronic version of the article can be found at DOI:10.10002/syn.21757.

# Chapter 3

# Electrophysiological characterization of dopamine neuronal activity in the ventral tegmental area across the light-dark cycle

Sergio Domínguez-López, Rebecca Dean Howell, Martha Graciela López- Canúl, Marco Leyton and Gabriella Gobbi

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**ABSTRACT**

Direct evidence that dopamine (DA) neurotransmission varies during the 24 h of the day has been lacking. Here, we have characterized the firing activity of DA neurons located in the ventral tegmental area (VTA) using single-unit extracellular recordings in anesthetized rats kept on a standard light-dark cycle. DA neuronal firing and burst activity were measured under basal conditions and in response to intravenous administration of increasing doses of amphetamine (AMPH: 0.5, 1, 2 and 5 mg/kg), apomorphine (APO: 25, 50, 100 and 200 μg/kg) and melatonin (MLT: 0.1, 1 and 10 mg/kg) at different time intervals of the light-dark cycle. A 24 h rhythm was detected in the number of spontaneously active DA neurons and a 12 h rhythm was detected in DA cell firing rates. The inhibitory effect of AMPH on DA firing rate was similar in the light and the dark phase and significant at doses higher than 1 mg/kg. The inhibitory effect of APO at the low dose of 25 μg/kg was more potent in the dark phase than in the light phase, whereas APO effects at higher doses were similar in both phases. Finally, MLT administration at 1 mg/kg produced a moderate inhibition of DA cell firing activity in both phases. Together, these experiments demonstrate, for the first time, a daily oscillation of firing activity in VTA DA neurons and a higher response of $D_2$ autoreceptors in the dark phase.

## 3.1 Introduction

Evidence from several studies has suggested the presence of diurnal activity rhythms in the mesoaccumbens dopaminergic pathway. For instance, the expression of the enzyme tyrosine hydroxylase (TH) in dopamine (DA) producing neurons in the ventral tegmental area (VTA) is increased during the light phase (Webb *et al*., 2009; Weber *et al*., 2004). In the nucleus accumbens (NAcc), an area containing dense axonal projections from the VTA, robust increases in the levels of DA, its precursor L-3,4-dihydroxyphenylalanine, and other indices of DA metabolism and release (homovanillic acid and 3,4-dihydroxyphenylacetic acid) occur during the dark phase (Castaneda *et al*., 2004; Kafka *et al*., 1986; O'Neill and Fillenz, 1985; Owasoyo *et al*., 1979; Paulson and Robinson, 1996). Since DA release is associated with DA cell firing (Andersson *et al*., 1994; Gonon, 1988; Grillner and Mercuri, 2002; Panin *et al*., 2012; Sombers *et al*., 2009), we hypothesized that VTA DA neuronal firing activity would exhibit rhythmic changes across the day.

The DA system is an important target for several psychostimulant, antipsychotic and antiparkinsonism drugs (Diana and Tepper, 2002; Ondo, 2011; Boyd and Mailman, 2012). Mesolimbic DA cell firing has been extensively used as an index of changes in DA neurotransmission in response to pharmacologic agents (Mercuri et al., 1992; Valenti and Grace, 2010; Panin et al., 2012), in particular those that activate somatodendritic DA $D_2$ autoreceptors (Ford 2014). For example, the $D_2$ receptor agonist apomorphine (APO) induces a strong inhibition of VTA DA cell firing (Skirboll *et al*., 1979; White and Wang, 1984b), which is associated with a decrease of DA release in the NAcc (Chai and Meltzer, 1992; Radhakishun *et al*., 1988) and in the VTA itself (Zhang *et al*., 1994). The psychostimulant amphetamine

(AMPH), in comparison, increases extracellular DA levels in NAcc and also inhibits DA cell firing (Di Chiara and Imperato, 1988; Diana and Tepper, 2002). To our knowledge the effects of these two drugs on DA cell firing activity have not been compared between the light and dark phases.

DA release can also be inhibited by MLT administration (Cardinali *et al*., 1975; Zisapel, 2001), a neurohormone involved in circadian and seasonal regulation (Simonneaux and Ribelayga, 2003), and an inverse correlation between striatal DA content and MLT secretion has been reported (Castillo Romero *et al*., 1992; Khaldy *et al*., 2002). In a previous study, we documented a daytime inhibitory effect of MLT administration on serotonin (5-HT) neurons recorded in the dorsal raphe nucleus (DRN) and we provided evidence of a tonic inhibitory influence of endogenous MLT secretion on 5-HT cell firing activity during the dark phase (Dominguez-Lopez et al., 2012). Since G-protein coupled MLT $MT_1$ receptors were detected in tyrosine hydroxylase positive VTA cells (Uz et al., 2005) we have hypothesized the possibility of an influence of MLT over DA neuronal activity too.

Based on the above observations, we addressed two main questions. First, does VTA DA neuron basal firing activity exhibit a diurnal rhythm? Second, do the acute effects of AMPH, APO and MLT administration on VTA DA cell firing activity change over the light and dark phases?

## 3.2 Materials and Methods

### 3.2.1 Animals

Adult male Sprague Dawley rats (Charles River, Saint-Constant, Quebec, Canada) weighing 300-330g were housed under standard laboratory conditions with a 12 h light-dark cycle (lights

on at 07:00 h) with *ad libitum* access to food and water. Experiments performed in the dark phase were conducted under dim red light provided by an infrared (IR) heating lamp (Philips, Infrared Heat, wavelength peak: 800-1200 nm). All procedures were in accordance with the guidelines set by the Canadian Institute of Health Research for animal care and scientific use and the Animal Care Committee of McGill University.

### 3.2.2 Drugs

D-amphetamine sulphate (AMPH, Sigma-Aldrich, UK), R-(-)-apomorphine hydrochloride hemihydrate (APO) and chloral hydrate (Sigma-Aldrich, Oakville, Canada) were dissolved in physiological solution of NaCl 0.9% (saline) as vehicle (VEH). MLT (Sigma-Aldrich, Oakville, ON, Canada) was dissolved in a VEH of 30% propyleneglycol (MP Biomedicals, Solon, OH, USA) in saline. All drugs were freshly prepared the day of the experiments just before being used. Intravenous (i.v.) administration of all drugs was carried out using a catheter inserted into the lateral vein of the tail. The maximum volume used for a single i.v. injection was 0.1 ml (infused in approximately 1 min).

### 3.2.3 In vivo electrophysiological recordings

Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.) in their housing room. For experiments performed during the dark phase, anesthesia was given under infrared illumination (GE, Infrared Reflector, $\lambda$ = 800-1200 nm) and black duct tape was used to cover the eyes of the rats (rats remain this way during the whole recording procedure). Animals were transported in light-free boxes to the procedural room. Surgeries and electrode placement were done with stereomicroscope assistance and a fiber optic dim light source with an integrated red light filter,

which was used during night time recordings. Rats were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and a hole was drilled through the skull. Body temperature of the animals was measured using a rectal thermometer (Yellow Springs Instrument Co., Yellow Springs, OH, USA) and was maintained at 35-36.5 °C using a IR heating lamp (Philips, Infrared Heat) which also provided dim red light illumination.

### 3.2.4 Recording of VTA DA neurons

Single-barrel glass micropipettes with an internal diameter of 1.5 mm (Harvard Apparatus, St-Laurent, QC, Canada) were pulled to a shank length of approximately 1 cm in a Narashige PE-2 pipette puller (Tokyo, Japan) and filled with 2% pontamine sky blue dye (Sigma-Aldrich, Oakville, ON, Canada) dissolved in sodium acetate (0.5 M, pH 7.5). The electrodes, with resistances ranging from 6 to 9 MΩ, were descended into the VTA using a hydraulic micropositioner (David Kopf Instruments, Tujunga, CA, USA) within the stereotaxic coordinates described in the rat brain atlas of Paxinos and Watson (2007); A-P: 3.4 to 4.1 mm from the interaural line; lateral: 0.6 to 1.1 mm from the midline; ventral: 7.5 to 8.8 mm from the brain surface. One to 5 electrode descents were done per rat. Spontaneous electrical activity of single cells was recorded using an MDA-3 amplifier system (Bak Electronics, Mount Airy, MD, USA). Spike frequencies and waveforms were collected and stored in a computer using the Spike2 software (CED, Cambridge, UK). Putative DA neurons were identified based on well-established electrophysiological properties: a wide action potential (> 2.5 ms), biphasic or triphasic waveform and slow firing (0.5–10 Hz) (Grace and Bunney, 1983; Ungless and Grace, 2012). Neuronal activity was measured by calculating the mean firing rate frequency, expressed as the number of spikes per second or Hz, and the number of spontaneously active neurons per

electrode descent or track (neurons/track). Additionally, the burst activity of DA neurons was analyzed using a script for the Spike 2 software (available on line at www.ced.co.uk). Based on criteria previously described, a burst was defined as a train of at least two spikes with an initial interspike interval (ISI) ≤ 80 ms and a maximum ISI of 160 ms, within a regular low-frequency firing pattern and decreased amplitude from the first to the last spike within the burst (Grace and Bunney, 1983; Ungless and Grace, 2012). The parameters analyzed with the Spike 2 script included the percentage of spikes fired in burst, the number of spikes per burst, the mean burst ISI (ms) and mean burst length (ms). In the final track of each rat, pontamine sky blue dye was injected iontophoretically by passing a constant positive current of 20 μA for 5 min through the recording pipette to mark the recording site. Then rats were decapitated and their brains extracted and placed in formaldehyde solution (4%) for at least two days. Subsequent localization of the labeled site was made by visual inspection of 40μm-think brain frontal sections.

### 3.2.5 Experimental procedure

A series of single-unit extracellular recordings were performed in groups of rats assigned to one of six time intervals across the light-dark cycle, three intervals belonging to the light phase (07:00-11:00 h, 11:00-15:00 h and 15:00-19:00 h) and three belonging to the dark phase (19:00-23:00 h, 23:00-03:00 h and 03:00-07:00 h). Firing activity of DA neurons was compared between phases and across the different time intervals. In addition, a COSINOR analysis (non-linear curve fitting analysis) was also performed on the data to identify rhythms in DA neuronal firing activity. Based on methodology described elsewhere (Barnett and Dobson, 2010; Lentz, 1990), mean values of firing rate and neurons per track were used to fit the equation: $y = M + a \cos (2\pi x/Prd) + b \sin (2\pi x/Prd)$, where $M$ is the MESOR (Midline Estimating Statistic of

Rhythm, mean of the sample), *a* and *b* are the coefficients to be calculate, *Prd* is the fixed period of the cycle, *x* is a time point in the cycle, and *y* is the value of the firing activity parameter under evaluation.

In some rats, the effects of i.v. administration of cumulative doses of AMPH (0.5, 1, 2 and 5 mg/kg), APO (25, 50, 100, 200 μg/kg) and MLT (0.1, 1 and 10 mg/kg) on DA cell firing activity were assessed. For this, a stable DA neuron was chosen and its basal firing activity (CTRL) was recorded for at least five minutes. Then rats were injected first with VEH and every five minutes with sequential doses of one of the three drugs (AMPH, APO or MLT) until all doses were tested. Only one neuron was tested per rat and only one drug was tested per neuron.

### 3.2.6 Statistical analysis

Data were analyzed using SigmaPlot statistical suit (Systat Software, Inc.). One-way ANOVA was used to compare recordings in basal conditions across different time intervals and two-tailed Student *t*-tests were used for comparisons between light and dark phases. SigmaPlot dynamic fit function for curve fitting and non-linear regression analysis was used in the COSINOR analysis. One-way ANOVA for repeated measures was used to compare neuronal responses to cumulative administration of drugs (AMPH, APO or MLT). Two-way ANOVA was used to determine interactions between phase of the cycle and dose of drug administered (Phase x Dose), and time interval x Dose. For post hoc comparisons after ANOVAs, Bonferroni corrected *t*-tests were used. Neuronal responses to drugs were compared between time intervals of the light-dark cycle as percentage change from CTRL. All data are reported as mean ± standard error of the mean (SEM). Statistical values of $p \leq 0.05$ were considered significant.

## 3.3 Results

### 3.3.1 Basal firing activity of DA neurons in the VTA across the light-dark cycle

A total of 285 DA neurons were recorded in the VTA from 68 rats. Of these neurons, 145 were recorded in the light phase (36 rats) and 140 were recorded in the dark phase (32 rats). Recording sites are shown in Figure 1. Comparing light and dark phases, no differences were found in DA firing rate (light phase: 2.9 ± 0.2 Hz; dark phase: 3.0 ± 0.2 Hz; $t_{(283)} = 0.18$, $p = 0.859$) or in the number of spontaneously active neurons per track (light phase: 2.2 ± 0.1 neurons/track in 67 tracks; dark phase: 2.3 ± 0.1 neuron/track in 61 tracks; $t_{(126)} = 0.64$, $p = 0.520$). Data analysis by intervals of time revealed a significant increase ($F_{(5,279)} = 2.33$, $p = 0.043$) in DA cell firing rate at 19:00-23:00 h compared to lower firing rates observed at 11:00-15:00 h ($p = 0.029$), as shown in Figure 2A. The number of spontaneously active DA neurons recorded per track oscillates across time intervals but no significant changes were detected with ANOVA ($F_{(5,122)} = 2.29$, $p = 0.12$), as shown in Figure 2B. Of all DA neurons recorded, approximately 64% were burst-firing neurons (184 neurons) exhibiting a significantly higher frequency of discharge than single-firing neurons (single-firing: 1.9 ± 0.1 Hz; burst-firing: 3.5 ± 0.2 Hz; $t_{(283)} = 5.44$, $p < 0.001$). The percentage of burst-firing DA neurons did not change across the light-dark cycle ($\chi^2_{(5)} = 1.079$, $p = 0.95$), as shown in Table 1. A significant increase in the percentage of spikes fired in burst mode ($F_{(5,178)} = 3.23$, $p = 0.008$) was detected at 15:00-19:00 h compared with all the other intervals except for 19:00-23:00 h, as indicated in Table 1. No other changes were observed in burst activity parameters.

### 3.3.2 Rhythms in DA firing activity

After visual inspection of the data, fixed periods of 12 h and 24 h were used to fit DA firing rate and neurons per track values across time intervals, respectively. Adjusted coefficients of determination (adj$R^2$) obtained with COSINOR analysis, indicates that DA firing rate (adj$R^2$ = 0.88; $F_{(1,4)}$ = 39.7, p = 0.003) and DA neurons per track data (adj$R^2$ = 0.73; $F_{(1,4)}$ = 14.7, p = 0.019) can be both expressed as rhythmic functions of time according with parameters given in Table 2. For DA cell firing rates, peaks of activity were estimated to occur within the first three hours of each phase, at 08:40 h and at 20:40 h (Figure 2A). The peak in number of spontaneously active DA neurons was estimated at 05:54 h, near the end of the dark phase (Figure 2B).

### 3.3.3 AMPH inhibits DA neurons firing activity independently of the phase of the cycle

The acute effect of AMPH administration was tested in 16 neurons in the light phase and 15 neurons in the dark phase. AMPH induced a dose-dependent inhibition of DA cell firing ($F_{(4,116)}$ = 93.61, p < 0.001) but there was not an effect of Phase (F(1,116) = 0.1, p = 0.751) or a Phase by Dose interaction ($F_{(4,116)}$ = 0.222, p = 0.92). A significant reduction of DA firing rate was observed after the dose of 1 mg/kg with additional decreases after 2 and 5 mg/kg of AMPH (p < 0.001 vs. VEH, in all cases). Representative examples are shown in Figure 9. Similarly, only a main effect of Dose was observed in the burst activity parameters (percentage of spikes in burst: $F_{(4,108)}$ = 37.25, p < 0.001; number of spikes per burst: $F_{(4,108)}$ = 48.6, p < 0.001; burst interspike interval: $F_{(4,108)}$ = 46.31, p < 0.001; burst length: $F_{(4,108)}$ = 47.83, p < 0.001), as shown in Figure 4), independently of the phase of the cycle. In most cases, indices of burst activity were significantly reduced after administration of 2 mg/kg of AMPH and further reduced with the

dose of 5 mg/kg ($p < 0.001$ vs. VEH, in all cases). Data broken down by time intervals are shown in supplementary Tables S1 (for firing rate) and S2 (for burst activity).

### 3.3.4 APO inhibition of DA neurons firing activity was stronger in the dark phase

The acute effect of APO administration was tested in 12 neurons in the light phase and 14 neurons in the dark phase. A significant Phase by Dose interaction was found in DA cell firing ($F_{(4,96)} = 3.45$, $p = 0.011$). In both phases, inhibition of DA firing frequency was dose-dependent and significant with all doses of APO tested ($p < 0.001$ vs. VEH, in all cases), as shown in Figure 5. Between phases, the reduction of DA neuronal firing rate induced by the lowest dose of APO (25 μg/kg) was more pronounced in the dark phase as compared with the effect observed in the light phase ($p = 0.005$). Representative examples are shown in Figure 9. APO administration also resulted in a dose-dependent decrease in all DA cell burst activity parameters (percentage of spikes in burst: $F_{(4,84)} = 77.1$, $p < 0.001$; number of spikes per burst: $F_{(4,84)} = 53.83$, $p < 0.001$; burst interspike interval: $F_{(4,84)} = 64.87$, $p < 0.001$; burst length: $F_{(4,84)} = 61.58$, $p < 0.001$, as shown in Figure 6), but no effect of Phase or Phase by Dose interaction was detected. The percentage of spikes in burst and burst length were significantly reduced with APO at 25 mg/kg ($p < 0.001$ and $p = 0.043$ vs. VEH, respectively). In general, all burst indices were substantially reduced after administration of APO at 50 μg/kg with no additional significant reductions at doses of 100 and 200 μg/kg ($p < 0.001$ vs. VEH, in all cases). Data broken down by time intervals are shown in supplementary Tables S3 (for firing rate) and S4 (for burst activity).

### 3.3.5 MLT inhibits DA firing activity independently of the phase of the cycle

The acute effect of MLT administration was tested in 15 neurons in the light phase and 12 neurons in the dark phase. MLT induced a dose-dependent decrease in DA cell firing frequency ($F_{(3, 75)} = 11.29$, $p < 0.001$) but there was not an effect of Phase ($F_{(1, 75)} = 0.31$, $p = 0.58$) or Phase by Dose interaction ($F_{(3, 75)} = 0.32$, $p = 0.81$) (Figure 7). The decrease in firing rate was significant after administration of 1 mg/kg of MLT, with no further decrease following 10 mg/kg ($p < 0.001$ vs. VEH, in both cases). Representative examples are shown in Figure 9. A main effect of Dose was also detected in the burst activity parameters (percentage of spikes in burst ($F_{(3,51)} = 4.38$, $p = 0.008$); number of spikes per burst ($F_{(3,51)} = 5.83$, $p = 0.002$); burst interspike interval ($F_{(3,51)} = 5.39$, $p = 0.003$); and burst length ($F_{(3,51)} = 5.52$, $p = 0.002$) as shown in Figure 8). Decreases of burst activity parameters were statistically significant following administration of 1 mg/kg MLT ($p < 0.05$ vs. VEH, in all cases) with no additional decreases after 10 mg/kg. Data broken down by time intervals are shown in supplementary Tables S5 (for firing rate) and S6 (for burst activity).

