FORM AND FUNCTION OF A SUPRACHIASMATIC NUCLEUS - VENTRAL TEGMENTAL AREA CIRCUIT

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DEDICATIONS

To Suge and Irie, the best little helpers anyone could ask for.

To the wonderful friends I’ve made at Penn, both in the Aston-Jones lab and in the Neuroscience Graduate Program. I could not have spent this special part of my life with a more caring group of people.

And most of all, to my fiancé Ed, none of this was possible without your unending love and support.
ABSTRACT

FORM AND FUNCTION OF A SUPRACHIASMATIC NUCLEUS - VENTRAL TEGMENTAL AREA CIRCUIT

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The Earth’s pervasive 24 hr light/dark cycle has resulted in a fundamental rhythmicity in nearly all living organisms. This rhythmicity depends on the synchronization of endogenous circadian oscillators (i.e. entrainment), but also the direct influences of environmental timing cues (i.e. masking), such as light. In the mammalian circadian system, it is well established that the suprachiasmatic nucleus (SCN) is the central pacemaker whose signals contribute to temporal regulation of numerous physiological and behavioral processes. Using anatomical tract-tracing and electrophysiological techniques, I describe a novel output pathway from SCN to ventral tegmental area (VTA), a midbrain nucleus with a pivotal role in motivation and arousal. Chapter two will delineate the anatomical structure of this SCN-VTA circuit. Using the trans-synaptic, retrograde tract tracer, pseudorabies virus (PRV; Bartha strain), I found there exists a synaptically linked circuit from SCN that impinges on VTA through the medial preoptic nucleus (MPON). Using single-unit, extracellular recording techniques in the anesthetized rat, I further characterized resulting diurnal and circadian fluctuations in VTA impulse activity. Chapter three describes a novel population of VTA neurons that selectively fires during the active circadian phase. These neurons were fast-firing, low-
bursting, with wide action potential widths, and were non-responsive to footpad stimulation. Neurochemically, they were non-dopaminergic, non-GABAergic, but sensitive to changes in dopaminergic neurotransmission. Chapter four describes diurnal and circadian variations in classic dopaminergic and GABAergic VTA neurons. The two cell types were differentially influenced by the daily cycle with a unique pattern of topographically and functionally segregated populations firing a different phases. At one level, the results of this thesis present a novel synaptic circuit, which may drive organisms' adaptations to the ubiquitous 24 hr cycle. At another, they increase our appreciation for the immense complexity of the ventral tegmental area, both at a cellular and functional level.
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CHAPTER ONE

GENERAL INTRODUCTION

As earth rotates around its axis in a 24 hr period, half the earth is illuminated by the sun and half is in darkness at any one time. Nearly all living organisms have adapted to this pervasive 24 hr light/dark cycle. Circadian rhythms are processes that exhibit a periodicity of about 24 hrs without exogenous influences. Numerous processes exhibit circadian rhythms, including body temperature, hormone release, and the sleep/wake. In the laboratory, we can observe these circadian rhythms because we remove environmental time cues (e.g. housing under constant light or dark). The result is rhythms that are about, but not exactly, 24 hrs (Aschoff, 1981). Processes that persist under constant conditions are therefore termed “circadian”, and ones that are only expressed under light/dark conditions are termed “diurnal”. The role of light, a primary zeitgeber (time-giver), is at least two-fold. One is to “entrain” an endogenous circadian pacemaker such that the circadian rhythm is exactly 24 hrs (Aschoff, 1981; Pittendrigh, 1981). The second is to directly influence behavior itself, as evidenced by the light-induced inhibition of locomotor activity in nocturnal rodents. This phenomenon is termed “masking” as experimentally it often masks the indirect influence of “entrainment” (Mrosovsky, 1999). Masking is thought to be an important determinant of temporal niche (e.g. diurnality or nocturnality), although the underlying neuro-circuitry of masking is still relatively ill-defined (Redlin and Mrosovsky, 2004; Mrosovsky and
Hattar, 2005). Regardless, masking and entrainment are thought to be complementary phenomena needed for full expression of biological rhythms.

Characteristics of the suprachiasmatic nucleus

In the mammalian system, it is well established that the suprachiasmatic nucleus (SCN) is the principal endogenous circadian pacemaker. The SCN is a small nucleus (~10,000 cells each hemisphere) located in the anterior hypothalamus, just dorsal to the optic chiasm. Bilateral lesions of SCN abolish circadian rhythms in most processes including hormone levels, locomotor activity, drinking behavior, and sleep-wake (Moore and Eichler, 1972; Stephan and Zucker, 1972; Ibuka et al., 1977). Important to note is that SCN lesions do not abolish the process itself, but rather its temporal consolidation. SCN's 24 hr oscillations persist after knife cut isolations from the rest of brain (Inouye and Kawamura, 1979), within slice preparations (Groos and Hendriks, 1982), and even in dissociated cell cultures (Welsh et al., 1995). Furthermore, transplantation of donor SCN to SCN-lesioned recipients restore their circadian rhythms (Lehman et al., 1987; Viswanathan and Davis, 1995).