## 3.4 Discussion

Our results provide the first direct evidence of rhythms in VTA DA cell firing activity. The number of active DA neurons varies with a 24 h rhythm with a peak occurring toward the end of the dark phase. DA cell firing rate, in comparison, exhibits a period of 12 h with peaks occurring within the first three hours of the light and the dark phase. We also detected an increase in the percentage of spikes fired in burst occurring at 15:00-19:00 h in the light phase. A previous attempt to characterize diurnal patterns of DA neuronal firing activity did not identify differences in DA cell firing activity between light and dark phases (Luo *et al*., 2008). However, in this

previous study, recordings were restricted to eight hours within either the light or the dark phase of the cycle, and the hours before and after phase transitions were not recorded. In comparison, we were able to detect a 24 h period in spontaneously active neurons and a 12 h period in firing rate by grouping recordings in four-hour intervals and including the phase transitions.

The number of spontaneously active DA neurons reported in our experiments is relatively high (2.2 ± 0.15 neurons/track on average) compared with what is usually reported in the literature (≈ 1 neuron/track), using a grid sample method (Bunney and Grace, 1978; Chiodo and Bunney, 1983). The number of cells we recorded per track ranged from 0 - 6, which has also been reported by others (Ungless *et al*., 2004). The disparity in average number of neurons per track can be due to differences in techniques, such as using a new electrode for each track, especially when a large number of tracks are being performed (Morzorati *et al*., 2010), or differences in recording sites. Indeed, neurons in caudal regions of the VTA seem to be less active (i.e. decreased c-fos expression, an index of cellular activity) than neurons located in middle and rostral parts (Balfour *et al*., 2004) where our recordings were made. In concordance with our findings, it has been reported that the number of TH immunoreactive neurons co-expressing c-fos in the VTA decreased during the light phase and increased in the middle and to the end of the dark phase (Baltazar *et al*., 2013). This pattern of cellular activity in the VTA is similar to the one observed for spontaneously active DA neurons in our experiments, identified using well-established electrophysiological parameters (Ungless and Grace, 2012) and by their positive responses to AMPH or APO administration. Luo *et al*. (2008) also reported a tendency in the number of active VTA neurons to increase during the dark phase which was attributed to a novel

class of neurons that were not identified as DA or GABAergic neurons, but were possibly glutamatergic neurons (Dobi *et al*., 2010; Hnasko *et al*., 2012).

A more extensive time sampling in the present study allowed us to unveil rhythmic changes in DA cell firing activity in the VTA. However, it remains to be established how these rhythms are generated. Our results with MLT administration may suggest a subtle inhibitory influence of MLT on DA cell firing (see discussion below). However, neither periodical changes in firing frequency or number of spontaneously active neurons seem to be associated with the diurnal pattern of MLT secretion, usually reported to increase at night (Liu and Borjigin, 2006). Alternatively, there is evidence that the VTA receives indirect innervation originating from the suprachiasmatic nucleus (SCN) of the hypothalamus (Luo and Aston-Jones, 2009). The complex molecular-cellular mechanism that exists in the SCN, involving clock gene expression and light photonic input from the retina, is believed to act as a central master clock, synchronizing circadian rhythms in the body (Morin, 2013). Neuronal activity in the SCN is higher during the light phase, in both nocturnal and diurnal species, following a rhythm of activity with a period close to 24 h that is reset and entrained by light (Houben *et al*., 2009; Inouye and Kawamura, 1979; Reppert and Weaver, 2001). Therefore, it is possible that the 24 h rhythm detected in the number of active DA neurons in the VTA may be related to a regulatory influence of the SCN, but out of phase by approximately six hours, indicative of an indirect regulation. This may involve the medial preoptic nucleus as a relay structure, as previously suggested by Aston-Jones and colleagues (Luo *et al*., 2008; Luo and Aston-Jones, 2009). The SCN has been also proposed to influence, to some extent, the rhythmic expression of the DA transporter (DAT) and TH, not only in the VTA but also in mesolimbic terminal areas such as the NAcc (Sleipness *et al*., 2007;

Webb *et al*., 2009; Weber *et al*., 2004). Interestingly, TH expression in the VTA seems to have an opposite pattern of diurnal expression compared with TH expression in the NAcc (Webb *et al*., 2009), suggesting time differences in DA synthesis at somatodendritic and terminal regions that can potentially affect DA cell firing activity. In addition, DA neuron activity in the VTA is also modulated by several neurotransmitters (e.g. GABA, glutamate, 5-HT, NA and acetylcholine) and neuronal circuits (Adell and Artigas, 2004), of which diurnal influence on local DA release and cell firing remains to be evaluated. Theoretically, all these regulatory factors could be contributing to a long 24 h rhythm in the number of active DA neurons and a short ultradian rhythm of 12 h in their firing rates. The dynamics governing the desynchronization between firing frequency and spontaneously active neurons remains to be explored. Furthermore, it remains to be established if the photoperiod is necessary for the rhythmic changes of DA cell firing or if these rhythms are of a circadian nature (i.e. self sustained, self generated), as suggested by the influence of the SCN and the presence of seemly functional clock genes in the VTA (McClung *et al*., 2005; Mukherjee *et al*., 2010; Webb *et al*., 2009).

In our experiments, the inhibitory effects of AMPH administration on DA neuronal firing rate and burst activity were equipotent between the light and dark phases. The effects of AMPH on DA neurotransmission are mediated in part by its interaction with the DAT (i.e. reuptake competitive inhibition and reverse transport of DA) which results in a net increase of extracellular DA levels (Eriksen *et al*., 2010; Sulzer *et al*., 2005). The overflow of DA activates $D_1$ and $D_2$ receptors in terminal areas and $D_2$-like ($D_2$ and $D_3$) autoreceptors at the somatodendritic level contributing to the inhibitory effects of AMPH on DA cell activity in

midbrain neurons (Bunney and Aghajanian, 1978; Bunney *et al*., 1973; Diana and Tepper, 2002; Shi, 2009). Initially, our results suggest that the increase in extracellular DA induced by AMPH is not influenced by the light-dark cycle. This is at odds with reported diurnal changes in DAT protein expression and DA clearance in terminal areas including the NAcc (Sleipness *et al*., 2007; Sleipness *et al*., 2008) and with reported diurnal changes in behavioral response to AMPH administration (Wolfe *et al*., 1977; Gaytan *et al*., 1998a, b; Webb *et al*., 2009). However, Sleipness *et al*., (2008) also reported that diurnal changes in DA clearance are flattened in the presence of cocaine, whose mechanism of action is also mediated by the DAT. In addition, AMPH also promotes extracellular DA levels by several other mechanisms that include activation of TH, inhibition of the enzyme monoamine oxidase and increasing vesicular release of DA in the cytosol (Sulzer, 2011). It is then possible that the broad effect of AMPH on DA neurotransmission obliterated any existing rhythmic change, resulting in a similar inhibitory effect in the firing activity of VTA DA neurons in the light and dark phase. The diurnal effect observed with APO administration indicate that the effects of AMPH in VTA DA cell firing are not influenced by diurnal $D_2$ receptor availability (see below). Recently, it has been proposed that AMPH can also increase extracellular DA by up-regulating vesicular DA release and enhancing phasic-like transients of DA release (Daberkow *et al*., 2013). This suggests that diurnal differences may still exist in the effects of AMPH on DA neurotransmission, independently of its effects on VTA DA cell firing.

The inhibitory effect of APO administration on DA cell firing rate was significantly more potent in the dark phase, particularly with the lower doses tested (25 μg/kg). The strong inhibitory effect of APO was also observed in DA burst activity, but differences between light and dark

phase were not significant. Although APO is a $D_2$ receptor agonist ($D_{2S}$ Ki = 35nM; $D_{2L}$ Ki = 83nM) with lower affinity for the $D_1$ receptor ($D_1$ Ki = 372 nM) (Millan *et al*., 2002), its inhibitory effects on midbrain DA cell firing is thought to be mediated primarily by $D_2$ somatodendritic autoreceptor activation (Diana and Tepper, 2002; Shi, 2009; White and Wang, 1984b). Indeed, systemic APO administration at doses lower than 50 μg/kg, are considered to preferentially activate $D_2$ autoreceptors (Jeziorski and White, 1989; Skirboll *et al*., 1979; Valenti and Grace, 2010). However, some VTA and SN DA neurons only respond to doses of APO higher than 50 μg/kg (Chiodo *et al*., 1984; Freeman and Bunney, 1987; White and Wang, 1984a). It is possible that at higher doses (> 50 μg/kg) APO also activates postsynaptic $D_2$ and $D_1$ receptors in DA terminal areas such as the caudate nucleus, NAcc and globus pallidus, and can lead to long loop inhibitory feedback (Carlson *et al*., 1987; Rebec *et al*., 1979; Shi *et al*., 2000; Skirboll *et al*., 1979). Therefore, it is reasonable to assume that a higher sensitivity of VTA DA neurons to APO administration can be associated with a higher density of $D_2$ somatodendritic autoreceptors in the dark phase, even if some contribution of post-synaptic $D_2$ heteroreceptors cannot be ruled out (Anzalone *et al*., 2012). To our knowledge, there are no available reports on a putative diurnal expression of $D_2$ receptors in the VTA and few studies have reported a diurnal expression of DA receptors in terminal areas. For example, in rat striatum, $D_2$ receptor binding peaks in the middle of the dark phase with lower levels occurring in the light phase (Torner *et al*., 1998). Accordingly, $D_2$ receptor mRNA expression in the caudate-putamen region is also reported to be higher during the dark phase than the light phase, while $D_1$ receptor mRNA expression seems to be constant across the light-dark cycle (Weber *et al*., 2004). The diurnal response of $D_2$ autoreceptors to APO suggested that these receptors may contribute to the basal rhythm of firing activity detected in DA neurons, though, out of phase.

More research will be necessary to determine if a higher expression of $D_2$ autoreceptors also occurs in the VTA at night, which could help to explain a stronger effect of APO in DA cell firing in the dark phase, and to understand how diurnal changes in $D_2$ autoreceptors could contribute to rhythmic changes of DA neurons activity.

Finally, we observed that acute MLT administration induced a moderate decrease in the firing activity of VTA DA neurons. Interestingly, injection of MLT at 10 mg/kg did not induce further decreases in DA cell firing rate and burst activity than that achieved by the previous dose of 1 mg/kg. Although, this could be due to a saturation effect of MLT administration, it is more likely that the dose of 10 mg/kg was indeed devoid of effect. This is because we evaluated the effect of MLT using sequential-cumulative doses in the same DA neuron. Indeed, a previous study reported that a single dose of MLT at 4 mg/kg produced no significant effects on DA cell firing rate (Millan *et al*., 2003). We observed a similar response in 5-HT neurons recorded in the dorsal raphe nucleus where firing activity was inhibited by single doses of MLT from 0.5 and 1 mg/kg but not at higher doses (5 to 20 mg/kg) (Dominguez-Lopez *et al*., 2012). Therefore, the acute inhibitory effects of MLT on monoaminergic neurons occur selectively at low doses (1 mg/kg). This raises the possibility that different cellular mechanisms are activated by higher doses of MLT counteracting its own inhibitory effects on monoaminergic cells. For example, a recent study showed that a two-day administration of MLT at a dose of 20 mg/kg/day induced an increase of spontaneously active DA neurons without affecting their firing rate and burst activity (Chenu *et al*., 2014). Also agomelatine, a MLT receptor agonist with 5-$HT_{2B}$ and 5-$HT_{2C}$ receptor antagonist properties, is reported to induce excitatory effects on DA and 5-HT neurons (Chenu *et al*., 2013; Chenu *et al*., 2014; Millan *et al*., 2003).

There is evidence that the action of MLT on DA neurons could be mediated by MLT receptors. In mice, neurons located in the striatum and in the NAcc expressing $D_2$ receptors, and neurons located in the VTA expressing TH, are also positive for $MT_1$ mRNA expression (Uz *et al.*, 2005). $MT_1$ and $MT_2$ receptor mRNA expression have been reported in human SN (Adi et al., 2010). In addition, recent evidence generated by our group suggests the presence of somatodendritic $MT_1$ and $MT_2$ receptors in the SN and VTA of rats (Lacoste *et al*., 2014, unpublished). MLT receptors activation initiates several transduction mechanisms and intracellular responses (Dubocovich *et al*., 2010), including $MT_1$-mediated increase in potassium conductance via inwardly rectifying potassium channels Kir3 (formerly GIRK) (Nelson *et al*., 1996). The presence of Kir3 channels in DA neurons in the VTA (Eulitz *et al*., 2007) suggests that MLT could induce hyperpolarization of DA neurons through increased potassium conductance, similar to what has been observed in hippocampus and SCN neurons (Zeise and Semm, 1985; Mason and Brooks, 1988; Jiang *et al*., 1995). The relevance of all these observations and the contribution of MLT secretion from the pineal gland to rhythm changes of DA neurons activity require further evaluation.

### 3.5 Conclusion

In conclusion, our results demonstrate for the first time the existence of rhythmic changes of mesolimbic DA neuronal firing activity. The number of DA active neurons has a 24 h period of activity and their firing rate oscillates in a 12 h rhythm. These rhythms are probably the result of a complex interaction between different regulatory factors that remain to be identified. In addition, we observed that APO administration induced a more potent inhibition of DA firing

activity during the dark phase. Since this effect was observed at a dose that selectively activated $D_2$ autoreceptors (25 μg/kg), we speculate that $D_2$ autoreceptors could be expressed at a higher density in VTA DA neurons during the dark phase. A diurnal change in somatodendritic $D_2$ autoreceptor could be also contributing to the rhythms observed in basal DA cell firing activity. In comparison, the inhibitory effect of AMPH administration in VTA DA neurons seems to be independent of the light-dark cycle, probably reflecting the robust disruption induced by AMPH in DA neurotransmission. Finally, the acute effect of MLT on DA firing activity at low doses (1 mg/kg) is inhibitory and independent of the light-dark cycle. A direct action of MLT on its receptors in DA neurons is possible but remains to be determined.

# Figures and Tables

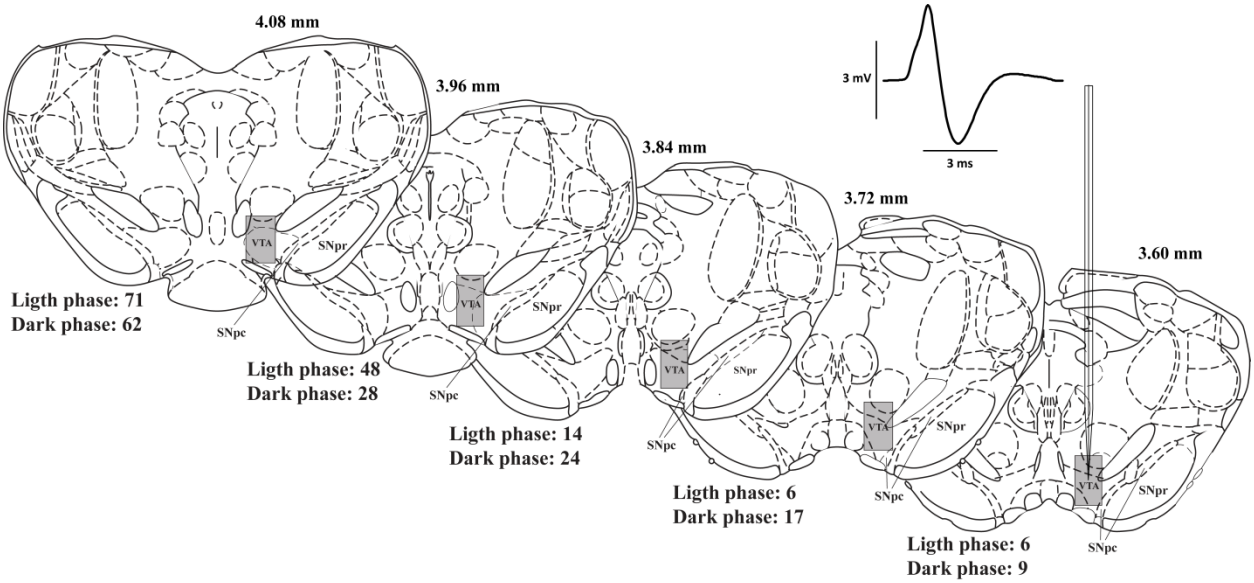


**Figure 1.** Recording sites of dopamine (DA) neurons in the ventral tegmental area (VTA). The shadowed area indicates the sites in which electrodes were descended into the VTA. The insert at the top is a representative spike-waveform from a recorded DA cell. The number of DA neurons recorded in each phase of the cycle is indicated in the bottom of each diagram. Numbers on the top of each diagram indicate the anterior-posterior coordinate with reference to the interaural line, based on the rat stereotaxic atlas of Paxinos and Watson (2007). SNpc: Substantia nigra pars compacta; SNpr: Substantia nigra pars reticulata.

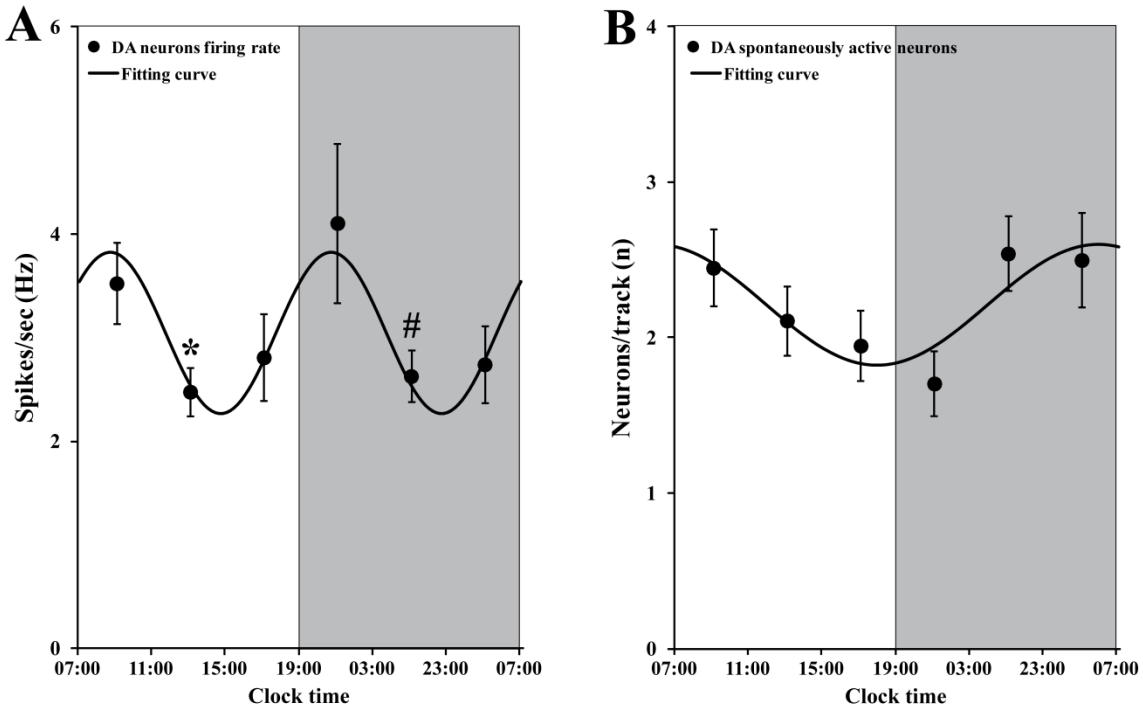


**Figure 2.** Firing activity of dopamine (DA) neurons in the ventral tegmental area (VTA) across the light-dark cycle in anesthetized rats. A) The mean firing rate of DA neurons recorded in the VTA varies across different intervals of the light-dark cycle with a period of 12 h detected through COSINOR analysis (adj$R^2$ = 0.88). Two peaks for DA firing rate were detected at 0840 and 2040 h. B) A diurnal rhythm of 24 h was detected for the number of spontaneously active DA neurons per electrode track (adj$R^2$ = 0.73) with a peak detected at 0554 h. The total number of neurons recorded and the tracks performed by time interval are as follows (neurons/track): 07:00-11:00 h = 49/20, 11:00-15:00 h = 57/27, 15:00-19:00 h = 39/20, 19:00-23:00 h = 29/17, 23:00-03:00 h = 61/24 and 03:00-07:00 h = 50/20. Points represent mean ± SEM within the time interval that is indicated. In both graphs, the shadowed background indicates the dark phase. ANOVA was also performed in the data with Bonferroni *t*-test post hoc comparison: * $p$ = 0.029 and # $p$ = 0.057 vs. 19:00-23:00h.

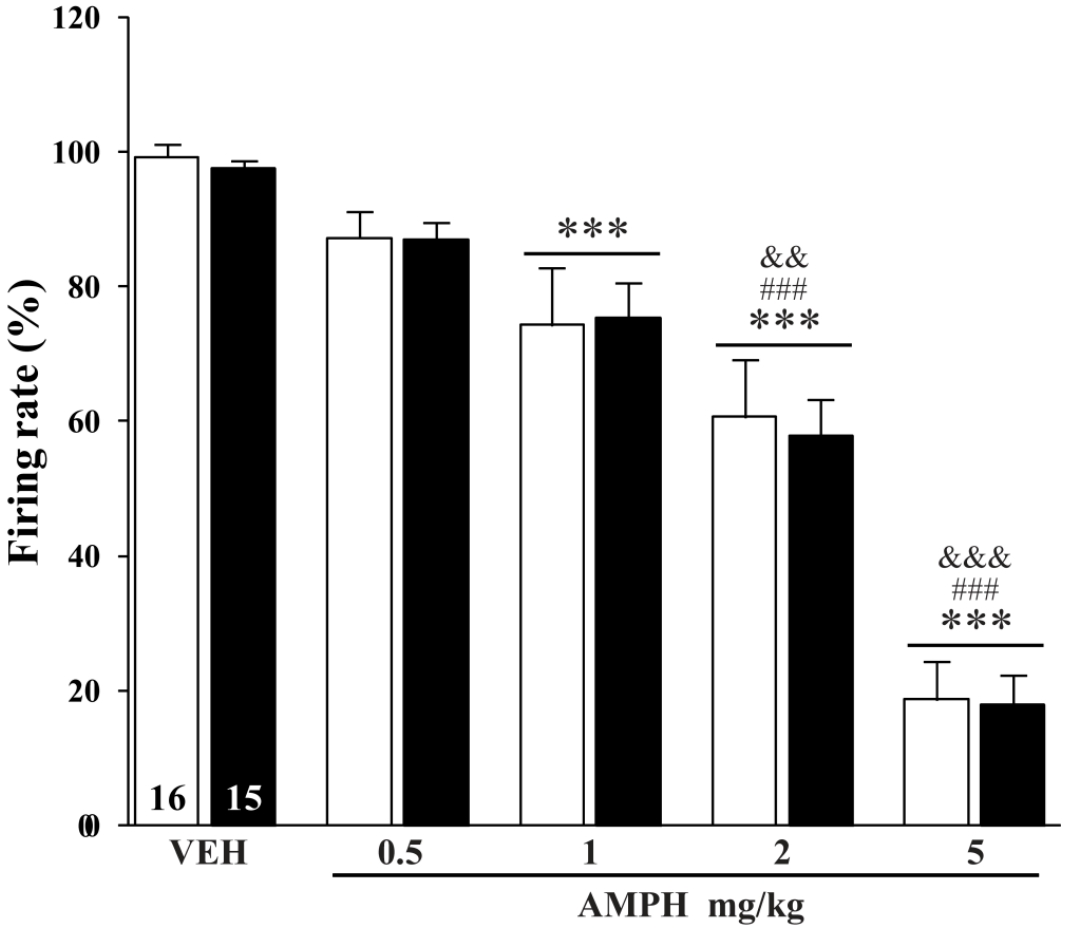


**Fig. 3.** Inhibitory effects of intravenous amphetamine (AMPH) administration on the firing rate of ventral tegmental area (VTA) dopamine (DA) neurons in the light and dark phase. Independent of the phase of the cycle, the firing rate of VTA DA neurons was dose-dependently inhibited by AMPH administration. In both phases, a significant decrease of DA cell firing rate was achieved with AMPH administration at 1 mg/kg, and a more pronounced decrease was observed at 2 mg/kg reaching a maximum effect with the 5 mg/kg dose. Bars represent mean ± SEM expressed as percentage of basal firing rate, white bars represent data from the light phase and black bars represent data from the dark phase. Numbers within bars indicate the number of neurons tested in each phase. Bonferroni *t*-test post hoc comparisons: *** $p < 0.001$ vs. VEH; ### $p < 0.001$ vs. 0.5 mg/kg; &&& $p < 0.001$ vs. 1 mg/kg and vs. 2 mg/kg; && $p < 0.01$ vs. 1 mg/kg.

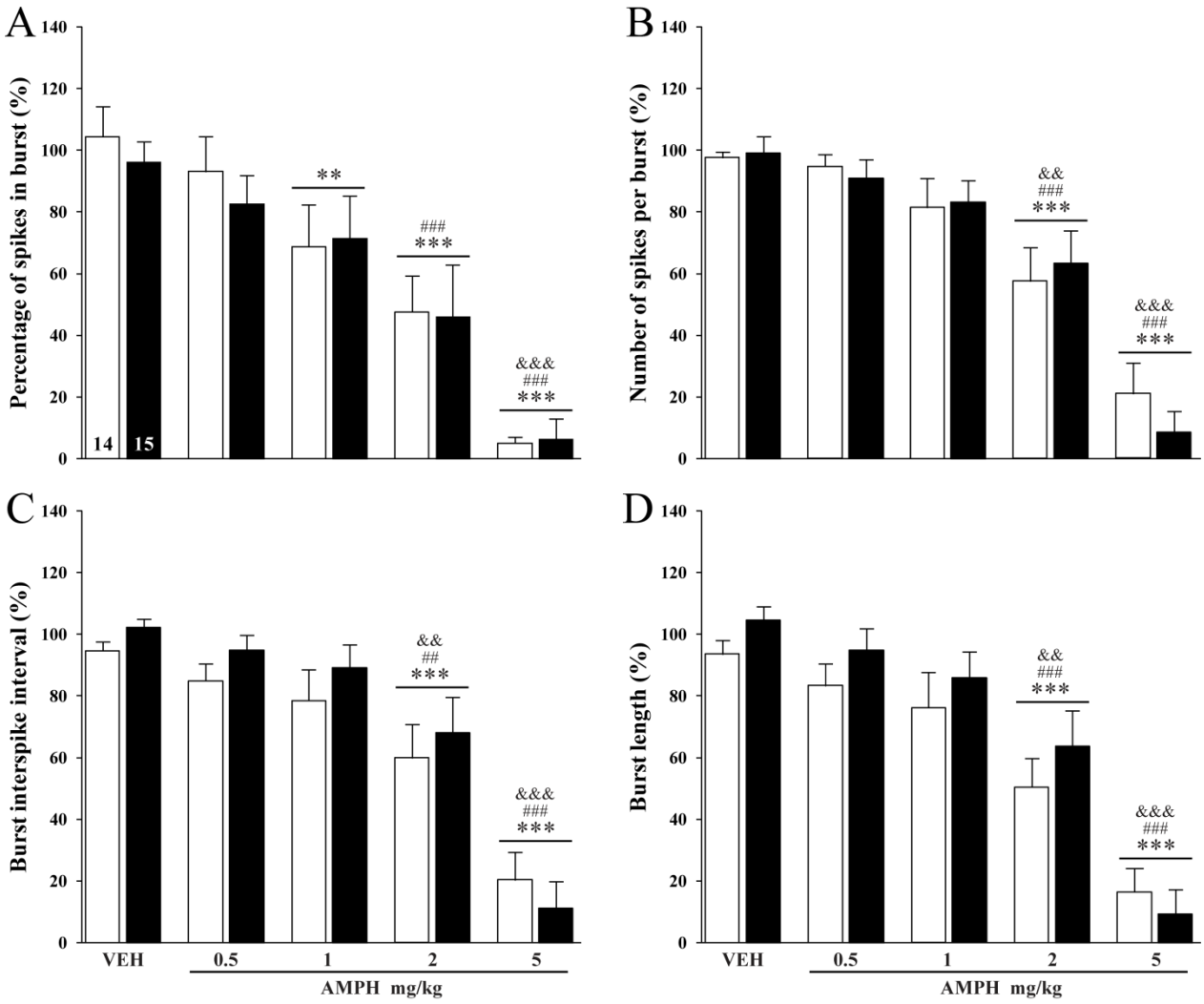


**Fig. 4.** Inhibitory effects of intravenous amphetamine (AMPH) administration on burst activity of ventral tegmental area (VTA) dopamine (DA) neurons in the light and dark phase. In both phases, all parameters of burst activity in VTA DA neurons were dose-dependently reduced after AMPH administration: A) the percentage of spikes fired in burst; B) number of spikes per burst; C) burst interspike interval and; D) burst length. A significant decrease in the percentage of spikes fired in burst mode was observed after AMPH administration at 1 mg/kg. All burst activity parameters were reduced with the dose and 2 mg/kg and almost completely inhibited with the dose of 5 mg/kg. Bars represent mean ± SEM expressed as percentage of basal firing rate, white bars represent data from the light phase and black bars represent data from the dark phase. Numbers within bars in A indicate the number of neurons in each phase. Bonferroni *t*-test post hoc comparisons: *** $p < 0.001$ and ** $p < 0.01$ vs. VEH; ### $p < 0.001$ and ## $p < 0.01$ vs. 0.5 mg/kg; &&& $p < 0.001$ vs. 1 mg/kg and vs. 2 mg/kg; && $p < 0.05$ vs. 1 mg/kg.

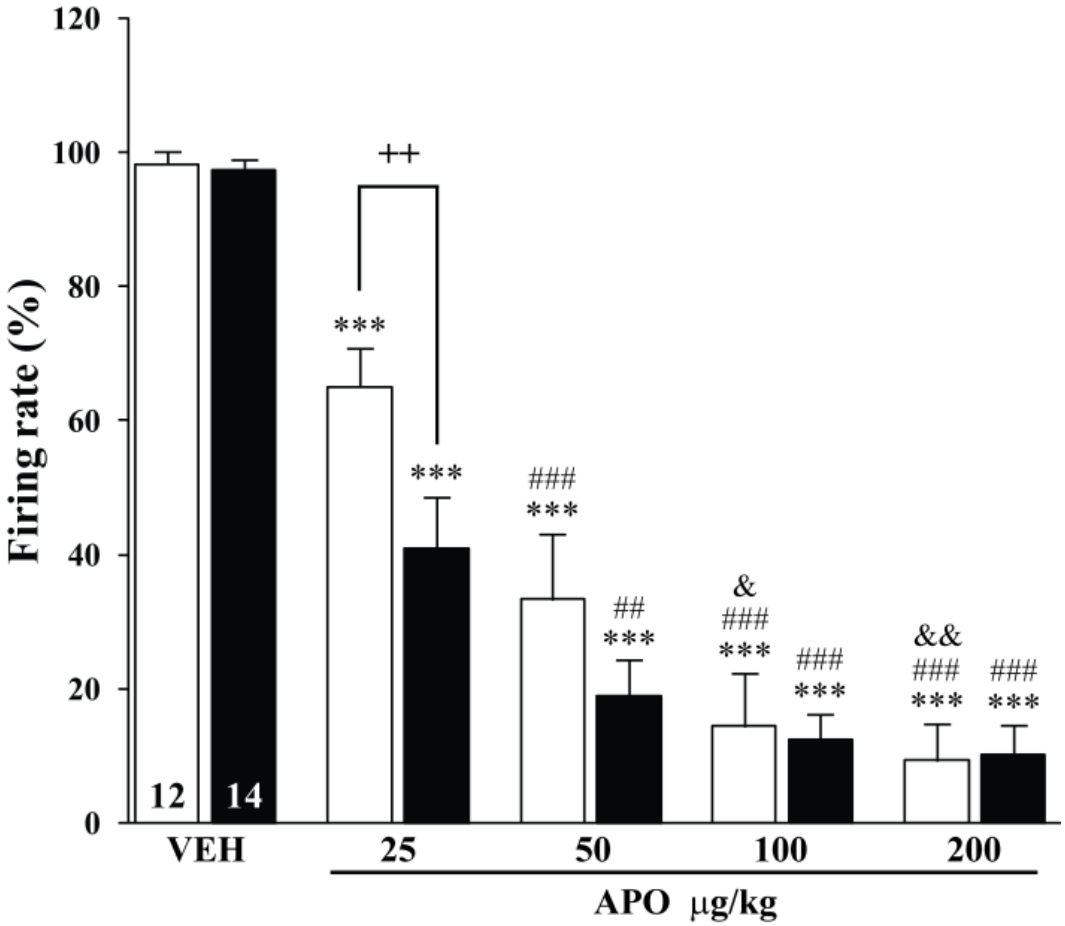


**Fig. 5.** Inhibitory effects of intravenous apormorphine (APO) administration on the firing rate of ventral tegmental area (VTA) dopamine (DA) neurons in the light and dark phase. APO administration induces a strong dose-dependent inhibition in the firing rate of VTA DA neurons. This inhibition of DA cell firing was of higher magnitude during the dark phase at the 25 μg/kg dose. In both phases, a further inhibition of DA firing was observed with the dose of 50 μg/kg and a maximum effect was observed with the dose of 100 μg/kg. Bars represent mean ± SEM expressed as percentage of basal firing rate, white bars represent data from the light phase and black bars represent data from the dark phase. Numbers within bars indicate the number of neurons tested in each phase. Bonferroni *t*-test post hoc comparisons: *** $p < 0.001$ vs. VEH; ### $p < 001$ and ## $p < 0.01$ vs. 25 μg/kg; && $p = 0.001$ and & $p < 0.05$ vs. 50 μg/kg; ++ $p < 0.01$ vs. light phase.

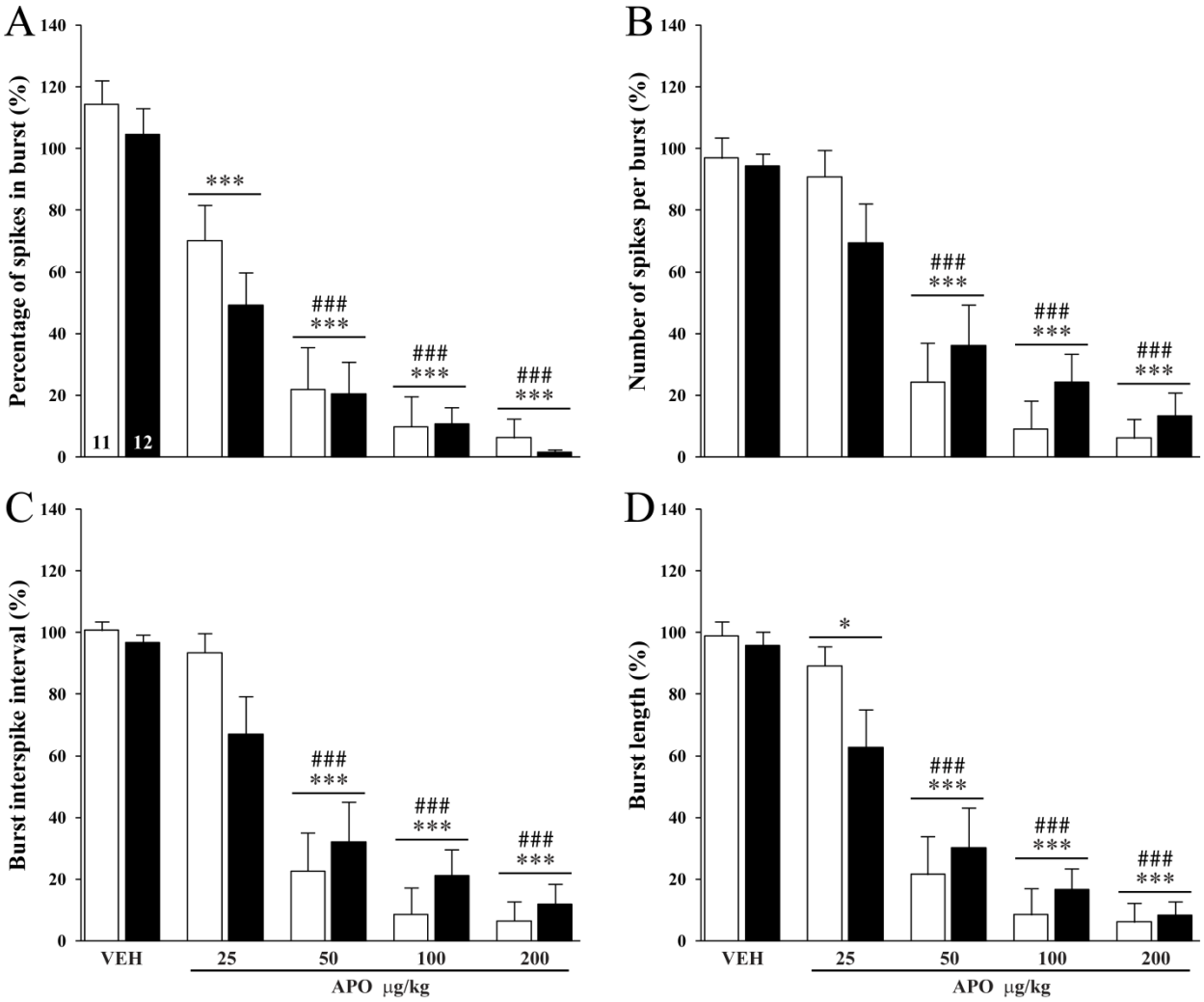


**Fig. 6.** Inhibitory effect of apomorphine (APO) intravenous administration in burst activity of ventral tegmental area (VTA) dopamine (DA) neurons in the light and dark phase. Regardless of the phase of the cycle, all parameters of burst activity in VTA DA neurons were dose-dependently reduced after APO administration: A) the percentage of spikes fired in burst; B) number of spikes per burst; C) burst interspike interval and; D) burst length. Slight but significant decreases in the percentage of spikes fired in burst mode and burst length were observed after administration of 25 μg/kg of APO. All burst parameters were more reduced at the dose of 50 μg/kg. Additional, but non-significant reductions were observed with doses of 100 and 200 μg/kg of APO which nearly inhibits all DA burst activity. Bars represent mean ± SEM expressed as percentage of basal firing rate, white bars represent data from the light phase and black bars represent data from the dark phase. Numbers within bars in A indicate the number of neurons in each phase. Bonferroni *t*-test post hoc comparisons: *** $p < 0.001$ and * $p < 0.05$ vs. VEH; ### vs. 25 μg/kg.