Circadian rhythmicity in SCN originates from molecular transcription/translation feedback systems. The clock is made of positive and negative components that together form an auto-regulatory loop. The mammalian positive limb contains the transcription factors CLOCK (CLK) and BMAL1 which heterodimerize and stimulate transcription of three mammalian PERIOD (mPER) genes and two mammalian cryptochrome (mCRY) genes (Gekakis et al., 1998; Bunger et al., 2000). The resulting mCRY and mPER proteins also heterodimerize, and once these repressor complexes reach a critical
concentration, they block the stimulatory action of CLK and BMAL1. As a result, transcription of mPERs and mCRYs are diminished, as are their corresponding proteins, allowing the auto-regulatory loop to continue (Kume et al., 1999; Vitaterna et al., 1999).

Overall the SCN exhibits it highest firing rates during subjective day and its lowest during subjective night (Groos and Hendriks, 1982; Shibata et al., 1982). However, the SCN is not simply a homogenous grouping of cell-autonomous circadian clocks. For example, SCN neurons can differ in their efferent/afferent circuitry, pacemaking ability, response to exogenous timing cues, and neuropeptide expression.

The SCN has been anatomically and to large extent, functionally sub-divided into the “ventrolateral” or “core”, and “dorsomedial” or “shell” region (Moore, 1996b). Notably, SCN core, but not shell receives visual information via the retinohypothalamic tract (RHT) and the geniculohypothalamic tract (GHT) from the intergeniculate leaflet (IGL) of the lateral geniculate nucleus (LGN). The core also receives a strong projection from median raphe (Moga and Moore, 1997). In contrast, the shell primarily receives inputs from the thalamus, hypothalamus, basal forebrain, and limbic cortices; the majority of which are also efferent targets of SCN (Watts and Swanson, 1987; Watts et al., 1987; Moga and Moore, 1997; Leak and Moore, 2001). Synaptic connectivity within SCN is largely uni-directional, with core projecting to shell but not vice versa (Leak et al., 1999). However, synaptic information flow may not be the primary means for intra-SCN communication, as gap junctions and diffusible factors are important for SCN synchrony (Aton and Herzog, 2005).

It has long been thought that nearly all SCN neurons express γ-aminobutyric acid (GABA), although ultra-structural studies now suggest the proportion is closer to 40-70%
Regardless, GABA is still the most prominent neurotransmitter within SCN and is expressed in both core and shell regions. Therefore, the sub-regions are most often neurochemically distinguished by their differing neuropeptide expression. SCN core neurons express vasoactive intestinal peptide (VIP) and a dense neuropeptide-Y innervation from the IGL. In contrast, SCN shell neurons express arginine vasopressin (VP) (Moore et al., 2002). Multiple other neuropeptides are also differentially expressed including gastrin-releasing peptide and calretinin, however, the VIP/VP distinction is most often used to define the boundaries of core and shell (Moore et al., 2002).

The SCN is also physiologically heterogeneous. A prevailing view is that SCN is made up of endogenously oscillating neurons that synchronize to form a monophasic output (Liu et al., 1997). However, more recent evidence suggests that not all SCN neurons fire, or express clock proteins rhythmically, and even when they are rhythmic, the oscillations are not necessary in phase (Hamada et al., 2001; Quintero et al., 2003). One theory is that SCN core contains rhythmic and non-rhythmic “gate” cells that respond to photic input. In contrast, the shell contains “oscillator” cells primarily responsible for intrinsic pacemaking. Through reciprocal interactions, the two components create a cohesive output (Antle and Silver, 2005).

SCN slices from light/dark housed animals differ from those housed under dark/dark (Quintero et al., 2003). In particular, rhythmic PER1 neurons are more prevalent in SCN core from light/dark animals. Diurnal rhythms are a product of both masking effects and SCN entrainment. The role of the SCN in masking is still unresolved. CRY mutants and SCN lesioned animals still show nocturnal locomotor
activity if housed under light/dark conditions (Redlin and Mrosovsky, 1999; Albus et al., 2002). However, CLOCK mutants, which lack circadian rhythms (Vitaterna et al., 1994), and Melanopsin mutants, which lack photic entrainment, also exhibit impaired masking responses (Mroovsky and Hattar, 2003; Redlin et al., 2005). Interestingly, lesions of IGL and visual cortex, likely candidate sites to regulate masking, tend to increase masking (Redlin et al., 1999; Redlin et al., 2003). Furthermore, the results by Li et al., (2005) conflicts with Redlin and Mrosovsky (1999) as they find light-masking deficits in SCN lesioned animals. The authors suggest that perhaps their lesion affected masking by also abolishing the SCN projection to subparaventricular zone (SPZ). Redlin and Mrosovsky (1999) did not report whether their lesions also severed this pathway. Presently it is not known whether the underlying substrates of masking involve the SCN itself, downstream effector systems, or completely bypasses the SCN system although.

Output pathways of a central circadian timing system

Indeed, we know relatively little about how SCN communicates with the rest of the brain and body to impart overt circadian rhythms. SCN projects directly to a limited number of areas, with its strongest projections to other hypothalamic areas (Swanson and Cowan, 1975; Watts and Swanson, 1987; Watts et al., 1987). These intra-hypothalamic projections may account for some processes, such as the circadian fluctuation in hormone levels (Funabashi et al., 2000; Palm et al., 2001), and body temperature (Osborne and Refinetti, 1995; Ruby et al., 2002). However, these direct inputs do not likely account for circadian influences on other phenomena, such as sleep/wake, reward and motivation. Rather, studies have shown that indirect circuit projections are an important means by
which SCN temporally regulates behaviors such as the sleep-wake cycle (Aston-Jones et al., 2001; Deurveilher and Semba, 2005; Gonzalez and Aston-Jones, 2006).