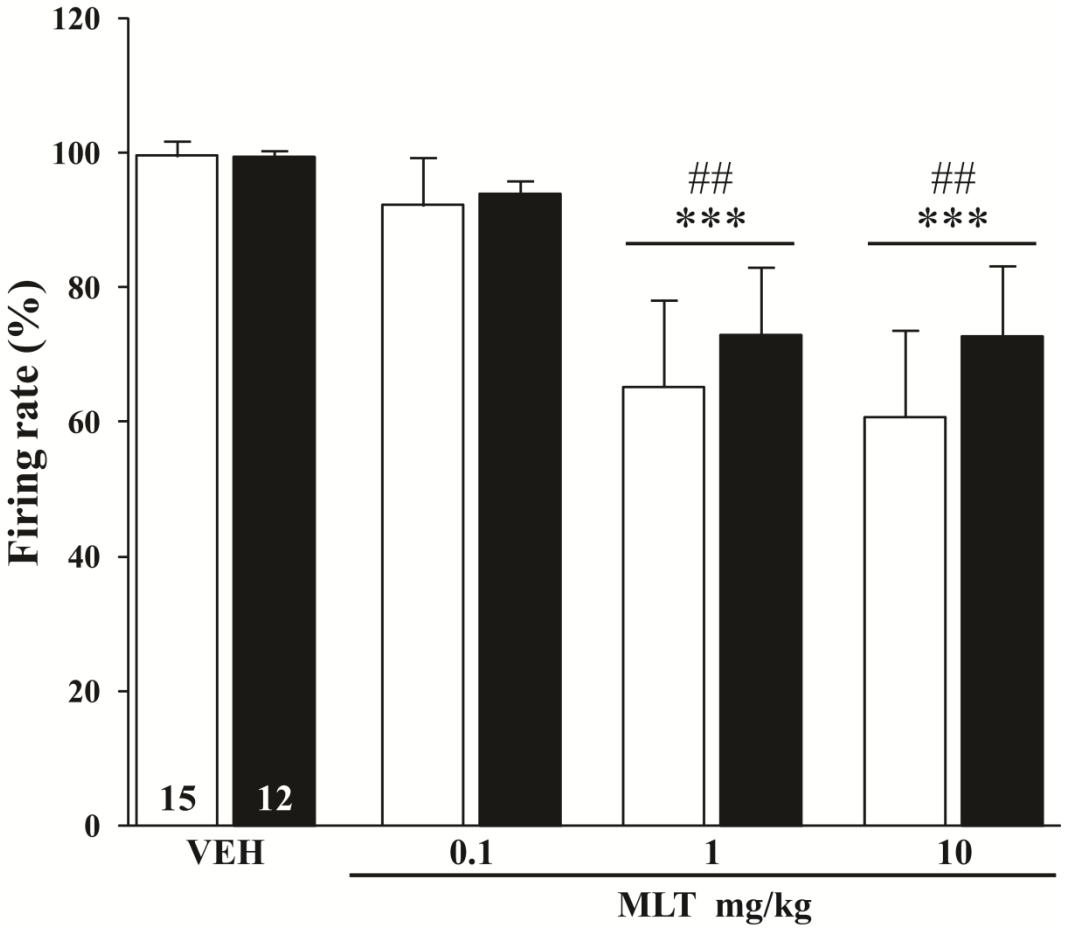


**Fig. 7.** Inhibitory effects of intravenous melatonin (MLT) administration on the firing rate of ventral tegmental area (VTA) dopamine (DA) neurons in the light and dark phase. In both phases, a significant decrease in cell firing was observed in VTA DA neurons with the doses of 1 mg/kg of MLT. No further effect was observed with the dose of 10 mg/kg. Bars represent mean ± SEM expressed as percentage of basal firing rate, white bars represent data from the light phase and black bars represent data from the dark phase. Numbers within bars indicate the number of neurons tested in each phase. Bonferroni *t*-test post hoc comparisons: *** $p < 0.001$ vs. VEH; ## $p < 0.01$ vs. 0.1 mg/kg.

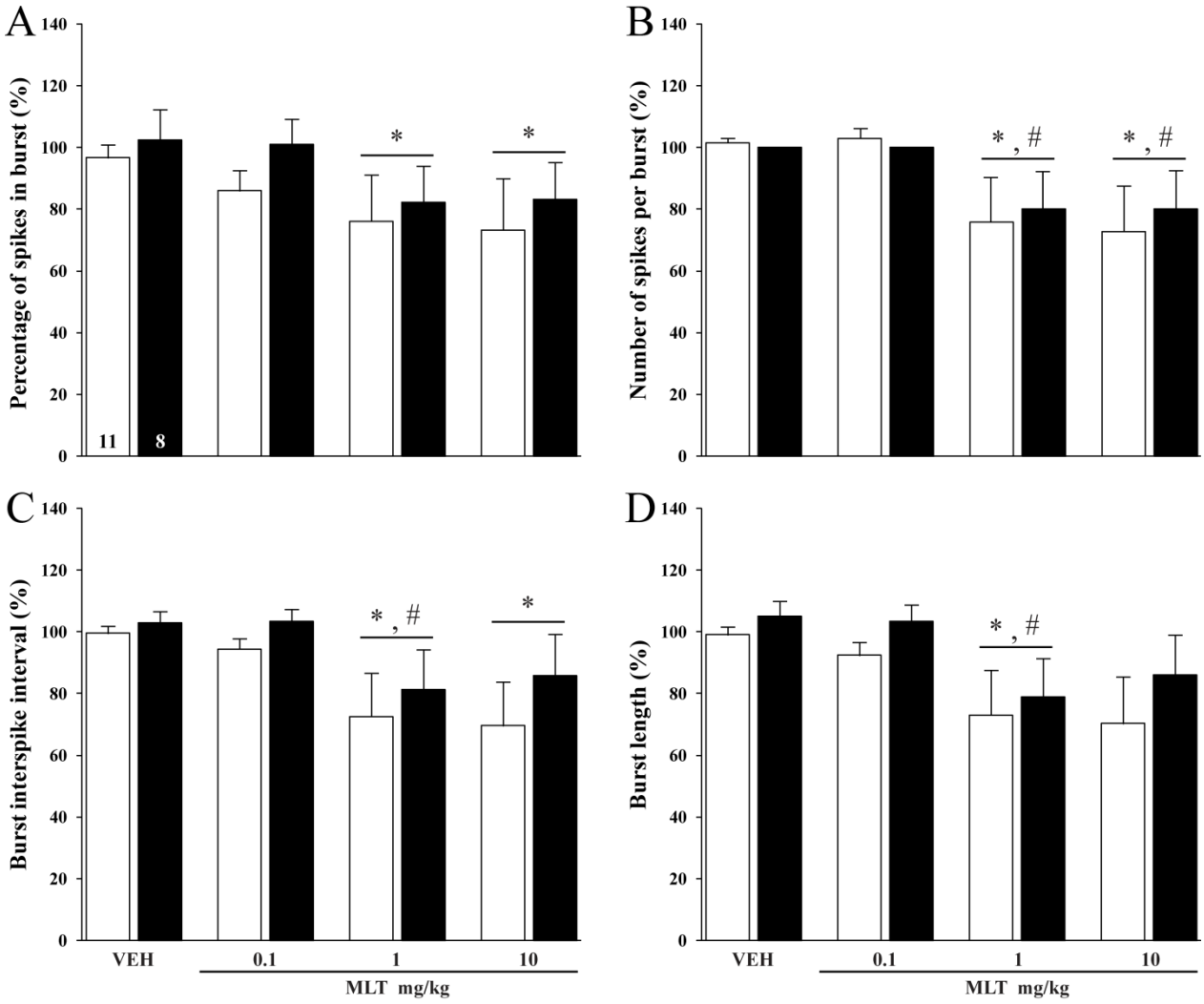


**Fig. 8.** Inhibitory effect of intravenous melatonin (MLT) administration in burst activity of ventral tegmental area (VTA) dopamine (DA) neurons in the light and dark phase. Independent of the phase of the cycle, all parameters of burst activity in VTA DA neurons were decreased with MLT administration: A) the percentage of spikes fired in burst; B) number of spikes per burst; C) burst interspike interval and; D) burst length. Slight but significant decreases in all parameters of DA cell burst activity were observed with the dose of 1 mg/kg. No further effect was observed with the dose of 10 mg/kg and the decrease in burst length was not significant with this dose of MLT. Bars represent mean ± SEM expressed as percentage of basal firing rate, white bars represent data from the light phase and black bars represent data from the dark phase. Numbers within bars in A indicate the number of neurons in each phase. Bonferroni *t*-test post hoc comparisons: * $p < 0.05$ vs. VEH; # $p < 0.05$ vs. 0.1 mg/kg.

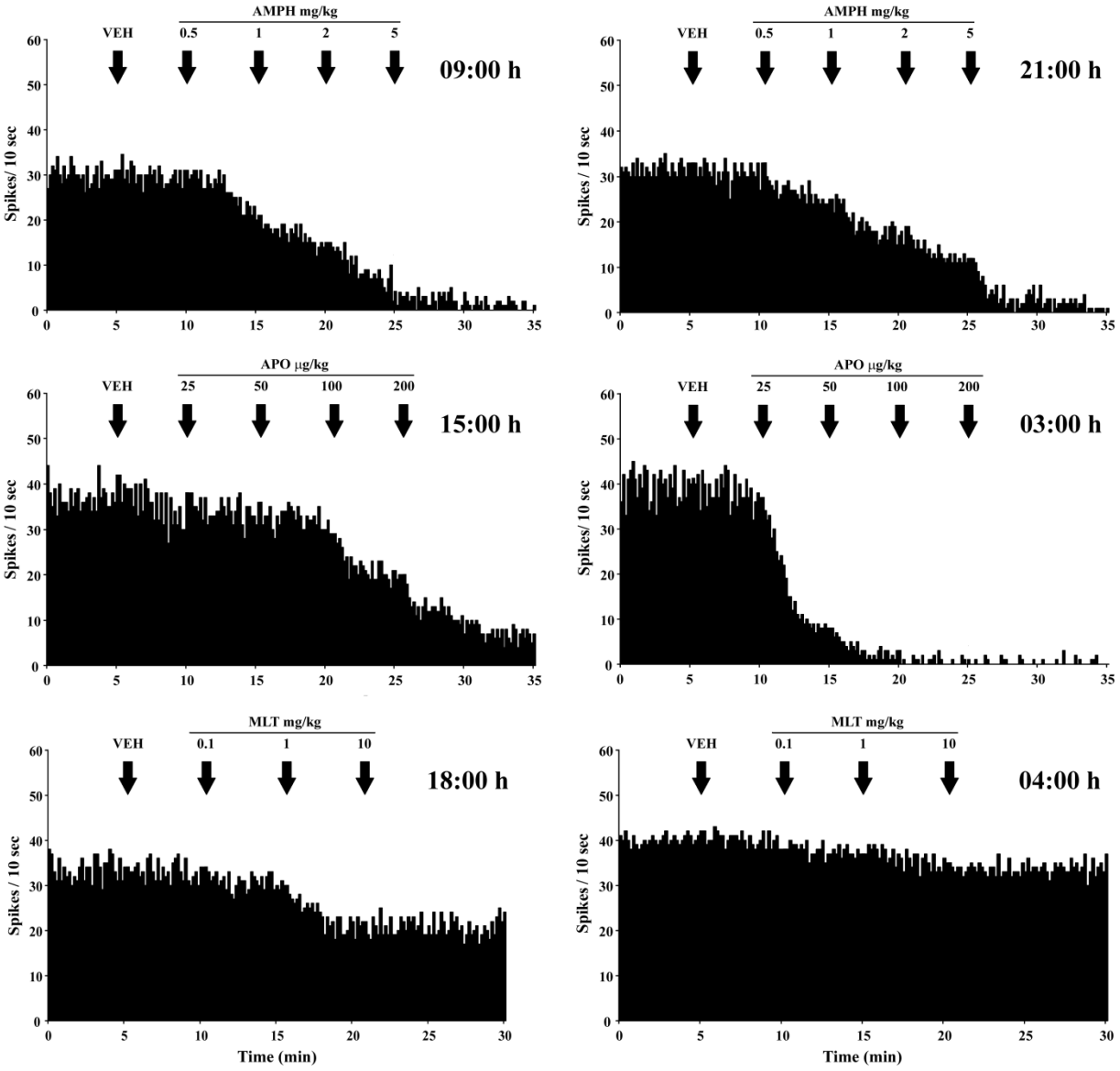


**Fig. 9.** Representative integrated firing rate histograms showing the acute response of dopamine (DA) neurons to intravenous administration of amphetamine (AMPH), apomorphine (APO), melatonin (MLT) or vehicle (VEH) at different time of the day. Arrows indicate sequential injections of doses of AMPH (0.5 + 0.5 + 1 + 3 mg/kg), APO (25 + 25 + 50 + 100 μg/kg) or MLT (0.1 + 0.9 + 9 mg/kg). The cumulative dose reached is provided on top of each arrow. Clock time for the first injection is indicated in each example. A similar dose-dependent decrease in firing frequency can be observed in the two neurons tested with AMPH. APO also induces an inhibitory effect on DA cell firing, but note the stronger effect of the 25 μg/kg dose on the neuron tested at 03:00 h. MLT administration have a moderate effect on DA cell firing which can be better observed in the neuron tested at 18:00 h.

Table 1. Burst activity of dopamine neurons recorded in the ventral tegmental area across the light-dark cycle.[1]

| Time interval | Burst-firing neurons (%) | Firing rate (Hz) | Spikes in burst (%) | Spikes per burst (n) | Burst interspike interval (ms) | Burst length (ms) |
|---|---|---|---|---|---|---|
| 07:00-11:00h | 75.5 | 3.8 ± 0.4 | 36.5 ± 4.2* | 3.2 ± 0.2 | 40.0 ± 2.7 | 146.7 ± 20.9 |
| 11:00-15:00h | 54.4 | 3.1 ± 0.3 | 29.4 ± 3.9** | 2.8 ± 0.1 | 40.4 ± 2.7 | 128.2 ± 15.8 |
| 15:00-19:00h | 64.1 | 3.3 ± 0.6 | 56.4 ± 5.5 | 3.9 ± 0.3 | 37.7 ± 3.1 | 158.3 ± 24.5 |
| Light phase | 64.1 | 3.4 ± 0.3 | 39.5 ± 2.8 | 3.3 ± 0.1 | 39.5 ± 1.6 | 143.7 ± 11.8 |
| 19:00-23:00h | 65.5 | 5.2 ± 1.0 | 38.2 ± 7.0 | 3.7 ± 0.5 | 44.9 ± 2.8 | 202.6 ± 40.2 |
| 23:00-03:00h | 60.6 | 3.2 ± 0.3 | 34.0 ± 4.5** | 3.1 ± 0.2 | 40.8 ± 2.3 | 135.9 ± 16.3 |
| 03:00-07:00h | 70 | 3.2 ± 0.4 | 36.0 ± 4.5* | 3.1 ± 0.3 | 35.2 ± 3.1 | 138.7 ± 25.9 |
| Dark phase | 65 | 3.6 ± 0.3 | 35.7 ± 2.9 | 3.2 ± 0.2 | 39.5 ± 1.7 | 150.9 ± 14.7 |

[1] Values represent mean ± SEM. Bonferroni t-test post-hoc comparisons: * $p$ <0.05 and ** $p$ < 0.01 vs. 1500-1900h.

Table 2. COSINOR curve fitting parameters calculated for 24 h firing activity of dopamine neurons.

| | Period | MESOR | *a* | *b* | Amplitude | Peak | Trough | ANOVA results |
|---|---|---|---|---|---|---|---|---|
| Firing rate (Hz) | 12 h | 3.05 | 0.49 | 0.6 | 0.78 | 08:40 h<br>20:40 h | 14:40 h<br>02:40 h | $F_{(1,4)} = 39.7$, $p = 0.003$ |
| Neurons per track (n) | 24 h | 2.12 | 0.37 | -0.11 | 0.39 | 05:54 h | 17:54 h | $F_{(1,4)} = 14.3$, $p = 0.019$ |

# Supplementary Tables

**Table S1**. Firing rate of DA neurons recorded in the VTA across the light-dark cycle after AMPH administration.

| | | | AMPH (mg/kg) | | | |
|---|---|---|---|---|---|---|
| Time interval | CTRL | VEH | 0.5 | 1 | 2 | 5 |
| 0700-1100h | 4.0 ± 0.6 | 4.1 ± 0.6 | 3.5 ± 0.7 | 3.3 ± 0.9 | 2.9 ± 0.8 | 0.7 ± 0.6*** |
| 1100-1500h | 3.1 ± 0.4 | 2.9 ± 0.4 | 2.6 ± 0.3 | 2.1 ± 0.5* | 1.6 ± 0.4*** | 0.6 ± 0.2*** |
| 1500-1900h | 3.5 ± 0.4 | 3.6 ± 0.5 | 3.3 ± 0.5 | 2.9 ± 0.4 | 2.3 ± 0.6* | 0.7 ± 0.4*** |
| 1900-2300h | 4.2 ± 0.5 | 4.0 ± 0.4 | 3.3 ± 0.4 | 2.7 ± 0.6 | 1.5 ± 0.6*** | 0.03 ± 0.0*** |
| 2300-0300h | 3.8 ± 0.8 | 3.6 ± 0.8 | 3.5 ± 0.7 | 3.5 ± 0.7 | 2.8 ± 0.4 | 1.0 ± 0.4*** |
| 0300-0700h | 4.0 ± 0.7 | 3.9 ± 0.6 | 3.5 ± 0.6 | 2.9 ± 0.4** | 2.1 ± 0.4*** | 0.8 ± 0.2*** |

Values represent mean ± SEM. Bonferroni t-test post-hoc comparisons: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. CTRL.

**Table S2.** Burst activity of DA neurons recorded in the VTA across the light-dark cycle after AMPH administration.

| Time interval | Treatment (mg/kg) | Spikes in burst (%) | Spikes per burst (n) | Burst interspike interval (ms) | Burst length (ms) |
|---|---|---|---|---|---|
| 0700-1100h | CTRL | 32.8 ± 9.1 | 3.2 ± 0.3 | 50.5 ± 5.4 | 167.1 ± 38.5 |
| | VEH | 39.3 ± 11.3 | 3.2 ± 0.3 | 49.6 ± 5.9 | 171.8 ± 38.2 |
| | AMPH (0.5) | 34.6 ± 9.2 | 2.8 ± 0.4 | 47.1 ± 8.4 | 157.7 ± 38.2 |
| | AMPH (1) | 27.0 ± 11.0 | 2.6 ± 0.6 | 46.4 ± 12.4 | 159.3 ± 44.2 |
| | AMPH (2) | 18.1 ± 8.8 | 1.6 ± 0.6** | 29.2 ± 12.4 | 88.7 ± 40.1* |
| | AMPH (5) | 1.0 ±1.0*** | 0.4 ± 0.4*** | 8.71 ± 8.7*** | 21.0 ± 21.0*** |
| 1100-1500h | CTRL | 15.4 ± 3.9 | 2.4 ± 0.2 | 42.1 ± 3.7 | 110.2 ± 15.1 |
| | VEH | 12.6 ± 3.3 | 2.4 ± 0.2 | 36.9 ± 4.8 | 91.5 ± 15.3 |
| | AMPH (0.5) | 12.2 ± 3.0 | 2.4 ± 0.2 | 35.1 ± 6.3 | 90.0 ± 20.6 |
| | AMPH (1) | 7.03 ± 3.0 | 1.8 ± 0.4 | 28.4 ± 8.5 | 66.8 ± 23.5 |
| | AMPH (2) | 4.85 ± 2.1* | 1.8 ± 0.4 | 29.4 ± 8.1 | 65.1 ± 20.2 |
| | AMPH (5) | 0.79 ± 0.4* | 0.8 ± 0.4* | 12.2 ± 7.5* | 26.1 ± 16.0** |
| 1500-1900h | CTRL | 14.2 ± 5.3 | 2.9 ± 0.4 | 51.5 ± 5.9 | 154.8 ± 30.8 |
| | VEH | 14.1 ± 4.8 | 2.6 ± 0.2 | 51.5 ± 5.2 | 146.4 ± 25.8 |
| | AMPH (0.5) | 15.7 ± 5.9 | 2.9 ± 0.4 | 43.2 ± 8.9 | 128.0 ± 42.1 |
| | AMPH (1) | 15.9 ± 6.0 | 2.6 ± 0.2 | 41.3 ± 6.6 | 112.5 ± 25.0 |
| | AMPH (2) | 9.3 ± 4.2 | 1.5 ± 0.6** | 27.9 ± 11.2* | 71.4 ± 29.0* |
| | AMPH (5) | 2.0 ± 1.5** | 0.3 ± 0.2*** | 5.92 ± 4.4*** | 14.2 ± 10.5*** |
| 1900-2300h | CTRL | 20.4 ± 5.4 | 2.5 ± 0.2 | 46.2 ± 3.4 | 121.6 ± 14.1 |
| | VEH | 19.0 ± 5.0 | 2.5 ± 0.2 | 45.8 ± 3.8 | 118.6 ± 13.7 |
| | AMPH (0.5) | 14.2 ± 3.8 | 2.1 ± 0.1 | 38.7 ± 3.4 | 93.5 ± 7.2 |
| | AMPH (1) | 10.0 ± 4.0 | 1.6 ± 0.3 | 31.6 ± 6.4 | 70.5 ± 14.5* |
| | AMPH (2) | 4.5 ± 1.9** | 1.3 ± 0.4* | 25.5 ± 8.2* | 58.8 ± 30.8** |
| | AMPH (5) | 0.0 ± 0.0*** | 0.0 ± 0.0*** | 0.0 ± 0.0*** | 0.0 ± 0.0*** |
| 2300-0300h | CTRL | 24.1 ± 11.1 | 2.8 ± 0.3 | 41.2 ± 6.2 | 121.2 ± 33.5 |
| | VEH | 27.3 ± 13.1 | 3.0 ± 0.5 | 44.9 ± 5.0 | 141.2 ± 34.3 |
| | AMPH (0.5) | 27.8 ± 12.4 | 2.6 ± 0.4 | 43.8 ± 4.6 | 136.7 ± 32.9 |
| | AMPH (1) | 27.3 ± 11.2 | 2.6 ± 0.4 | 42.9 ± 4.9 | 129.1 ± 32.2 |
| | AMPH (2) | 10.7 ± 6.0 | 2.0 ± 0.6 | 33.8 ± 9.3 | 93.1 ± 31.6 |
| | AMPH (5) | 1.2 ± 1.2 | 0.8 ± 0.5** | 12.2 ± 7.5*** | 28.3 ± 17.9** |
| 0300-0700h | CTRL | 11.4 ± 2.2 | 2.3 ± 0.2 | 40.6 ± 4.2 | 93.2 ± 13.6 |
| | VEH | 9.6 ± 1.2 | 2.0 ± 0.0 | 38.4 ± 3.8 | 86.0 ± 12.6 |
| | AMPH (0.5) | 6.3 ± 1.6** | 2.0 ± 0.0 | 36.4 ± 3.4 | 78.7 ± 9.3 |
| | AMPH (1) | 2.2 ± 0.4*** | 2.0 ± 0.0 | 36.6 ± 2.0 | 76.0 ± 5.8 |
| | AMPH (2) | 0.4 ± 0.1*** | 1.5 ± 0.5 | 5.4 ± 8.4 | 50.9 ± 16.9* |
| | AMPH (5) | 0.0 ± 0.0*** | 0.0 ± 0.0*** | 0.0 ± 0.0*** | 0.0 ± 0.0*** |

Values represent mean ± SEM. Bonferroni t-test post-hoc comparisons: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. CTRL.

**Table S3**. Firing rate of DA neurons recorded in the VTA across the light-dark cycle after APO administration.

| | | | APO (μg/kg) | | | |
|---|---|---|---|---|---|---|
| Time interval | CTRL | VEH | 25 | 50 | 100 | 200 |
| 0700-1100h | 4.0 ± 0.9 | 3.7 ± 0.7 | 2.3 ± 0.4* | 0.9 ± 0.5*** | 0.3 ± 0.3*** | 0.2 ± 0.2*** |
| 1100-1500h | 4.2 ± 0.7 | 4.0 ± 0.6 | 2.7 ± 0.5* | 1.4 ± 0.5*** | 0.5 ± 0.2*** | 0.3 ± 0.2*** |
| 1500-1900h | 3.2 ± 0.6 | 3.4 ± 0.7 | 2.1 ± 0.5 | 1.2 ± 0.7 | 0.7 ± 0.7* | 0.5 ± 0.5** |
| 1900-2300h | 3.7 ± 0.5 | 3.5 ± 0.4 | 2.0 ± 0.3*** | 0.8 ± 0.3*** | 0.7 ± 0.3*** | 0.5 ± 0.2*** |
| 2300-0300h | 2.5 ± 0.3 | 2.3 ± 0.2 | 1.1 ± 0.4*** | 0.6 ± 0.3*** | 0.3 ± 0.2*** | 0.3 ± 0.3*** |
| 0300-0700h | 2.9 ± 0.2 | 3.0 ± 0.3 | 0.8 ± 0.4*** | 0.4 ± 0.3*** | 0.2 ± 0.2*** | 0.2 ± 0.2*** |

Values represent mean ± SEM. Bonferroni t-test post-hoc comparisons: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. CTRL.

**Table S4**. Burst activity of DA neurons recorded in the VTA across the light-dark cycle after apomorphine (APO) administration.

| Time interval | Treatment (μg/kg) | Spikes in burst (%) | Spikes per burst (n) | Burst interspike interval (ms) | Burst length (ms) |
|---|---|---|---|---|---|
| 0700-1100h | CTRL | 28.4 ± 13.0 | 3.2 ± 0.4 | 39.3 ± 11.4 | 133.4 ± 47.8 |
| | VEH | 24.1 ± 9.6 | 2.7 ± 0.2 | 41.0 ± 11.8 | 126.7 ± 41.0 |
| | APO (25) | 9.7 ± 2.0 | 2.3 ± 0.3 | 34.9 ± 11.7 | 99.6 ± 36.6 |
| | APO (50) | 1.7 ± 1.7* | 0.5 ± 0.5*** | 1.82 ± 1.8** | 3.7 ± 3.7** |
| | APO (100) | 0.0 ± 0.0* | 0.0 ± 0.0*** | 0.0 ± 0.0** | 0.0 ± 0.0** |
| | APO (200) | 0.0 ± 0.0* | 0.0 ± 0.0*** | 0.0 ± 0.0** | 0.0 ± 0.0** |
| 1100-1500h | CTRL | 26.7 ± 21.6 | 3.0 ± 0.5 | 48.1 ± 8.1 | 153.3 ± 52.7 |
| | VEH | 25.2 ± 19.5 | 2.6 ± 0.6 | 43.7 ± 8.1 | 127.4 ± 45.5 |
| | APO (25) | 15.0 ± 11.5 | 3.0 ± 0.5 | 46.9 ± 7.8 | 135.5 ± 41.0 |
| | APO (50) | 0.44 ± 0.4 | 0.6 ± 0.6* | 8.0 ± 8.0** | 17.6 ± 17.6* |
| | APO (100) | 0.0 ± 0.0 | 0.0 ± 0.0** | 0.0 ± 0.0*** | 0.0 ± 0.0* |
| | APO (200) | 0.0 ± 0.0 | 0.0 ± 0.0** | 0.0 ± 0.0*** | 0.0 ± 0.0* |
| 1500-1900h | CTRL | 17.3 ± 3.6 | 2.2 ± 0.2 | 38.2 ± 5.1 | 89.2 ± 16.4 |
| | VEH | 23.8 ± 4.9 | 2.5 ± 0.2 | 40.6 ± 5.5 | 99.3 ± 19.1 |
| | APO (25) | 15.0 ± 6.1 | 2.2 ± 0.4 | 34.8 ± 7.3 | 85.8 ± 22.8 |
| | APO (50) | 7.9 ± 7.9 | 0.7 ± 0.7* | 11.2 ± 11.2* | 28.5 ± 26.5* |
| | APO (100) | 6.8 ± 6.8 | 0.7 ± 0.7* | 10.8 ± 10.8* | 7.4 ± 27.4* |
| | APO (200) | 4.2 ± 4.2* | 0.5 ± 0.5** | 8.1 ± 8.1** | 9.7 ± 19.7* |
| 1900-2300h | CTRL | 21.5 ± 13.1 | 3.0 ± 0.4 | 45.4 ± 3.8 | 132.2 ± 27.3 |
| | VEH | 18.9 ± 10.0 | 2.7 ± 0.4 | 46.1 ± 4.4 | 124.9 ± 23.8 |
| | APO (25) | 13.4 ± 7.7 | 2.7 ± 0.4 | 43.6 ± 5.0 | 118.7 ± 26.8 |
| | APO (50) | 5.8 ± 5.8 | 1.0 ± 1.0** | 12.0 ± 12.0** | 43.1 ± 46.1** |
| | APO (100) | 3.1 ± 3.1 | 0.7 ± 0.7*** | 9.6 ± 9.6*** | 29.7 ± 29.7*** |
| | APO (200) | 0.2 ± 0.2* | 0.2 ± 0.2*** | 5.4 ± 5.4*** | 12.2 ± 12.2*** |
| 2300-0300h | CTRL | 9.4 ± 3.4 | 2.5 ± 0.3 | 41.2 ± 3.1 | 102.5 ± 15.1 |
| | VEH | 7.7 ± 2.4 | 2.2 ± 0.2 | 37.1 ± 1.3 | 87.0 ± 6.7 |
| | APO (25) | 6.1 ± 2.4 | 1.7 ± 0.6 | 27.3 ± 9.6 | 64.1 ± 23.3 |
| | APO (50) | 4.0 ± 2.1 | 1.7 ± 0.6 | 26.4 ± 10.5 | 66.7 ± 30.3 |
| | APO (100) | 2.0 ± 1.0* | 1.0 ± 0.4** | 14.5 ± 6.1** | 30.0 ± 12.6** |
| | APO (200) | 0.3 ± 0.3** | 0.5 ± 0.5*** | 7.1 ± 7.1*** | 14.4 ± 14.4*** |
| 0300-0700h | CTRL | 27.6 ± 9.8 | 2.5 ± 0.2 | 41.2 ± 3.0 | 109.4 ± 12.4 |
| | VEH | 37.6 ± 15.5 | 2.5 ± 0.2 | 40.2 ± 3.3 | 115.2 ± 18.5 |
| | APO (25) | 4.6 ± 3.4 | 1.2 ± 0.7 | 17.5 ± 10.1** | 42.1 ± 24.6** |
| | APO (50) | 0.3 ± 0.3 | 0.5 ± 0.5** | 4.6 ± 4.6*** | 9.2 ± 9.2*** |
| | APO (100) | 0.3 ± 0.3 | 0.5 ± 0.5** | 4.6 ± 4.6*** | 9.2 ± 9.2*** |
| | APO (200) | 0.3 ± 0.3 | 0.5 ± 0.5** | 4.2 ± 4.2*** | 9.2 ± 9.2*** |

Values represent mean ± SEM. Bonferroni t-test post-hoc comparisons: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. CTRL.

**Table S5**. Firing rate of DA neurons recorded in the VTA across the light-dark cycle after MLT administration.

| | | MLT (mg/kg) | | | |
|---|---|---|---|---|---|
| Time interval | CTRL | VEH | 0.1 | 1 | 10 |
| 0700-1100h | 3.1 ± 0.3 | 3.1 ± 0.3 | 2.9 ± 0.3 | 2.1 ± 0.4* | 1.6 ± 0.7* |
| 1100-1500h | 2.7 ± 0.8 | 2.7 ± 0.9 | 2.7 ± 1.0 | 1.8 ± 1.1 | 1.7 ± 1.0 |
| 1500-1900h | 2.9 ± 0.7 | 2.8 ± 0.7 | 2.0 ± 0.4 | 1.4 ± 0.6 | 1.4 ± 0.6 |
| 1900-2300h | 2.9 ± 0.2 | 2.8 ± 0.2 | 2.7 ± 0.1 | 1.9 ± 0.6 | 2.0 ± 0.7 |
| 2300-0300h | 3.3 ± 0.4 | 3.2 ± 0.4 | 3.1 ± 0.4 | 2.4 ± 0.9 | 2.3 ± 0.9 |
| 0300-0700h | 3.9 ± 0.5 | 4.0 ± 0.6 | 3.6 ± 0.3 | 3.4 ± 0.3 | 3.3 ± 0.1 |

Values represent mean ± SEM. Bonferroni t-test post-hoc comparisons: * $p < 0.05$ vs. CTRL.

**Table S6.** Burst activity of DA neurons recorded in the VTA across the light-dark cycle after MLT administration.

| Time interval | Treatment (mg/kg) | Spikes in burst (%) | Spikes per burst (n) | Burst interspike interval (ms) | Burst length (ms) |
|---|---|---|---|---|---|
| 0700-1100h | CTRL | 26.3 ± 11.3 | 2.5 ± 0.2 | 40.6 ± 3.6 | 103.1 ± 22.4 |
| | VEH | 29.2 ± 13.9 | 2.5 ± 0.2 | 40.0 ± 2.8 | 100.8 ± 19.5 |
| | MLT (0.1) | 25.7 ± 11.4 | 2.5 ± 0.2 | 39.7 ± 3.3 | 98.9 ± 20.2 |
| | MLT (1) | 28.8 ± 12.6 | 2.5 ± 0.2 | 40.0 ± 3.0 | 96.9 ± 15.4 |
| | MLT (10) | 19.5 ± 9.7 | 2.0 ± 0.0 | 33.8 ± 2.2 | 73.8 ± 5.9 |
| 1100-1500h | CTRL | 36.4 ± 11.1 | 2.2 ± 0.2 | 30.2 ± 6.2 | 80.0 ± 23.8 |
| | VEH | 35.8 ± 12.8 | 2.4 ± 0.3 | 30.5 ± 5.6 | 82.9 ± 25.6 |
| | MLT (0.1) | 35.1 ± 14.4 | 2.5 ± 0.5 | 29.2 ± 7.1 | 82.5 ± 30.2 |
| | MLT (1) | 32.3 ± 16.9 | 2.0 ± 0.8 | 21.3 ± 9.3 | 68.2 ± 39.6 |
| | MLT (10) | 32.5 ± 15.8 | 2.0 ± 0.8 | 2.1 ± 9.6 | 68.1 ± 37.3 |
| 1500-1900h | CTRL | 45.2 ± 15.6 | 2.6 ± 0.3 | 41.2 ± 6.0 | 115.3 ± 26.7 |
| | VEH | 41.5 ± 16.0 | 2.6 ± 0.3 | 39.6 ± 6.8 | 106.9 ± 25.8 |
| | MLT (0.1) | 32.4 ± 14.9 | 2.6 ± 0.3 | 38.0 ± 6.3 | 94.6 ± 21.5 |
| | MLT (1) | 19.0 ± 19.0 | 1.0 ± 1.0 | 13.0 ± 13.0 | 34.4 ± 34.4 |
| | MLT (10) | 22.3 ± 22.3 | 1.0 ± 1.0 | 14.2 ± 14.2 | 44.8 ± 44.8 |
| 1900-2300h | CTRL | 36.8 ± 17.5 | 2.5 ± 0.3 | 38.8 ± 1.6 | 98.8 ± 12.9 |
| | VEH | 35.6 ± 18.9 | 2.5 ± 0.3 | 38.3 ± 2.6 | 97.0 ± 17.1 |
| | MLT (0.1) | 31.1 ± 15.6 | 2.5 ± 0.3 | 38.2 ± 1.0 | 91.2 ± 4.6 |
| | MLT (1) | 28.6 ± 16.4 | 1.8 ± 0.9 | 24.5 ± 12.3 | 61.5 ± 31.3 |
| | MLT (10) | 33.6 ± 19.3 | 1.8 ± 0.9 | 27.6 ± 13.8 | 79.8 ± 40.8 |
| 2300-0300h | CTRL | 39.2 ± 20.2 | 3.0 ± 0.2 | 40.8 ± 1.8 | 112.7 ± 13.0 |
| | VEH | 40.4 ± 19.7 | 3.0 ± 0.2 | 40.6 ± 2.3 | 113.2 ± 15.2 |
| | MLT (0.1) | 39.5 ± 19.5 | 3.0 ± 0.2 | 41.1 ± 2.1 | 112.6 ± 15.3 |
| | MLT (1) | 38.8 ± 19.9 | 3.0 ± 0.2 | 34.2 ± 6.4 | 99.0 ± 25.9 |
| | MLT (10) | 39.3 ± 19.5 | 3.0 ± 0.2 | 35.9 ± 5.2 | 104.4 ± 19.8 |
| 0300-0700h | CTRL | 42.8 ± 21.0 | 3.5 ± 0.5 | 39.0 ± 9.5 | 128.6 ± 42.4 |
| | VEH | 52.6 ± 26.1 | 3.5 ± 0.5 | 43.6 ± 5.7 | 157.6 ± 44.4 |
| | MLT (0.1) | 52.6 ± 26.0 | 3.5 ± 0.5 | 43.4 ± 6.0 | 155.6 ± 46.3 |
| | MLT (1) | 41.6 ± 20.5 | 3.5 ± 0.5 | 41.7 ± 6.4 | 129.1 ± 32.9 |
| | MLT (10) | 38.2 ± 18.5 | 3.5 ± 0.5 | 41.6 ± 5.7 | 121.5 ± 30.7 |

Values represent mean ± SEM.

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## Foreword to Chapter 4

In the previous chapters, we characterized the diurnal patterns of firing activity in DRN 5-HT neurons and VTA DA neurons. Perhaps the most surprising outcome was an apparent desynchronization between a long 24 h rhythm of spontaneously active DA neurons and a shorter 12 h rhythm in their discharge frequency (section 3.3.1 and 3.3.2). Our observations indicate that the number of active neurons and their firing rates are higher at the beginning of the light phase and then they decrease towards the end of the light phase. $D_2$ autoreceptor activation by APO administration inhibited DA cell firing, and these effects were greatest at night (section 3.3.4). In comparison, the inhibitory effect of AMPH on DA neurons seemed to be constant at all times though possibly slightly less potent around midnight (section 3.3.3). In this last chapter, additional experiments are presented that evaluated whether the locomotor effects induced by APO and AMPH administration varied across the light-dark cycle. The open field test was chosen because it is a well validated and sensitive test to contrast locomotor inhibitory effects of lower doses of APO and locomotor activating effects of AMPH.

# Chapter 4

# Apomorphine and amphetamine effects on behavioral activity in the open field across the light-dark cycle

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## ABSTRACT

We recently reported that ventral tegmental area (VTA) dopamine (DA) cell inhibitory responses to low autoreceptor selective doses of apomorphine (APO) vary throughout the day, with the largest effects seen at night. Inhibitory effects of AMPH, in comparison, are largely unaffected by the time of day though tended to be slightly weaker at midnight. Here, we tested whether time of day influenced the behavioral effects of these two drugs. Locomotor activity was measured in an open field during both the light and dark phase (light phase: 0700-1100 h, 1100-1500 h and 1500-1900 h; dark phase: 1900-2300 h, 2300-0300 h and 0300-0700 h). At each time interval, rats were randomly assigned to receive a single i.p. dose of APO (50, 100 or 200 μg/kg), AMPH (0.5, 1, 2 or 5 mg/kg) or VEH. In the dark phase, APO at the lowest dose (50 μg/kg) was less effective in reducing distance traveled, probably suggestive of changes in $D_2$ receptor availability. Independent of dose, APO administration induced a stronger increase in immobility during the light phase. AMPH induced -increase in distance traveled was marked during the light phase and at the higher dose (5 mg/kg) the difference with the dark phase was significant. Independent of dose, AMPH administration induced a stronger decrease in immobility during the dark phase. Together, this study demonstrated time of day differences in the behavioral effects of APO and AMPH.