**Neurobiological substrates of behavioral arousal**

A modern, prevailing theory of sleep/wake regulation is that two complementary processes drive its structure. “Process S” is the homeostatic pressure to sleep, which accumulates during wake and dissipates during sleep. “Process C” is the circadian process, which is the temporal drive by the endogenous circadian pacemaker. It is largely independent of prior sleep/wake (i.e. Process S), and determines time periods with higher or lower propensities for sleep (Borbely, 1982; Daan et al., 1984). The two-process model has been highly successful in describing and predicting outcomes in a variety of experimental protocols, includes sleep deprivation and forced desynchrony (Daan et al., 1984; Dijk and Czeisler, 1995). The biological locus of Process C is largely agreed upon to be the SCN. The neurobiological systems involved in the homeostatic regulation (Process S) of sleep/wake continue to be an active area of research.

Early in the last century, neurologist Von Economo carefully studied the anatomical correlates of encephalitis lethargica and found that cases of persistent insomnia or coma-like somnolence exhibited lesions in differing brain areas. He concluded that sleep promoting areas were located in the anterior hypothalamus and basal forebrain, and wake promoting areas were located in the posterior hypothalamus and rostral midbrain (Von Economo, 1931). Later it was found that lesions of dopamine containing neurons in the ventral tegmentum produced a state of behavioral unresponsiveness. This behavioral somnolence was not necessarily correlated with...
cortical activity (EEG activity), and it was therefore concluded that the midbrain
tegmentum was important for behavioral arousal but not necessary for cortical arousal
(Jones et al., 1973).

Characteristics of the ventral tegmental area

The ventral tegmental area (VTA) is a nucleus located within the midbrain tegmentum.
Because VTA is surrounded by other dopaminergic cell groups with no obvious
anatomical demarcation, the defined boundaries of VTA vary between studies. However,
generally speaking, the VTA is rostrally bound by the posterior hypothalamus, caudally
by the rostral linear nucleus of the raphe, laterally by the substantia nigra pars compacta,
and dorsally by the red nucleus and tegmental decussations (Oades and Halliday, 1987).

The afferent/efferent circuitry of VTA positions it as a highly integrative
structure. The VTA receives inputs from a multitude of sources, including, but not
limited to, the prefrontal and cingulate cortices, lateral septum, nucleus accumbens,
ventral pallidum, basolateral amygdala, preoptic area, dorsal and median raphe,
pedunculopontine nucleus and laterodorsal tegmental nucleus (Phillipson, 1979; Geisler
and Zahm, 2005). In addition, generally no one input projects more strongly to the VTA
than another (Geisler and Zahm, 2005). VTA axons primarily ascend through a fiber
system termed the "medial forebrain bundle", and terminate in several of the same
structures from which the VTA receives inputs. The two most prominent projection
streams are commonly termed the "mesocortical" and "mesolimbic" pathways (Swanson,
1982). The mesocortical pathway typically refers to VTA neurons that project to the
prefrontal (i.e. prelimbic and infralimbic) and cingulate cortices. The mesolimbic
pathway typically refers to VTA neurons that project to the nucleus accumbens, olfactory tubercle, and ventral pallidum. Interestingly, VTA axons are largely non-collateralized, indicating separate populations of the VTA neurons project to separate target areas (Fallon and Loughlin, 1982; Albanese and Minciacchi, 1983). This characteristic is in contrast to the other monoamine containing nuclei; the raphe and locus coeruleus are noted for their extensive collateralization (Fallon and Loughlin, 1982).

The VTA is often regarded as synonymous with the A10 dopaminergic cell group (Dahlstrom and Fuxe, 1964), although it also contains GABA neurons (Hökfelt et al., 1984; Mugnaini and Oertel, 1985; Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000). Co-localization of dopamine and glutamate within VTA neurons has also been reported (Kaneko et al., 1990; Dal Bo et al., 2004), in addition to co-localization with cholecystokinin and neurotensin (Seroogy and Fallon, 1989; Jayaraman et al., 1990; Bayer et al., 1991). Most recently, the co-localization of dopamine and glutamate has been re-evaluated as reports indicate that glutamatergic neurons may comprise a separate, third population of VTA neurons (Kawano et al., 2006; Yamaguchi et al., 2007).

The electrophysiological characteristics of VTA neurons are based upon seminal intra- and extracellular studies on substantia nigra dopamine and GABA neurons (Guyenet and Aghajanian, 1978; Grace and Bunney, 1983; Lacey et al., 1989). Similar characteristics were found in VTA neurons (Yim and Mogenson, 1980; Johnson and North, 1992a; Steffensen et al., 1998), and therefore the same criteria were (Cameron et al., 1997) ascribed to them. The characteristics of VTA DA neurons include, 1) long duration action potentials (2-4 ms), 2) low spontaneous firing rates (2-9 spikes/sec), 3) pronounced \( I_h \) current, 4) burst firing in vivo, 5) slow conduction velocity (mean = .046
m/sec to nucleus accumbens), and 6) DA agonist-induced inhibition. These are in contrast to presumed VTA GABA neurons that are characterized by, 1) short duration action potentials (0.5-2 ms), 2) fast spontaneous firing rates (10-50 spikes/sec), 3) lack of I_h current, 4) lack of bursting activity, 5) faster conduction velocity (mean = 2.2 m/sec to nucleus accumbens), and 6) DA agonist-induced excitation (Yim and Mogenson, 1980; Wang, 1981; Shinba et al., 1985; Johnson and North, 1992a; Steffensen et al., 1998).

As mentioned above, VTA DA neurons fire relatively slowly in a single-spike pattern, with intermittent bursts. The burst firing pattern exhibited by VTA DA neurons in vivo is theorized to be physiologically and functionally important to the organism. Burst firing results in a supralinear increase in synaptic DA release (Gonon, 1988; Wightman and Zimmerman, 1990) and is thought to act as a reward learning signal (Montague et al., 1996; Hollerman and Schultz, 1998). Burst activity patterns are dependent on glutamatergic afferent inputs, as burst firing is not exhibited in the de-afferented in vitro slice preparation but can be induced by application of glutamatergic agonists (Grace and Bunney, 1984; Johnson et al., 1992).

The ventral tegmental area and behavioral arousal

Recent studies have shown the activity of VTA dopamine (DA) and GABA neurons varies with behavioral state, with activity lowest during slow-wave sleep, higher during active wake, and highest during paradoxical (REM) sleep (Lee et al., 2001; Maloney et al., 2002). In particular, VTA DA neurons show increased bursting activity during REM sleep, similar to that observed during appetitive stimuli presentation (Dahan et al., 2006). Furthermore, hyperdopaminergic DA transporter (DAT) knockout mice exhibit a REM-
like state during wakefulness when exposed to a novel environment (Dzirasa et al., 2006). This REM-like state can be blocked by D2 receptor agonist pre-treatment, in agreement with previous studies showing D2 agonist in VTA promotes slow-wave sleep (Bagetta et al., 1988).

The function of VTA in behavioral arousal has been extended to include an integral role in motivated learning and reward. Animals trained in a classical conditioning task show phasic DA burst firing increases following rewards and reward-predicting stimuli (Schultz et al., 1998). These phasic increases have been theorized to act as a neurobiological learning signal (Montague et al., 1996; Schultz, 2002). Furthermore, it has long been known that most addictive drugs elevate levels of dopamine originating from VTA (Di Chiara and Imperato, 1988). In particular, the rewarding properties of cocaine, amphetamine and nicotine are due to these elevated levels (Yokel and Wise, 1976; De Wit and Wise, 1977; Corrigall et al., 1992). In addition to VTA DA neurons, GABA neurons have also been implicated in reward, as their firing increases just before intra-cranial brain stimulation reward (Steffensen et al., 2001).

Modulation of arousal and reward processing by circadian factors

As with most behaviors, reward processing is also influenced by circadian factors. Behavioral sensitization (Andretic et al., 1999; Gaytan et al., 1999; Abarca et al., 2002), conditioned place preference (Abarca et al., 2002) and drug self-administration (Baird and Gauvin, 2000), are modulated by time of day. Several lines of animals with mutant circadian clock genes also show altered responsiveness to drug reward (Abarca et al., 2002).
2002; Liu et al., 2005; McClung et al., 2005; Spanagel et al., 2005). One of these, the mouse clock mutant, exhibits increased preference for cocaine that is concomitant with increased VTA dopamine neuron excitability (McClung et al., 2005). The mechanism by which circadian clock genes modulate reward circuitry is presently not known.

However, it is possible that animals with compromised SCN function due to mutated clock genes affect downstream reward regulatory sites, such as the VTA. Given this possibility and the VTA’s role in behavioral arousal, we hypothesized that the VTA is a downstream effector system of SCN. We utilized multiple tools to examine the structure and function of a possible SCN-VTA circuit. Our experiments reveal that a synaptic circuit from the SCN to the VTA exists via the MPON. Concomitantly, daily fluctuations in VTA impulse activity occur that differentially regulate DA and GABA neurons, including a newly characterized novel population of VTA neurons.
CHAPTER TWO

CIRCUIT PROJECTION FROM SUPRACHIASMATIC NUCLEUS TO VENTRAL TEGMENTAL AREA: RELAY IN MEDIAL PREOPTIC NUCLEUS

ABSTRACT

The suprachiasmatic nucleus (SCN) is a circadian pacemaker that synchronizes a number of vital physiological and behavioral processes. Although a great deal of research has focused on input pathways to SCN and on the central clock itself, relatively little is known about SCN output signaling pathways. The ventral tegmental area (VTA) has been extensively studied for its influence in motivated learning, and more recently for a potential role in arousal and sleep-wake regulation. Possible circadian influences on VTA neurons have received little attention, in part because the SCN does not directly project to VTA. Here we present data that SCN indirectly projects to VTA via the medial preoptic nucleus (MPON). Microinjection of the retrograde, trans-synaptic tracer pseudorabies virus (PRV; Bartha strain) in the VTA consistently resulted in labeling of the SCN at time-points indicative of an indirect circuit projection (48 hrs post-injection or longer). To specify intermediate relay nuclei between the SCN and VTA, putative nuclei were lesioned with ibotenic acid one week prior to PRV injections in the VTA. Unilateral lesions of the MPON reduced PRV labeling (48 hr survival time) in the SCN by 81.6% in the ipsilateral hemisphere and 75.8% in the contralateral hemisphere. Bilateral lesions of the caudal-dorsal lateral septum (cd-LS), another putative relay nuclei and dorsal injection control, did not significantly reduce in PRV labeling in the SCN. These results
show a novel SCN output pathway to the VTA that may function in the circadian regulation of numerous behavioral processes including arousal and motivation.