## 4.1 Introduction

Midbrain dopamine (DA) neurons located in the ventral tegmental area (VTA) potently influence the locomotor activity effects induced by apomorphine (APO) and amphetamine (AMPH) administration (Morgenstern, 1980; Dunnet *et al*., 1984; Oades *et al*., 1986; Louis and Clarke, 1998). In previous experiments, we demonstrated that lower doses of APO, which have preferential effects on DA autoreceptors, have a stronger inhibitory effect on VTA DA cell firing when administered during the dark phase. The effects of AMPH on DA cell firing parameters were more stable throughout the day, but tended to be less inhibitory around midnight (Chapter 3). This prompted us to ask if time of day sensitivity to APO and AMPH could also be observed in locomotor activity.

The effects of APO and AMPH administration on rodent locomotor activity have been well documented. The effects of APO are biphasic, with low doses of APO (50 to 500 μg/kg) decreasing locomotor activity and higher doses (> 500 μg/kg) increasing locomotor activity and the occurrence of stereotypic behaviors (Strömbom, 1976; Havemann *et al.*, 1986: Depoortere *et al*., 1996). In comparison, AMPH induces a preferential increase in locomotor activity at doses ranging from 0.5 to 5 mg/kg, with an increased frequency of stereotypic behaviors at doses higher than 2 mg/kg (Segal, 1975; Porrino *et al*., 1984; Kuczenski and Segal 1989).

Time of day differences have been reported for behavioral responses seen following the administration of stimulating doses of APO (> 500 μg/kg) (Nakano *et al*., 1980; Kuribara and Tadokoro, 1982). To our knowledge, no data are available about diurnal effects of lower doses of

APO on locomotor activity. For AMPH, time of day differences in locomotor-activating effects have been observed in some (Wolfe *et al*., 1977; Webb *et al*., 2009) but not all studies (Gaytan *et al*., 1998a, b). Thus, the aim of the present experiments was to evaluate the acute effects of AMPH and lower doses of APO on locomotor activity at different times of the day.

## 4.2 Materials and methods

### 4.2.1 Animals

Adult male Sprague Dawley rats (Charles River, Saint-Constant, Quebec, Canada) weighing 300-330 g were housed under standard laboratory conditions with a 12 h light-dark cycle (lights on at 07:00 h) with *ad libitum* access to food and water. Rats remained in the animal facilities for at least one week before behavioral testing. All procedures were in accordance with the guidelines set by the Canadian Institute of Health Research for animal care and scientific use.

### 4.2.2 Drugs

D-amphetamine sulphate (AMPH, Sigma-Aldrich, UK), R-(-)-apomorphine hydrochloride hemihydrate (APO) and chloral hydrate (Sigma-Aldrich, Oakville, Canada) were dissolved in physiological solution of NaCl 0.9% (saline) as vehicle (VEH). All drugs were freshly prepared the day of the experiments and administered in a 0.5 ml volume by intraperitoneal (i.p.) injection.

### 4.2.3 Behavioral activity in the open field test

The animals in their home cages were placed for habituation in the procedural room 12 h before testing with food and water *ad libitum*. Experiments during the dark phase were conducted under

dim red light provided by an infrared heating lamp (Philips, Infrared Heat, wavelength peak: 800-1200 nm). Experiments during the light phase were performed under normal dim light. The open field consisted of a black painted closed box (80 x 80 x 40 cm) in which animals were tested individually for a period of 10 min. The first five minutes of the test were considered habituation time. The remaining five minutes were taken for analysis. Behavioral analysis included locomotion or distance traveled (m) and immobility time (s). All behavioral sessions were video recorded and analyzed off-line using a digital movement analysis system (Videotrack, Viewpoint Life Science, Montreal, Quebec, Canada).

### 4.2.4 Experimental design

Different cohorts of rats were tested in one of six time intervals across the light-dark cycle (light phase: 07:00-11:00 h, 11:00-15:00 h and 15:00-19:00 h; dark phase: 1900-2300 h, 2300-0300 h and 0300-0700 h). At each time interval, rats were randomly assigned to receive a single i.p. dose of APO (50, 100 or 200 µg/kg), AMPH (0.5, 1, 2 or 5 mg/kg) or VEH (n = 4 - 5 / time interval / dose. Total N = 223). All drugs were administered 10 min before testing began in the open field.

### 4.2.5 Statistical analysis

Data were analyzed using SigmaPlot statistical suit (Systat Software, Inc.). Two-way ANOVA was used to determine interactions between phase of the cycle and dose of drug administered (Phase x Dose), and time interval x Dose, on behavioral activity. For post hoc comparisons after ANOVAs, Bonferroni corrected t-tests were used. All data were presented as mean ± standard error of the mean (SEM). Statistical values of $p \leq 0.05$ were considered significant. Data

analyses were performed to compare behavioral activity between light and dark phases and between time intervals as percentage of change from VEH.

## 4.3 Results

### 4.3.1 Locomotor activity in the open field test across the light-dark cycle

A total of 28 rats were tested in the control group (VEH), half in the light and half in the dark phase. No significant difference between dark and light phase was found in immobility ($t_{(26)} = 0.910$, $p = 0.371$) or in distance traveled ($t_{(26)} = 1.037$, $p = 0.309$). A significant increase in distance traveled ($F_{(5,22)} = 4.265$, $p = 0.007$) was observed in rats tested at 2300-0300 h compared with rats tested at 1500-1900 h ($p = 0.029$) and at 1900-2300 h ($p = 0.003$), as shown in Figure 1A. No significant changes between time intervals were detected for immobility ($F_{(5,22)} = 0.63$, $p = 0.68$; see Figure 1B).

### 4.3.2 APO inhibits locomotor activity: time of day effects

The effect of APO was tested in 82 rats, half in the light and half in the dark phase. Significant Dose by Phase ($F_{(3,102)} = 5.04$, $p = 0.003$) and Dose by Time Interval ($F_{(15,86)} = 2.2$, $p = 0.012$) interactions were found for distance traveled. As depicted in Figure 2A, APO dose-dependently reduced distance traveled in both phases. The dose of 50 μg/kg had a stronger inhibitory effect in the light phase ($p < 0.001$ vs. dark phase), particularly in rats tested at 0700-1100 h and at 1500-1900 h compared with rats tested at 2300-0300 h and at 0300-0700 h. Independent of Phase and time interval, rats treated with the dose of 50 μg/kg had increased immobility duration ($F_{(3,86)} = 5.56$, $p = 0.002$; see Figure 2B). Independent of Dose, APO administration had a stronger effect

on immobility in the light phase ($F_{(1,102)} = 10.18$, $p = 0.002$; see insert in Figure 2B), particularly at 0700-1100 h.

### 4.3.3 AMPH administration increases locomotor activity: time of day effects

The effect of AMPH was tested in 113 rats, 56 in the light and 57 in the dark phase. Independently of Phase or time interval, all doses of AMPH reduced immobility time ($F_{(4,131)} = 7.9$, $p < 0.001$), as indicated in Figure 3B. Independent of Dose, AMPH administration had a stronger effect on immobility during the dark phase ($F_{(1,131)} = 6.7$, $p = 0.011$), particularly at 1900-2300h (Figure 3B). The effect of AMPH on distance traveled depended on the time of day, as indicated by a significant Dose by Phase ($F_{(4,131)} = 6.8$, $p < 0.001$) and Dose by Time Interval interactions ($F_{(20,111)} = 5.68$, $p < 0.001$). A dose-dependent increase in distance traveled was observed preferentially in time intervals of the light phase (Figure 3A). The effect of the 5 mg/kg dose was significantly higher in the light phase ($p < 0.001$ vs. dark phase). In the dark phase, only the dose of 0.5 mg/kg induced a significant increase in distance traveled. No effect of AMPH administration on distance traveled was observed at 2300-0300 h.

## 4.4 Discussion

To our knowledge this is the first evaluation of the inhibitory effects of lower doses of APO on locomotor activity across the light-dark cycle. In general, APO administration decreased distance traveled while increasing immobility time. AMPH administration induced the opposite effects, decreasing immobility and increasing distance traveled. These results are good agreement with the effects reported for APO and AMPH, in the range of doses used, when time of day is not taken into consideration (Porrino *et al*., 1984; Depoortere *et al*., 1996; Schwarting and Huston, 1992).

The exact behavioral effects of APO and AMPH depended on the time of day. The lower doses of APO induced stronger inhibitory effects during parts of the light phase. These diurnal differences were most evident in rats receiving APO at 50 μg/kg. No diurnal differences were observed with the dose of 200 μg/kg which induced strong behavioral inhibition at all times. Similar to what we observed here, though, Nakano *et al*. (1980) reported a higher expression of stereotypic behaviors in rats tested during the light phase as compared to dark following the administration of quite high doses of APO (> 1000 μg/kg). In mice, time of day effects might differ, and Kuribara and Tadokoro (1982) reported greater increases in mouse locomotor activity during the dark phase with APO doses of 500 and 1000 μg/kg.

The behavioral effects of high doses of APO (> 500 μg/kg) are considered to reflect the activation of postsynaptic $D_2$ hetereoreceptors, mimicking enhanced DA neurotransmission (Strömbom, 1976; Havemann *et al.*, 1986; Depoortere *et al*., 1996). Locomotor depression observed with APO at lower doses (< 500 μg/kg) is thought to reflect activation of presynaptic $D_2$ autoreceptors and a subsequent inhibition of DA release (Bunney *et al*., 1973; Baudry *et al*., 1977; Havemann *et al.*, 1986; Jackson and Westlind-Danielsson, 1994). Our results then could be interpreted as reflecting increased activation of $D_2$ autoreceptors during the light phase. However, available information indicates that $D_2$ binding sites in striatal areas increase at night (Torner *et al*., 1998) and in our previous electrophysiological experiments we also detected a stronger effect of APO in VTA DA neurons tested during the dark phase (Chapter 3). Previous work indicates that doses of APO ranging from 50 to 200 μg/kg reduce extracellular DA levels to 50% of baseline values (Zetterström and Ungerstedt, 1984; Imperato *et al*., 1988; Andersen and Gazzara, 1993). Then, the weaker inhibitory effects of APO on behavioral activity may

derive from increased DA release that occurs at night (Paulson and Robinson, 1994; Feenstra *et al*., 2000; Hood *et al*., 2010).

Our results suggested diurnal differences in the behavioral activating effects of AMPH administration. In the light phase the effects of AMPH were observed as an increase in distance traveled, particularly with the dose of 5 mg/kg. In the dark phase, the effect of AMPH was observed as a decrease in immobility time more evident at 1900-2300 h. The distance traveled by rats administered AMPH at 2300-0300 h and 0300-0700 h was not different from VEH injected rats, which displayed a peak in distance traveled at these times. Webb *et al*. (2009) also reported a peak of locomotor activity at 2400 h in the control group and no further increase in locomotor activity of rats receiving AMPH administration (1 mg/kg). Interestingly these authors reported a higher response to AMPH administration at 0600 h. In our experiments, we also observed a higher effect of AMPH towards the end of the dark phase (at 0300-0700h) but only with the dose of 0.5 mg/kg. In contrast, Gaytan *et al*. (1998a, b) showed that the change in the distance traveled by rats, after administration of a given dose of AMPH (0.6, 1.25, 2.5, or 10 mg/kg), was of similar magnitude independent of time of day (0800, 1400, 2000 or 0200 h). We indeed observed a similar level of movement induced by the different doses of AMPH tested, particularly in the intervals included from 1500 to 2300 h, but not in latter time intervals of the dark phase. The differences in outcomes may be due to differences in the methodology used. For example, Gaytan et al., (1998a, b) used a smaller test chamber, a long habituation period (2 days) and a 5 h period of observation post-AMPH administration, and they reported diurnal differences in other behaviors (e.g. horizontal activity and stereotypy movements), that may be better observed in such test conditions (Mazurski and Beninger, 1988a, b).

Since AMPH administration promotes a dose-dependent release of DA in terminal areas (Kuczenski *et al*., 1991), it is likely that AMPH's behavioral effects are enhanced when DA tissue content is high. In rats, DA tissue content in DA terminal areas is higher during the light phase, decreasing at night when extracellular DA release is increased (Schade *et al*., 1993; Castaneda *et al*., 2004). Thus during the light phase, a higher availability of DA tissue content will produce a dose-dependent increase in extracellular DA that, in an otherwise hypodopaminergic state, would stimulate locomotor activity, as we observed. Although a decreased DA tissue content might be hypothesized to limit DA-releasing and locomotion effects of AMPH at night, we also observed a more pronounced decrease in immobility time during the dark phase. Of note, then, it has been reported that NAcc DAT expression and TH levels, two proteins target by AMPH, are higher at night, while DA clearance is decreased (Sleipness et al., 2007). Therefore a possible explanation is that the effects of AMPH are facilitated in the dark phase (as shown by a preferential decrease in immobility) by dynamics in the DA system that favors a hyper-dopaminergic state (increased basal DA release, DAT and TH expression and decreased DA clearance). In these conditions the expression of other behaviors (other than distance traveled) may be favored by AMPH administration in the dark phase, as previously suggested (Gaytan et al., (1998a, b). In addition, diurnal changes in 5-HT and NE may also account for the behavioral effects of AMPH (Kuczenski et al., 1989; Kuczenski and Segal, 1992).

### 4.5 Conclusion

Our results show, for the first time, a diurnal difference in the locomotor effects of APO and AMPH. The inhibitory effect of APO at 50 μg/kg on behavior was less effective in rats tested

during the night. The stimulatory effect of AMPH on distance traveled was in general stronger during the daytime particularly at higher doses (5 mg/kg). In the dark phase AMPH had more pronounced effects on immobility. Increased distance traveled in the control group during nighttime intervals may have masked effects produced by APO and AMPH administration in the middle of the dark phase. Although the behavioral effects and associated neurochemical mechanism of these two drugs have been well characterized, few studies have described the neurochemical dynamics occurring across the light-dark cycle. In addition, AMPH interacts with other neurotransmitters system (e.g. NE and 5-HT) and their diurnal dynamics may also account for the changes observed in behavior. Nevertheless, our study demonstrates time of day differences in the behavioral effects of APO and AMPH and adds to the evidence that diurnal changes in brain DA neurotransmission must be taken into consideration to better understand behavioral effects of drugs acting on the DA system.

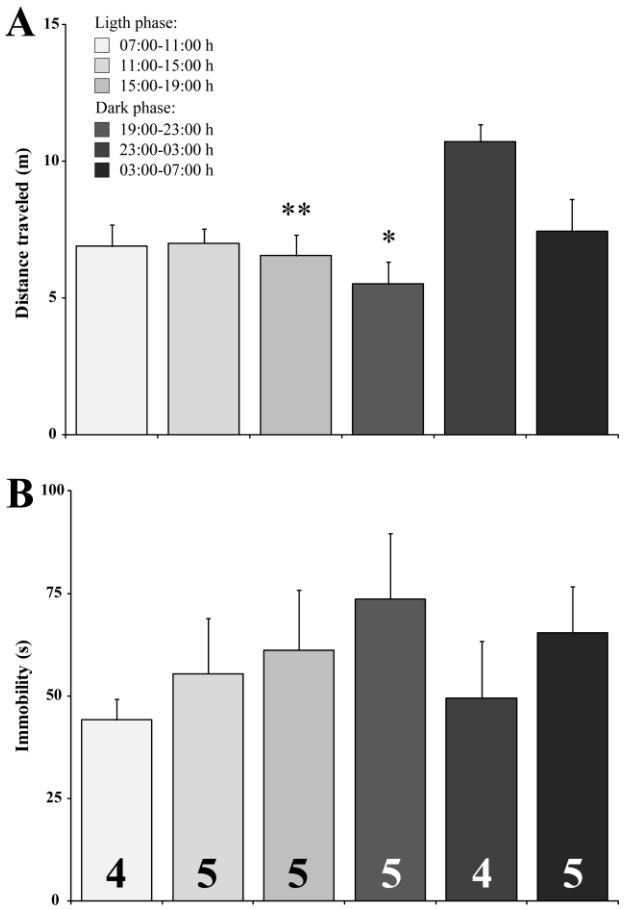


**Figure 1.** Locomotor activity of rats with intraperitoneal administration of saline (VEH). A) An increase in distance traveled was observed at 2300-0300h in the dark phase. B) No significant changes were observed in immobility duration between phases or time intervals. Bars represent mean ± SEM. Numbers within bars indicates number of rats in each time interval. Bonferroni t-test post hoc comparisons: ** $p < 0.01$ and $p < 0.05$ vs. 2300-0300 h.