INTRODUCTION

From unicellular organisms to mammalian vertebrates, nearly all life on Earth has adapted to our pervasive 24 hr light/dark cycle. These circadian rhythms allow the organism to anticipate changes in its environment and to optimally adjust its state accordingly. A circadian timing system consists of three main components: (i) an input pathway containing both photic and non-photic information, (ii) a central circadian pacemaker, and (iii) an output pathway by which downstream processes can be synchronized to the central pacemaker.

In the mammalian system, it is well established that the suprachiasmatic nucleus (SCN) is a central pacemaker, through ablation (Moore and Eichler, 1972; Stephan and Zucker, 1972; Ibuka et al., 1977), transplant (Lehman et al., 1987; Viswanathan and Davis, 1995), and in vitro physiological studies (Groos and Hendriks, 1982; Murakami et al., 1991; Welsh et al., 1995). Largely due to advances in molecular biology and genetics, we know a great deal about how information is conveyed to SCN, and about the molecular workings of SCN itself. In contrast, we know very little about how SCN communicates to other parts of the brain and body to impart a circadian rhythmicity to behavioral and physiological processes.

The SCN projects directly to a limited number of areas, with its strongest projections to other hypothalamic areas (Swanson and Cowan, 1975; Watts and Swanson, 1987; Watts et al., 1987). These intra-hypothalamic projections may account for some
processes, such as the circadian fluctuation in hormone levels (Funabashi et al., 2000; Palm et al., 2001), and body temperature (Osborne and Refinetti, 1995; Ruby et al., 2002). However, these direct inputs do not likely account for circadian influences on other phenomena such as sleep/wake, reward and motivation. Rather, studies have shown that indirect circuit projections are an important means by which the SCN temporally regulates behaviors such as the sleep-wake cycle (Aston-Jones et al., 2001; Deurveilher and Semba, 2005; Gonzalez and Aston-Jones, 2006).

The ventral tegmental area (VTA) has been strongly implicated in reward and motivation (for review: Wise, 2004; Schultz, 2006) and more recently in sleep/wake regulation (Lee et al., 2001; Maloney et al., 2002). Studies in mutant animals have revealed that drug reward is influenced by circadian clock genes (Andretic et al., 1999; Abarca et al., 2002). In particular, the clock mutant shows high cocaine preference along with increased excitability of VTA dopamine neurons (McClung et al., 2005). The activity of VTA dopamine and GABA cells also varies with arousal (Lee et al., 2001; Maloney et al., 2002), and lesions of the VTA decrease arousal (Jones et al., 1973).

In the present study we injected the retrograde, trans-synaptic tracer, pseudorabies virus (PRV; Bartha strain) into VTA. PRV-Bartha virions invade neurons at the site of injection and are trans-synaptically passed to infect primary afferent neurons. The virus then replicates and is again transported to secondary afferent neurons. The cycle of replication and transport continues to repeat in a time-dependent manner and is confined to synaptically linked neurons (Card et al., 1993; Jansen et al., 1993; Rinaman et al., 1993). These attributes have made PRV-bartha an effective tool for the identification of extended circuitry to nuclei of interest (O'Donnell et al., 1997; Aston-Jones et al., 2001).
We performed a time-course analysis of PRV labeling and found SCN labeling at time-points consistent with an indirect VTA afferent. To identify possible intermediate relay nuclei in the SCN-VTA circuit, the caudal-dorsal aspect of the lateral septum (cd-LS) and the medial preoptic nucleus (MPON) were lesioned prior to PRV injections in VTA. Ibotenic acid lesions of the MPON, but not the cd-LS, markedly reduced PRV labeling in both hemispheres of the SCN. Taken together, these results indicate a novel SCN output pathway and that may be involved in the circadian regulation of sleep/wake and motivated behaviors.

MATERIALS AND METHODS

Anatomical Methods

Animal surgery and microinjections. Thirty-eight adult male Sprague Dawley rats (250-415 gm; Charles River Laboratories, Wilmington, MA) were used. All housing and surgical procedures conformed to the BioSafety level II regulations for studies involving the use of infectious pathogens [United States Department of Health and Human Services Publication No. (Centers for Disease Control and Prevention) 88-8395; Biosafety in Microbiological and Biomedical Laboratories]] and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Animals were anesthetized with ketamine/xylazine (55 mg/kg ketamine, 10 mg/kg xylazine, i.p.) and placed in a stereotaxic apparatus. For tracer injections, a mixture of PRV (Bartha strain; 1.7 x 10^9 pfu/ml, gift from J.P. Card, University of Pittsburgh) and cholera toxin subunit B (CTb; 0.05%; Sigma, St. Louis, MO) was unilaterally injected into the VTA through glass micropipettes (30-35 μm diameter tip). A volume of 480 nl of PRV/CTb was empirically
found to give adequate transport while maintaining focal injection size. This volume of tracer solution was delivered over a 15 minute period by brief pneumatic pulses from a controlled pressure source (Picospritzer; Parker Hannifin, Cleveland, OH). The pipette was left in place for an additional 5 minutes to reduce upward leakage along the injection tract. For lesion studies, ibotenic acid (1%; Acros Organics, Morris Plains, NJ) was either unilaterally (for MPON lesions, 360 nl) or bilaterally (for cd-LS lesions, 300 nl each hemisphere) injected into the target nuclei by similar means as described for tracer injections.

**Perfusion and tissue processing.** For time-course analysis of PRV infection, animals were sacrificed at 24, 36, 48, 52, 60 or 72 hrs after tracer injections. Animals were deeply anesthetized with ketamine/xylazine and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and post-fixed overnight with the same fixative. Brains were then transferred to 20% sucrose. Brains was cut into coronal sections (40 μm) on a cryostat and collected such that each well consisted of the entire brain at 240 μm intervals. For PRV immunohistochemistry, sections were incubated in a polyclonal rabbit anti-PRV antibody (1:5000, DP 134; developed by R. Miselis) overnight at room temperature. Sections were then incubated in biotinylated donkey anti-rabbit antibody (1:1000, Jackson Laboratories, West Grove, PA) for two hours at room temperature followed by ABC Elite reagents (1:1000, Vector Laboratories, Burlingame, CA) for 90 minutes at room temperature. Visualization of the signal was accomplished by incubation in a DAB-nickel solution for 3-5 minutes.
Immunohistochemical protocols for all other antigens were performed similarly to the above method with the following differences: (1) CTB: polyclonal goat anti-CTB (1:10,000, List Biological, Campbell, CA), donkey anti-goat IgG (1:1000, Jackson Laboratories), DAB solution, (2) Neuronal Nuclei (NeuN): monoclonal mouse anti-NeuN (1:1000, Chemicon, Temecula, CA), donkey anti-mouse IgG (1:500, Jackson Laboratories), DAB-nickel solution, (3) Tyrosine hydroxylase (TH): monoclonal mouse anti-TH (1:6000, DiaSorin, Stillwater, MN), donkey anti-mouse IgG (1:500, Jackson Laboratories), blue substrate SG (Vector Laboratories). All sections were mounted on gelatin slides and counterstained with methyl green (Vector Laboratories). Control studies were performed in which the primary antibody was omitted, and resulted in the lack of respective staining.

Quantification of PRV+ Neurons

Openlab image processing software (Improvision, Ltd.; Coventry England) was used to quantify PRV+ neurons from color digital images. Regions of interest were first selected by using a freehand selection tool according to previously established anatomical boundaries (Swanson, 2004). The numbers of PRV+ neurons in regions of interest were counted with a point-counter tool. This tool simultaneously marked and counted each cell so that no cells could be counted twice and the total of the number of cells counted was available. Sections (240 μm apart) were taken from each animal that corresponded to the rostral/caudal extent of each nuclei. Two blinded observers determined the number of PRV+ numbers. Because there was > 90% agreement between the two observers, the
counts of one of the observers was used for subsequent statistical and graphical analysis. Data sets were analyzed by t-test or one-way ANOVA as appropriate.

RESULTS

PRV injections in VTA result in time-dependent, retrograde transport to SCN.

PRV-Bartha was unilaterally injected in the VTA. Because the process of PRV replication and transport (see Introduction) is strongly time-dependent and can vary with a variety of factors (e.g. innervation; Card et al., 1999), it is important to perform a systemic time course analysis for each circuit of interest. Therefore, survival time points at 24, 36, 48, 52, 60, and 72 hrs (n = 22 animals total, 2-7 animals per time-point) were examined. PRV labeling was found in the SCN beginning at the 48 hrs time point in all cases with accurate injections in the VTA. SCN labeling was not present in cases in which injection sites were localized to nearby VTA nuclei (e.g. red nucleus, substantia nigra pars compacta or reticulate; data not shown). Figure 2.1A-C shows a time-course for PRV labeling in the VTA at 36, 48 and 60 hrs. At 36 hrs, PRV labeling was absent in the SCN although known primary afferents, such as nucleus accumbens (NAC; see Figure 2.3), lateral hypothalamus (LH) and pedunculopontine nucleus (PPT) showed PRV labeling.
Figure 2.1 Frontal photomicrograph of PRV retrograde labeling in the SCN at increasing time points. A, At 36hrs post-injection when labeling primary afferents was present (see figures 3, 4, 5a-b), labeling was absent in the SCN. B, By 48hrs, labeling was consistently observed in the ipsilateral, and to a lesser extent, in the contralateral SCN. C, By 60 hrs, labeling was substantial in both the ipsilateral and contralateral SCN. Midline is center and dorsal is up for all images. Scale bar = 200 μm. 3V, third ventricle; OC, optic chiasm.

Because PRV is avidly transported away from the injection site, the conventional retrograde tracer, Cholera Toxin subunit B (CTb) was included in the injection mixture to localize injection sites, and to differentiate between direct and indirect afferents, as in other studies (Chen et al., 1999; Aston-Jones and Card, 2000; Aston-Jones et al., 2001). Figure 2.2A-B shows a representative case of CTb immunoreactivity at the largest extent of the injection site. Tyrosine hydroxylase (TH) staining of adjacent sections was used to define the boundaries of VTA. Figure 2.3 shows retrograde labeling of CTb and PRV at 36hrs within the same area of NAC. Labeling in these areas continued to increase at longer survival time-points, however PRV was not consistently observed in the SCN until 48 hrs later (Figure 2.1B), strongly suggestive of an indirect afferent. Labeling appeared to be more dense in the region corresponding to the ipsilateral “shell” (Moore, 1996a, 1997; Moore and Silver, 1998). By 60 hrs, labeling was dense in both the ipsilateral and contralateral hemispheres of the SCN (Figure 2.1C).

Figure 2.2 Frontal photomicrograph of PRV/CTb injection in VTA. A, B, Because PRV is avidly transported away from the site of injection, CTB and TH immunoreactivity was used to localize the injection. A, CTb immunoreactivity at the largest extent of injection site. B, Adjacent
section stained for TH and used to define the boundaries of VTA. Midline is center and dorsal is up for all images. Scale bar = 500 μm.