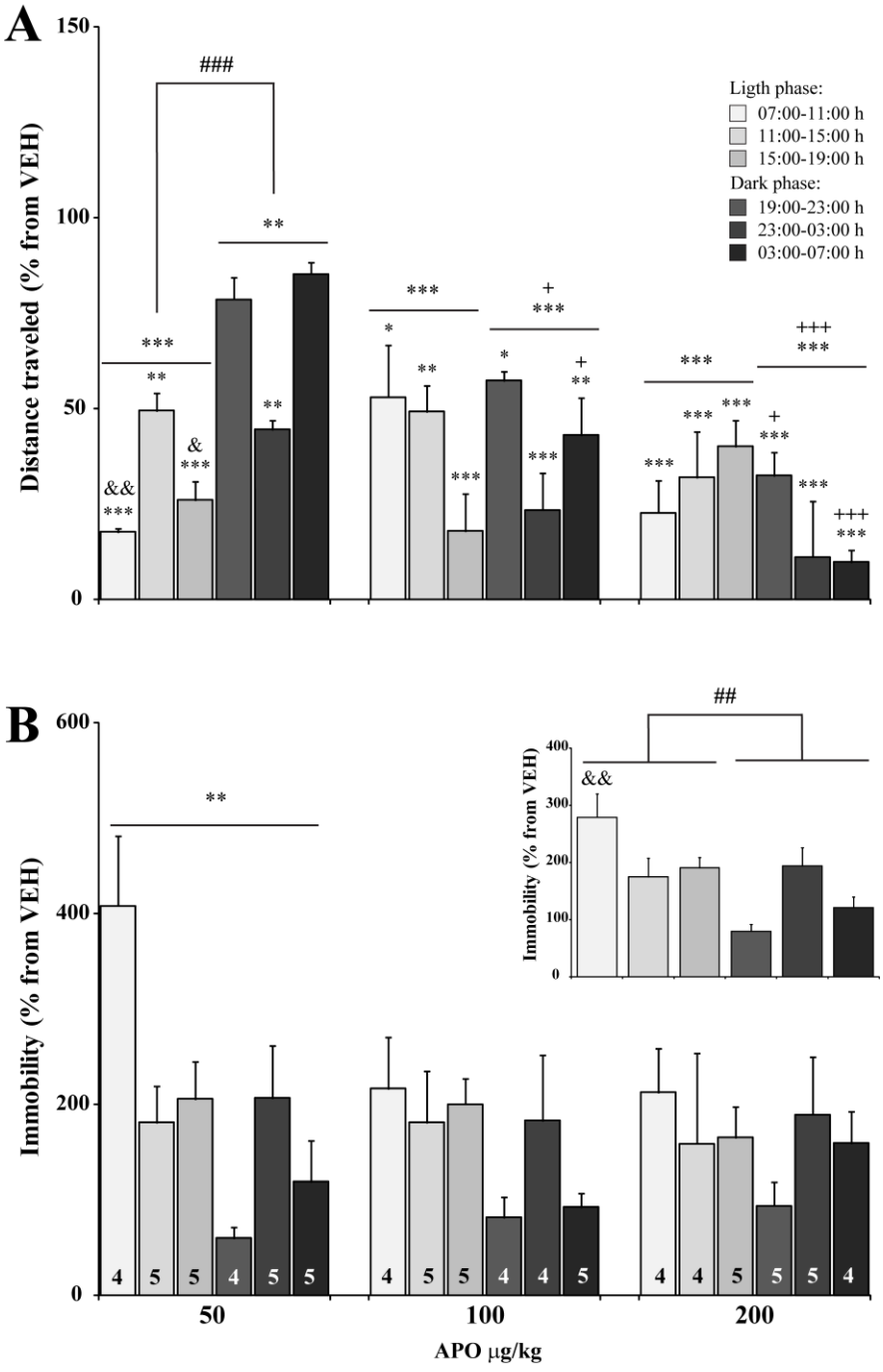
A
Distance traveled (% from VEH)
150
100
50
0
###
**
***
***
+
***
***
+++
***
Ligth phase:
07:00-11:00 h
11:00-15:00 h
15:00-19:00 h
Dark phase:
19:00-23:00 h
23:00-03:00 h
03:00-07:00 h
B
Immobility (% from VEH)
600
400
200
0
**
##
&&
4 5 5 4 5 5 4 5 5 4 4 5 4 4 5 5 5 4
50
100
200
APO μg/kg

**Figure 2.** Locomotor activity of rats after intraperitoneal apomorphine (APO) administration at different time intervals of the light and the dark phase. A) APO induced a dose-dependent decrease in distance traveled influenced by Phase and time interval. The effect of APO at the dose of 50µg/kg was stronger in intervals of the light phase, particularly at 0700-1100 h and at 1500-1900h. B) Immobility was increased with APO administration at the dose of 50 µg/kg independently of Phase and time interval. Insert: Immobility duration was increased by APO in the light phase as indicated. Bars represent mean ± SEM expressed as percentage change from vehicle (VEH) treated rats. Numbers within bars indicates number of rats in each time interval. Bonferroni t-test post hoc comparisons: *** $p < 0.001$, ** $p < 0.01$ and $p < 0.05$ vs. VEH; +++ $p < 0.001$ and + $p < 0.05$ vs. 50 µg/kg; && $p < 0.01$ and & $p < 0.05$ vs. 0300-0700 h and vs. 2300-0300; ### $p < 0.001$ and ## $p < 0.01$ vs. dark phase.

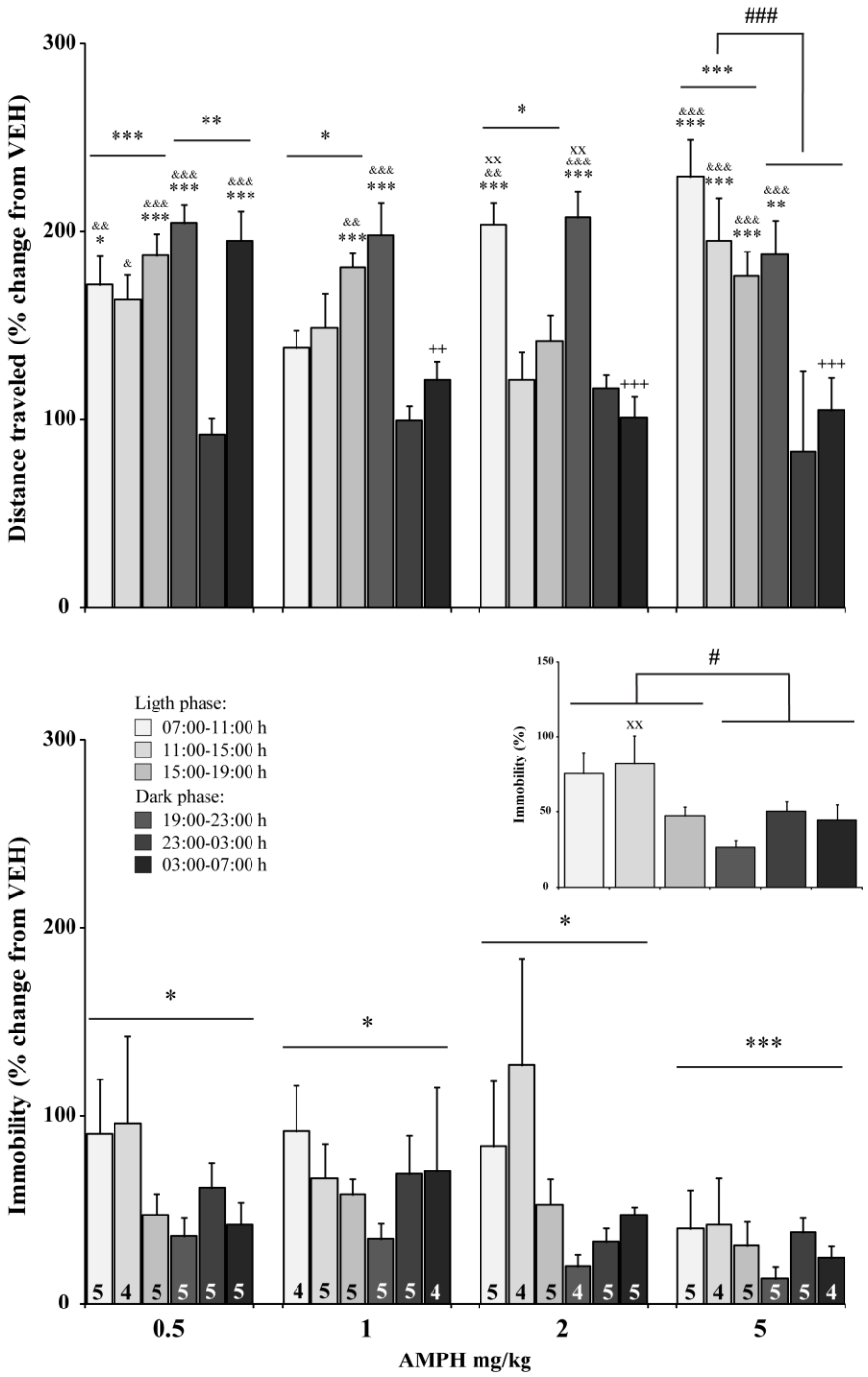
Distance traveled (% change from VEH)
300
200
100
0
###
***
**
*
*
*
***
**
Immobility (%)
150
100
50
0
#
XX
Ligth phase:
07:00-11:00 h
11:00-15:00 h
15:00-19:00 h
Dark phase:
19:00-23:00 h
23:00-03:00 h
03:00-07:00 h
Immobility (% change from VEH)
300
200
100
0
*
*
*
***
5 4 5 5 5 5
4 5 5 5 5 4
5 4 5 4 5 5
5 4 5 5 5 4
0.5
1
2
5
AMPH mg/kg

**Figure 3.** Locomotor activity of rats after intraperitoneal amphetamine (AMPH) administration at different time intervals of the light and the dark phase. A) AMPH induced a dose-dependent increase in distance traveled in most time intervals of the light phase. The effect of AMPH at the dose of 5 mg/kg was stronger in intervals of the light phase. No effects of AMPH were observed at 2300-0300 and at 0300-0700 h, except with the dose of 0.5 mg/kg. B) AMPH decreased immobility duration, independently of Phase or time interval. Independent of Dose, the effect of AMPH on immobility duration was stronger in the dark phase. Bars represent mean ± SEM expressed as percentage change from vehicle (VEH) treated rats. Numbers within bars indicates number of rats. Bonferroni t-test post hoc comparisons: *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs. VEH; +++ $p < 0.001$ and ++ $p < 0.01$ vs. 0.5 mg/kg; &&& $p < 0.001$, && $p < 0.01$ and & $p < 0.05$ vs. 2300-0300 h and vs. 0300-0700h (except in 0.5 mg/kg group); XX $p < 0.01$ vs. 1900-2300h; ### $p < 0.001$ and # $p < 0.05$ vs. dark phase.

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# Chapter 5
# General Discussion and Conclusions

In this doctoral thesis, the existence of diurnal rhythms in the basal activity of 5-HT and DA producing neurons located in the DRN and the VTA, respectively, were investigated using electrophysiological recordings in anesthetized rats. Effects of systemically administered MLT were measured across the light-dark cycle and the influence of the endogenous secretion of MLT on cell firing was examined in 5-HT neurons. For DA cells, the inhibitory effects of the direct and indirect DA agonists, APO and AMPH, were evaluated at different time intervals of the light-dark cycle, and, finally, the effects of APO and AMPH on rat's locomotor activity were assessed as an attempt to identify a behavioral association with the DA neuronal responses.

## 5.1 Diurnal rhythms in 5-HT and DA firing activity

Findings presented in Chapter 2 demonstrate that the population of active 5-HT neurons and their mean firing rates seem to fluctuate synchronously in a 24 h diurnal rhythm with peaks at noon and lower values occurring during the night (see Figure 1A). In Chapter 3, we demonstrated that the number of spontaneously active DA neurons also exhibits a 24 h diurnal rhythm with a peak occurring just before the transition from the dark to the light phase and the trough occurring toward the transition from the light to dark phase. Superimposed on this diurnal rhythm, mean firing rate values of DA neurons changed in a 12 h ultradian rhythm with peaks of activity occurring in the first hours of the light and the dark phases (see Figure 1B).

Together these observations confirmed our primary hypothesis that diurnal rhythms of firing activity in monoaminergic neurons can be observed in anesthetized animals, isolated from behavioral and sensory stimulation. Other authors have also suggested that in the study of biological rhythms, behavioral feedback must be minimized to avoid any influence on endogenously generated rhythms, similar to constant routine protocols used in humans (Minors and Waterhouse, 1984; Duffy and Dijk, 2002). In recent years, monoaminergic rhythms have become a topic of interest for understanding psychiatric disorders, such as depression (Salomon and Cowan, 2013). The next step would be to determine if the rhythms in monoaminergic neurotransmission observed in our experiments are altered in animal models of psychiatric disorders. However, our description of the diurnal rhythms of the firing activity of monoaminergic neurons in rats must be interpreted cautiously when being compared with humans; in particular we should keep in mind that we use a nocturnal animal in our experiments. It is then quite possible that different patterns of monoaminergic basal activity could be revealed in diurnal animals. This too could be studied in laboratory animals. Nevertheless, there is some evidence that 5-HT neurotransmission is affected the same way by sleep cycle, light environment and behavioral activity in nocturnal and diurnal rodents (Ferraro and Steger, 1990; Dudley *et al*., 1998; Birkett and Fite, 2005; Ashkenazy-Froling *et al*., 2010).

The diurnal pattern observed in the activity of 5-HT and DA neurons was suggestive of a strong influence of the photoperiod. This is because most of the changes in activity were observed just after and during the transition from one phase of the light-dark cycle to another. However, 5-HT neurons and their firing rates seem to be more tightly related to the 24 h light-dark cycle, whereas the DA neuron firing rate pattern indicates the presence of other regulatory influences.

There is indeed evidence of circadian control of DA neurotransmission, since functional expression of clock genes has been detected in several parts of the DA system (Hampp *et al.*, 2008; Hood *et al*., 2010; Mukherjee *et al.*, 2010). In addition, there is also evidence of an indirect projection from the SCN to the VTA (Luo and Aston-Jones, 2009). The diurnal difference in the expression of TH and DAT in NAcc, prefrontal cortex and caudate seems to depend of the presence of the SCN (Sleipness *et al*., 2007) and be out of phase with TH expression in the VTA suggesting multiple osccilation sources in DA neurotransmission (Weber *et al*., 2004; Sleipness *et al.*, 2007; Webb *et al.*, 2009). In comparison, it remains to be determined whether there is clock gene expression in the DRN. However, functional projections between the SCN and the raphe (DRN and MRN) have been described and 5-HT innervations are considered an important input modulating photic and non-photic regulation of the circadian system (Kawano *et al*., 1996; Morin *et al*., 2013). These findings together with our observations of the diurnal pattern of DA and 5-HT neuronal firing activity could suggest, firstly that clock genes and the SCN influence DA neurotransmission and secondly that 5-HT neurotransmission has a regulatory role on the master clock translating environmental light, as has previously been proposed (Amir *et al*., 1998; Ciarleglio *et al*., 2011). All this could be interpreted as a true circadian rhythm of DA neuron firing activity and a photoperiod dependent diurnal rhythm of 5-HT neuron firing activity.

**5.2 MLT influence on monoamine neurons firing activity**

As reviewed in the General Introduction (section 1.1), MLT is considered a neurohormone that synchronizes endogenous rhythms to the duration of the photoperiod. Given this, we tested whether MLT alters monoamine neuronal firing activity as an attempt to understand how the light-dark cycle influences their diurnal rhythms. First, we demonstrated that low doses of MLT

(0.5 – 1 mg/kg) have an acute inhibitory effect on both 5-HT and DA neuronal firing activity. This suggested that impulse-dependent release of 5-HT and DA may be affected by MLT. Indeed, our findings supported previous reports that MLT administration promotes tissue accumulation of DA and 5-HT and decreases their release (Anton-Tay *et al.*, 1968; Cardinali *et al.*, 1975; Miguez *et al.*, 1994; Zisapel, 2001). Of likely relevance to these effects, we recently reported immunohistochemical evidence of somatodendritic $MT_1$ receptors in the DRN and $MT_1$ and $MT_2$ receptors in the SN and VTA (Lacoste *et al*., 2014). Indeed, experiments reported here suggested that inhibitory effects of MLT on 5-HT neuronal firing activity may be indeed mediated by $MT_1$ receptors (Chapter 2).

The inhibitory effect of MLT administration (1 mg/kg) was seen in 60% of 5-HT neurons but only 30% of DA neurons tested (see Chapters 2 and 3, respectively). Inhibitory effects of MLT on firing activity have been studied in some detail in hippocampus and SCN neurons. Within these cells, the inhibitory effects have been attributed to MLT's capacity to induce cell membrane hyperpolarization (Zeise and Semm, 1985; Mason and Brooks, 1988; Jiang *et al*., 1995). Through activation of $MT_1$ receptors, MLT can stimulate G-protein inwardly rectifying potassium channels (Kir3, formerly GIRK) (Nelson *et al*., 1996). The presence of Kir3 channels in DA neurons in the VTA and 5-HT neurons in the DRN suggested that MLT could be inducing hyperpolarization of DA and 5-HT neurons through an increase in potassium conductance and subsequent membrane hyperpolarization (Inanobe *et al*., 1999; Eulitz *et al*., 2007; Saenz del Burgo *et al*., 2008). The opening of Kir3 channels is also a mechanism of action activated by 5-$HT_{1A}$ and $D_2$ autoreceptors to induce membrane hyperpolarization of 5-HT neurons in the DRN and DA neurons in the VTA, respectively (Penington *et al*., 1993; Maejima *et al*., 2013;

Hamasaki *et al*., 2013; Beckstead *et al*., 2004). However, it remains to be determined if MLT receptors are indeed expressed on DRN 5-HT neurons and VTA DA neurons or if these receptors are localized in other neuronal populations (e.g. GABA or glutamate neurons) within these two regions.

Since MLT had greater effects on 5-HT than DA neurons, we pursued the serotonergic responses further. In experiments described in Chapter 2, we showed that removal of the pineal gland at night increased 5-HT firing activity, thus providing evidence that the endogenous secretion of MLT exerts a tonic inhibition on 5-HT neuron firing activity. These findings are evidence that basal 5-HT neurotransmission is under control of the photoperiod via its endocrine MLT signal in a mechanism likely implicating $MT_1$ receptors (section 2.4.4). At this point, determinations of plasma and brain MLT levels would be desirable to support our claim of an association between 5-HT firing activity and MLT secretion, especially to backup our results after removal of the pineal gland. Nevertheless, our results in the 5-HT system led us to propose that 5-HT neurotransmission could be modulated by changes in photoperiod duration, representing a mechanism of action by which light therapy could exert its antidepressant effect and an etiological cause for SAD. Findings in brain imaging studies also suggest that there is an association between 5-HT neurotransmission and light environment conditions (section 1.4.3). From a wider perspective, the relation of 5-HT neurotransmission with light could be derived from the evolutionary role of 5-HT as a photo-energy convertor molecule (Azmitia, 2007).

### 5.3 Time of day differences on DA neurons response to DA agonists

In Chapter 3, we tested the hypothesis that monoaminergic neurons will also display diurnal differences in their responses to pharmacological agents. For this, we tested the acute effects of APO and AMPH on VTA DA cell firing. We chose these two drugs for their different mechanisms of action: APO activates DA receptors directly whereas AMPH accomplishes this indirectly by inducing DA release and inhibiting reuptake, mainly by interacting with the DAT (White and Wang, 1984a; 1984b; Di Chiara and Imperato, 1988; Diana and Tepper 2002). Both drugs inhibit midbrain DA cell firing activity. Of note, though, low doses of APO (< 50 μg/kg) activate $D_2$ autoreceptors selectively. As detailed in Chapter 3, our results showed that the inhibitory effect of APO on VTA DA neurons was stronger during the dark phase. The diurnal differences in DA neuron responses to APO flattened as the dose of APO increased. In contrast, we did not detect a diurnal rhythm in the inhibitory effects of AMPH.

Based on the pattern of results, it was proposed that the diurnal effects of APO reflect an increased number of $D_2$ somatodendritic autoreceptors at night, similar to the diurnal pattern reported for $D_2$ receptor binding in the striatum (Torner *et al*., 1998). In contrast, the effects of AMPH seem to be modified by different factors that may be independent of $D_2$ receptor expression and likely related to its actions as a DA releaser. In light of our results with APO, if AMPH administration releases an equal amount of DA at any given time of the light-dark cycle, we would expect a higher inhibition of VTA DA neurons at night by increased $D_2$ autoreceptor activation. This does not seem to occur. Given this, it seems more reasonable that the amount of DA released by AMPH varies during the day. This might be linked to diurnal changes in the

expression of DAT and DA clearance, but also to diurnal changes in TH expression. Unfortunately, information on how these factors interact on a 24 h basis remains scarce and incompletely understood (Sleipness *et al.*, 2007; Sleipness *et al.*, 2008; Webb *et al.*, 2009). Finally, diurnal modifications in the neuronal populations involved in the feedback loop to VTA DA neurons could also affect DA neuronal firing rate inhibition (Shi, 2009).

Thus, although in our preparation we were not able to detect changes in VTA DA neuron responses to AMPH administration we cannot excluded the possibility that diurnal changes may still occur at nerve terminals. It would be necessary, for example, to assess the amount of DA that AMPH is able to release at a given time of the day. Incorporating the use of microdialysis or cyclic voltammetry to assess DA efflux in terminal areas along with diurnal recordings of DA midbrain monoaminergic neurons could be an improved strategy to assess diurnal effects of drugs targeting the DA system and other neurotransmitter systems (Panin *et al*., 2012; Daberkow *et al*., 2013). This proposed strategy can also be used to describe chemical and neuronal dynamics of monoamine systems in basal conditions and after chronic pharmacologic treatment across the light-dark cycle.

The neuronal response of midbrain monoaminergic neurons has been widely used as a model to test the effects of drugs acting on the central DA and 5-HT system since the late 60s (Aghajanian *et al*. 1968; 1970; Bunney *et al*., 1973a; b). Our work provided evidence that this preparation can also be used to assess time of day effects of central DA system and 5-HT system acting drugs. Several behavioral studies have already provided evidence that the efficacy of antipsychotics, antidepressants, mood stabilizers, benzodiazepines, barbiturates, and psychostimulants depend

on the time of administration (Nagayama, 1999; Bruguerolle, 2009). Recently, delivery systems have started to evolve from constant drug release rate systems to variable drug release systems with the goal of improved clinical efficacy (Youan, 2004; Ohdo, 2010). Thus, we believe that our model will be useful to define the time of maximal efficacy of drugs targeting monoamine transmission, which can later be translated into more optimal pharmacological treatments.

**5.4 Time of day differences on behavioral response to DA agonists**

In the last set of experiments we tested whether the behavioral effects of APO and AMPH are associated with the diurnal responses observed in the DA neurons. These studies indicated that the inhibitory effects of APO on locomotor activity were markedly more potent during daytime intervals, whereas in the dark phase the effect of the lowest APO dose (50 μg/kg) was blunted. This is, to our knowledge, the first description of locomotion-inhibiting effects of lower doses of APO across the light-dark cycle. The behavioral effects of AMPH were opposite to those of APO, inducing increases in locomotor activity and decreases in immobility. A dose-dependent effect of AMPH was observed during daytime intervals on distance traveled but not in the dark phase when the reduction of immobility was most potent.

The series of experiments summarized above provided evidence for time of day differences in the behavioral effects of APO and AMPH. In general, our results were in agreement with those reported in the literature for these two drugs when administered during the light phase (Porrino *et al*., 1984; Depoortere *et al*., 1996; Schwarting and Huston, 1992). An important observation was that the time of day effects of both drugs were better explained when their opposing effects on extracellular DA levels were also taken into consideration (Zetterström and Ungerstedt, 1984;

Radhakishun *et al*., 1988; Kuczenski and Segal, 1989; Ozaki *et al*., 1989; Kuczenski *et al*., 1991; Chai and Meltzer, 1992). Thus, this suggested that the behavioral effects of these drugs depend on the available reserves of DA and the levels of extracellular DA occurring at a given time of the day. The opposite effects on locomotor activity between the light and dark phases observed for APO (higher immobility in the day) and AMPH (lower immobility in the night) suggest that the ongoing increase in nocturnal behavioral activity (and associated changes in DA release) modifies the way in which DA pharmacological agents will interact with the brain's DA biochemistry.

Some of the diurnal DA neuronal responses to APO and AMPH observed in our preparation in anesthetized animals did not map directly to the behavioral responses seen in conscious animals. Data from our electrophysiological recordings provided information about the activity and response of VTA DA neurons in isolation from behavioral feedback. Then, the differences between diurnal behavioral responses and the responses observed in VTA DA neurons to AMPH and APO could be attributable to other factors still to be identified. For example, Webb *et al*. (2009) have provided evidence that the rhythmic expression of TH in Nacc and VTA can have different contributions to the expression of behavior across the day. We designed our experiments based on evidence that the locomotor effects of APO and DA are influenced by the DA mesolimbic pathway originating in the VTA (Morgenstern, 1980; Dunnet *et al*., 1984; Oades *et al*., 1986; Louis and Clarke, 1998). However, the diurnal effects of APO and AMPH in DA neurotransmission and behavior could also be modulated by other neurotransmitters and other neuronal populations; for example, 5-HT, NE, glutamate and GABA (Swerdlow and Koob, 1984; Olds, 1988; Kuczenski and Segal, 1989; Kim and Vezina *et al*., 1998; Gaytan *et al*.,

1998b; Elliot and Beveridge, 2005; Kehr *et al*., 2011). This underscores the need to explore how neurotransmission in other systems changes across the day and their implications for behavioral responses to drug challenges.

## 5.5 Conclusions

### *Limitations*

Although performing electrophysiological recordings around the clock over 24 h has its merits, several methodological considerations have to be pointed out that could have been improved in our experiments. As mentioned above, diurnal rhythms in a nocturnal animal such as the rat might not translate well to species that are awake during the day. Repeating our experiments in diurnal animals and in those that are closer to human physiology (e.g., non-human primates) would be informative. Our conclusions would be further supported if measurements of monoamine release in DRN and VTA cell projection areas were conducted in parallel with our electrophysiological recordings (e.g. microdialysis or cyclic voltammetry). This also applies to MLT release, especially in the experiments in PX rats. In our pharmacological challenges we used two DA agonists (APO and AMPH) with different mechanisms of action which we concluded was the reason for their different effects. However, the use of other $D_2$ agonists with similar mechanism of action, in addition to immunocytochemical studies to quantify diurnal expression of $D_2$ receptors expression in the VTA, would corroborate our proposals about changes in DA receptor availability. Similar studies will also help to better explain the behavioral results.

***Future directions***

A number of experiments were proposed above that would complement the work done in this thesis. In addition to these, it would be of interest to measure whether photoperiod duration affects diurnal firing activity rhythms of 5-HT and DA neurons. Certainly, results described in this thesis suggest that changes in the photoperiod associated with an increase of nocturnal MLT secretion (i.e. shorter photoperiod or longer dark phase duration) could influence negatively 5-HT neurotransmission. The effects on DA neurotransmission are more difficult to predict due to the dissociation in rhythms between spontaneously active DA neurons and their mean firing frequency. To further improve our understanding of these rhythms, recordings of 5-HT and DA neuronal activity should be performed in rats kept on different light-dark cycle schedules (i.e. short and long photoperiods). This approach would also benefit from a secondary method to assess neuronal activity such as cFOS immunolabeling, with co-labeling of 5-HT (i.e. Tph or 5-HTT) and DA (i.e. TH or DAT) neuronal markers. In addition, plasma determinations of MLT would be necessary to associate changes in its diurnal secretion pattern with changes in photoperiod duration and monoamine neuronal activity. Since we have the hypothesis that a decrease in impulse activity in monoaminergic (in particular 5-HT) neurotransmission may result from short photoperiods due to an increase of MLT secretion, behavioral testing aimed to determine changes in mood (e.g., depressive and anxiety like-behavior) would also be required.

Another important follow-up experiment could be to determine how long-term pharmacological treatment can affect the diurnal rhythm of monoaminergic neurotransmission. It would be interesting to see, for example, whether 5-HT, DA and behavioral diurnal rhythms could be

modified by acute and chronic treatment with antidepressants or drugs of abuse (e.g. amphetamines, cocaine, ethanol). The use of a diurnal animal in all these experiments might help the translation of findings to humans.

***Clinical significance***

The monoaminergic systems have been implicated in a wide variety of physiological functions and the etiology of several neuropsychiatric disorders. Despite the evidence that monoaminergic neurotransmission varies across the light-dark cycle, the number of studies conducted remains small and their relevance for diagnosis and treatment in psychiatry remains largely unexplored (Nagayama, 1999; Bruguerolle, 2009). This noted, in recent years there is increasing evidence that behavioral disturbances in psychiatric patients are associated with disruptions to circadian rhythms (Benedetti, 2012; Pritchett *et al*., 2012; Monti *et al*., 2013; Robillard *et al*., 2013). The importance of the diurnal pattern of 5-HT neuron firing activity and its relation with the photoperiod and MLT secretion were already discussed as relevant for SAD and major depression. Recent evidence has also suggested that changes in DA neurotransmission could be linked to SAD (Cawley *et al*., 2013; Praschak-Rieder and Willeit, 2012). Changes in diurnal 5-HT and DA neurotransmission associated with changes in photoperiod may also be important to understand seasonal patterns and the occurrence of bipolar disorder and schizophrenia in the population (Duncan *et al*., 2006; Sung *et al*., 2011; Disanto *et al*., 2012; Zeschel *et al*., 2013) and comorbid sleep disorders (Harvey, 2008). Time of day effects of psychostimulants and other abused drugs that affect DA neurotransmission might help to explain diurnal patterns of drug consumption and overdose (Raymond *et al*., 1992; Erickson *et al*., 1998; Gibbs and Rose, 2000),

but can also be relevant for DA related neurological diseases such as Parkinson's (Santiago *et al*., 2010; Willison *et al*., 2013).

If we are able to characterize normal diurnal rhythms of neurotransmitter systems, we will be better positioned to identify changes associated with pathological states. The pharmacological treatment might then be optimized to return brain function to a normal rhythm of activity using pharmacological doses and specific times of administration that are more efficacious and with fewer adverse effects. This approach has already proved useful in other disciplines, such as oncology, immunology and cardiology (Baraldo, 2008; Ohdo, 2010).

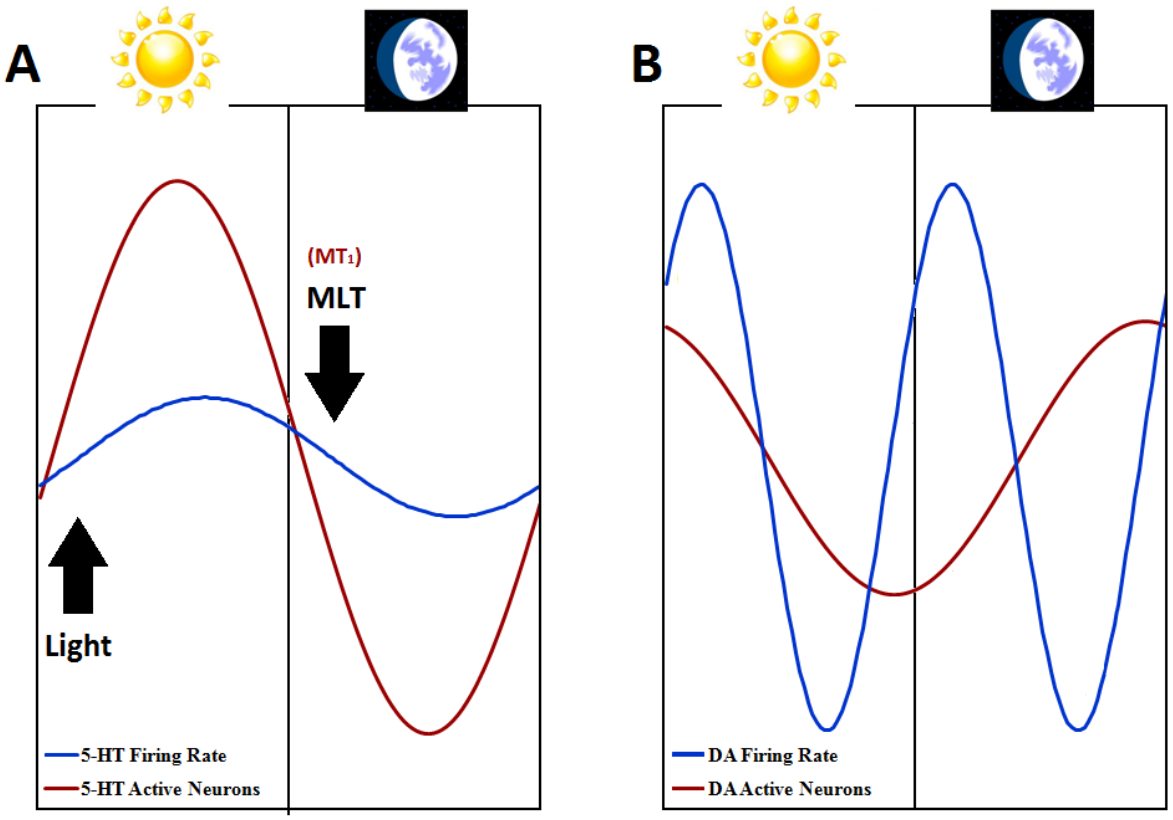


**Figure 1.** Summary of main findings. A) Serotonin (5-HT) neurons in the dorsal raphe nucleus have a 24 h rhythm of firing activity associated with the photoperiod. The population of active 5-HT neurons and their firing rate follows a parallel pattern that rises at the beginning of the light phase reaching their maximum values at midday. The secretion of melatonin (MLT) at night seems to exert a tonic inhibitory influence over 5-HT neurons firing activity, probably mediated by $MT_1$ receptor activation. B) Dopamine (DA) neurons in the ventral tegmental area have a complex pattern of activity that suggests additional influences other than the photoperiod. The population of active DA neurons varies with a 24 h diurnal rhythm out of synchrony with a 12 h rhythm in DA cell firing frequency.

# Appendix
## List of publications

**Papers**

Comai S, Ochoa-Sanchez R, **Dominguez-Lopez S**, Bambico F (2014) Melancholic depression-like behaviors and diurnal neurobiological abnormalities in melatonin MT1 receptor knockout mice. Mol Psychiatry. Under review.

Lacoste B, Angeloni D, **Dominguez Lopez S**, Fraschini F, Descarries L and Gobbi G (2014) Anatomical and subcellular localization of melatonin MT1 and MT2 receptors in the adult rat brain. J Comp Neurol: Under review.

**Dominguez Lopez S**, Howell RD, López- Canúl MG, Leyton M and Gobbi G (2014) Electrophysiological characterization of dopamine neuronal activity in the ventral tegmental area across the light-dark cycle. Synapse: accepted with revision.

Rainer Q, Speziali S, Rubino T, **Dominguez Lopez S**, Bambico FR, Gobbi G and Parolaro D (2014) Chronic nandrolone decanoate exposure during adolescence affects emotional behavior and monoaminergic neurotransmission in adulthood. Neuropharmacology: 83C; 79-88.

**Dominguez-Lopez S**, Mahar I, Bambico FR, Labonte B, Ochoa-Sanchez R, Leyton M and Gobbi G (2012) Short term effects of melatonin and pinealectomy on serotonergic neuronal activity across the light-dark cycle. J Psychopharmacol**:** 26; 830-844.

Morales-Medina JC, **Dominguez-Lopez S**, Gobbi G, Beck-Sickinger AG and Quirion R (2012) The selective neuropeptide Y Y5 agonist [cPP(1-7),NPY(19-23),Ala31,Aib32,Gln34]hPP differently modulates emotional processes and body weight in the rat. Behav Brain Res: 233(2); 298-304.

**Dominguez-Lopez S**, Howell R and G Gobbi (2012) Characterization of serotonin neurotransmission in knockout mice: implications for major depression. Rev Neurosci: 23(4); 29-43.

Labonte B, McLaughlin R, **Dominguez-Lopez S** , Bambico FR, Lucchino I, Ochoa-Sanchez R, Leyton M and Gobbi G (2012) Adolescent amphetamine exposure elicits dose-specific

effects on monoaminergic neurotransmission and behavior in adulthood. Int J Neuropsychopharmacol: 15(9); 1319-1330.

Ochoa-Sanchez R, Comai S, Lacoste B, Bambico FR, **Dominguez-Lopez S**, Spadoni G, Rivara S, Bedini A, Angeloni D, Fraschini F, Mor M, Tarzia G, Descarries L and Gobbi G (2011) Promotion of NREM sleep and activation of reticular thalamic neurons by a novel MT2 melatonin receptor ligand. J Neurosci: 31(50); 18439-18452.

Mahar I, Tan S, Davoli MA, **Dominguez-Lopez S**, Qiang C, Rachalski A, Turecki G, Mechawar N (2011) Subchronic peripheral neuregulin-1 increases ventral hippocampal neurogenesis and induces antidepressant-like effects. PLoS One: 6(10); e26610.

Bambico FR, Cassano T, **Dominguez-Lopez S**, Katz N, Walker CD, Piomelli D and Gobbi G (2010) Genetic deletion of fatty acid amide hydrolase alters emotional behaviour and serotonergic transmission in the dorsal raphe, prefrontal cortex, and hippocampus. Neuropsychopharmacology: 35(10); 2083-2100.

**Invited talks**

**Dominguez-Lopez S** (2011) Diurnal firing activity of monoaminergic neurons. Integrated Program of Neuroscience Retreat, McGill University, Montreal, Quebec, Canada.

**Dominguez-Lopez S** (2010) Melatonin decreases serotonergic activity in the dorsal raphe neurons through MT1 receptors and induces a depressive-like behavior in the forced swim test. V Congreso de Estudiantes de Posgrado e Investigadores Mexicanos en Canadá, CEIMEXCAN, Gatineau, Quebec, Canada.

**Abstracts**

Mahar I, Tan S, Labonte B, Davoli MA, **Domínguez-López S**, Qiang C, Rachalski A, Turecki G, Mechawar N. Neuregulin-1 administration increases ventral hippocampal neurogenesis and induces antidepressant effects. 12th Annual McGill Biomedical Graduate Conference, McGill University, Montreal, Quebec, Canada. January 2012.

**Dominguez-Lopez S**, López-Canul MG, Howell R, Leyton M, Gobbi G. Characterization of the dopaminergic firing activity in the ventral tegmental area and its response to amphetamine across the light-dark cycle. Abstract No. P-15-004. 10th World Congress of

Biological Psychiatry, Praga, Republica Checa. World Federation of Societies of Biological Psychiatry 2011: On line.

Mahar I, Davoli MA, Tan S, **Dominguez-Lopez S**, Mechawar N. Peripheral neuregulin-1 administration increases the number of adult-born dentate gyrus cells and produces antidepressant effects. Abstract No. 29.11/B1. 2010 Neuroscience Meeting Planner. San Diego, CA, USA; Society for Neuroscience 2010: On line.

**Dominguez-Lopez S**, Ochoa-Sanchez R, Comai S, Leyton M, Gobbi G. Melatonin decreases serotonergic activity in the dorsal raphe neurons through MT1 receptors and induces a depressive-like behaviour in the forced swim test. Abstract No. N10-136. 4nd Annual Canadian Neuroscience Meeting, Ottawa, Ontario, Canadá; Canadian College of Neuropsychopharmacology 2010: On line.

Mahar I, Davoli MA, Tan S, **Dominguez-Lopez S**, Mechawar N. Peripheral neuregulin-1 administration increases the number of adult-born dentate gyrus cells and produces antidepressant effects. Proceedings of the Canadian Society for Life Science Research 5th Annual Conference, Montreal, QC, Canadá; Canadian Society for Life Science Research 2010: 52-53.

**Dominguez-Lopez S**, Leyton M, Gobbi G. Melatonin decreases serotonergic activity in the dorsal raphe neurons by direct action on MT1 receptors. Abstract No. 750.24/W21. 2009 Neuroscience Meeting Planner. Chicago, IL, USA; Society for Neuroscience 2009: On line.

**Dominguez-Lopez S**, Leyton M, Gobbi G. Effect of melatonin on the serotonergic neural activity across the light-dark cycle. Abstract No. P-22-014. 9th World Congress of Biological Psychiatry, Paris, France; World Federation of Societies of Biological Psychiatry 2009: 211.

Labonte B, Luchino I, Bambico F, **Dominguez-Lopez S**, Leyton M, Gobbi G. Chronic administration of amphetamine during adolescence modifies monoamine transmission and induces behavioral disinhibition. Abstract No. 59.14/DD20. 2008 Neuroscience Meeting Planner. Washington, DC, USA; Society for Neuroscience 2008: On line.

**Dominguez-Lopez S**, Mahar I, Leyton M, Gobbi G. Modulation of serotonin neurotransmission by melatonin. Abstract No. B-E2170. 2nd Annual Canadian Neuroscience Meeting, Montreal, Quebec, Canadá; Canadian Association for Neuroscience 2008: On line.

**Dominguez-Lopez S**, Mahar I, Gobbi G. Modulation of serotonin neurotransmission by melatonin. Abstract No. 465.12/H24. 2007 Neuroscience Meeting Planner. San Diego, CA, USA; Society for Neuroscience 2007: On line.

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