Figure 2.3 Frontal photomicrographs of PRV (black) and CTb (brown) double immunoreactivity at 36 hrs post-injection in the NAC. A, Low-magnification photomicrograph of NAC. B, High-magnification photo of inset shown in (A). Blue arrows indicate PRV+ neurons and red arrows indicate CTb+ neurons. Midline is right edge of image and dorsal is up in (A). Scale bars A = 200 μm; B = 100 μm. AC, anterior commissure.

MPON and cd-LS are putative relay nuclei of the SCN-VTA circuit.

The time-course of PRV labeling in the SCN was consistent with that of an indirect afferent. In addition, CTb labeling was not present in any cases, and SCN has not been previously shown to directly project to the VTA (Phillipson, 1979; Geisler and Zahm, 2005). We therefore investigated possible intermediate nuclei that might serve as a relay in the SCN circuit projection to VTA. Criteria for candidate relay nuclei were (i) known direct target of SCN projections, (ii) known direct afferent to the VTA, and (iii) consistent PRV labeling at 36 hrs or less following injection; time-points prior to PRV labeling in the SCN. Relay nuclei that satisfied these criteria included the medial preoptic area (MPA), the lateral septum (LS), the bed nucleus of the stria terminalis (BNST), the paraventricular nucleus of the hypothalamus (PVN), and the lateral
hypothalamus (LH). Figure 2.4 shows that at 36 hrs, CTb and PRV+ neurons were intermingled within sub-regions of the MPA and LS, corresponding to the medial preoptic nucleus (MPON; Simerly et al., 1986; Simerly and Swanson, 1988) and the

![Image of Figure 2.4: PRV (black) and CTb (brown) double immunoreactivity of putative relay nuclei at 36 hrs post-injection. A, C Low-magnification photomicrographs of (A) caudal-dorsal aspect of lateral septum (cd-LS) and (C) medial preoptic nucleus (MPON). B, D High-magnification photomicrographs of insets shown in (A) and (C), respectively. Blue arrows indicate PRV+ neurons and red arrows indicate CTb+ neurons. Midline is right edge of image in (A) and center.](image)

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caudal-dorsal aspect of the lateral septum (cd-LS; Risold and Swanson, 1997,1997).

PRV+ labeling was higher at 36 hr survival in MPON and the cd-LS than in nearby nuclei, including known SCN efferents (PVT, paraventricular nucleus of the thalamus; PVN, paraventricular nucleus of the hypothalamus; VMH, ventromedial hypothalamus; p < 0.05 or smaller; ANOVA, Neuman Keuls post-test). Figure 2.5 shows labeling in the MPON and cd-LS at increasing time-points (36, 48, 60 hrs). The density of labeling increased with time and spread to adjacent areas. Because of the consistency and density of labeling in the MPON and cd-LS, these sub-regions were our primary candidate relay nuclei.

Figure 2.5 Frontal photomicrographs of PRV labeling in medial preoptic area (A-C) and lateral septum (D-F) at 36hrs (A,D), 48hrs (B,E), 60hrs (C,F), following PRV/CTb injection into VTA. Dashed lined in (B) and (E) indicate areas of heavy labeling corresponding to respective sub-regions, medial preoptic nucleus (MPON) and caudal-dorsal lateral septum (cd-LS), respectively. These sub-regions were targets of subsequent lesion studies (Figures 6 and

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Lesion of the MPON, but not cd-LS reduces PRV labeling in SCN

To test whether the MPON and/or the cd-LS are intermediate nuclei in the SCN-VTA pathway, we first lesioned these areas with ibotenic acid prior to PRV/CTB injections into the VTA. The logic was that if a nucleus is part of the SCN-VTA circuit, elimination of the corresponding somata should sever a pathway for PRV virions to be transported to the SCN. This lesion strategy has been successfully employed previously in our lab (Aston-Jones et al., 2001) and by others (Pickard et al., 2002; Smeraski et al., 2004) to delineate multi-synaptic circuits. Five to seven days after lesions, PRV/CTb was injected in the VTA and rats were sacrificed either 48 or 52hrs later. We picked these time-points because of the abundance of PRV labeling in the SCN of intact rats without significant PRV-induced cellular necrosis.

Figure 2.6 shows the results of excitotoxic lesions of either the MPON unilaterally (ipsilateral to PRV injection in the VTA), or the cd-LS bilaterally, on PRV labeling in the SCN at a 48 hr post-injection survival time-point. Such lesions of MPON resulted in an 81.6% reduction in the number of ipsilateral SCN neurons that became labeled with PRV (intact 177.1 ± 19.9 labeled cells per section, n = 5; lesioned 32.6 ± 16.5 labeled cells per section, n = 5; p < 0.001, t-test). PRV labeling in the contralateral SCN was also reduced, by 75.8% (intact 40.4 ± 24.8 labeled cells per section; lesioned 9.8 ± 4.8 labeled cells per section; p < 0.01). MPON-lesioned animals sacrificed at 52 hrs also had significantly reduced PRV labeling; a 61.5% reduction in the ipsilateral SCN (intact 194.5 ± 27.0 labeled cells per section, n = 8; lesioned 74.9 ± 3.6 labeled cells per
section, \( n = 12; \ p = 0.001 \) and a 69.2% reduction in the contralateral SCN (intact 71.4 ± 6.9 labeled cells per section; lesioned 22.0 ± 6.4 labeled cells per section; \( p < 0.01 \))

The cd-LS was also a possible relay nucleus in the SCN-VTA circuit, and in addition, serves as a control for upward leakage of ibotenate in MPON lesion cases because the cd-LS is dorsal to, and along the trajectory for, the MPON injection pipette. For cd-LS lesions, all animals were sacrificed at 48 hrs after PRV injections into the VTA because this time-point resulted in robust changes in MPON-lesioned animals. Lesions of the cd-LS resulted in a slight, but non-significant reduction in the number of PRV-labeled neurons in the SCN ipsilateral to the VTA injected 48 hrs earlier (15.0% reduction, intact 177.1 ± 19.9 labeled cells per section, \( n = 5 \); lesioned 150.6 ± 31.1 labeled cells per section, \( n = 8 \); \( p = 0.39 \), t-test). Interestingly, cd-LS lesioned animals had increased PRV labeling in the contralateral SCN (57.9% increase, intact 40.4 ± 24.8 labeled cells per section; lesioned 96.0 ± 27.7 labeled cells per section, \( p < 0.05 \)). This may be due to increased virion availability in the SCN-VTA circuit in lesioned animals, and will be discussed below. Regardless, lesion of the cd-LS did not result in reduced SCN labeling.
Figure 2.6  A-C, Frontal photomicrographs of PRV labeling in SCN 48 hrs after PRV/CTb injection into VTA from (A) intact, (B) MPON, or (C) cd-LS lesioned animals. Ipsilateral to the injection site is to the left. Scale bar = 200 μm. 3V, third ventricle; OC, optic chiasm. D, E, Average number of neurons labeling in the SCN with PRV in (D) MPON and (E) cd-LS lesioned animals. B, D, Lesion of the MPON reduced PRV labeling in both the ipsilateral (**, p < 0.001) and contralateral SCN (#, p < 0.01). C, E, Lesion of the cd-LS did not change PRV labeling in the ipsilateral SCN, but did increase labeling in the contralateral SCN (*, p < 0.05).

Figure 2.7A-D shows the representative extent of ibotenate lesions in the MPON or cd-LS. PRV immunoreactivity defined the lesioned area to be restricted to the MPON. Because PRV labeling in the LS was largely restricted to the cd-LS in the intact animal, a neuronal marker, NeuN, was used to define the lesioned area (insets of Figure 2.7A,B). Although MPON-lesioned animals exhibited substantial reductions in SCN labeling, Figure 2.7E,F shows that labeling in other afferent areas, such as the nucleus accumbens (NAC) did not significantly change (23.3% reduction, ipsilateral intact 163.2 ± 34.5 labeled cells per section, n = 5; ipsilateral lesioned 125.2 ± 42.4 labeled cells per section, n = 5; p = 0.50).

Figure 2.7  Frontal photomicrographs of PRV labeling in primary afferents at 48 hrs after injection into VTA in intact or lesioned animals. A, PRV labeling in the intact LS. B, After bilateral cd-LS lesions, PRV labeling was largely absent from the cd-LS. The inset photos in (A) and (B) are of NeuN immunoreactivity in adjacent sections of respective intact and lesioned LS. Blank areas in (B) inset mark the extent of the cd-LS lesion. C, PRV in the intact mPON. D, After ipsilateral MPON lesions, PRV labeling defined
the lesion site to be confined to the MPON. E, F, PRV labeling of the nucleus accumbens in an (E) intact and (F) MPON lesioned animals, respectively. Lesion of the MPON did not significantly change PRV labeling in the nucleus accumbens. Midline is center in (A-D) and the right edge of image in (E-F). Scale bar = 200 μm. LV, lateral ventricle; DD, corpus callosum; 3V, third ventricle; OC, optic chiasm; AC, anterior commissure.

DISCUSSION

In the present study we used the retrograde trans-synaptic tracer PRV-Bartha to define a multi-synaptic afferent circuit to the VTA from the SCN. Cells appeared in direct inputs at 36 hrs, well before SCN labeling. Injections of PRV into VTA resulted in SCN labeling at longer time-points (48 hrs or more). In addition, no SCN neurons were found to contain CTb label after a VTA injection. These findings, along with the lack of prior finding of a direct pathway from SCN to VTA, indicate that PRV labeling in SCN likely reflects an indirect circuit pathway.

The MPON and the cd-LS were considered primary candidate relay nuclei based on known efferent/afferent circuitry, and on PRV labeling in these structures at survival time-points prior to when PRV appeared in the SCN. Ibotenate-induced lesions of the MPON resulted in a marked (75-82%) reduction in PRV labeling in the SCN ipsilateral and contralateral to the PRV injection in VTA. In contrast, even bilateral lesions of the cd-LS did not significantly reduce PRV labeling in either the ipsilateral or contralateral SCN.

Technical considerations

PRV-Bartha is less virulent than wild-type PRV, and studies have confirmed its exclusive retrograde transport to chains of synaptically linked neurons, (Card et al., 1992; Card et al., 1993; Jansen et al., 1993; Geerling et al., 2006). However, as with any tracer,
methodological issues must be considered in the design and interpretation of PRV experiments.

Because PRV is readily transported away from the site of injection, CTb was co-injected to specify injection location and spread, and is compatible with PRV infectivity and transport (Aston-Jones and Card, 2000). CTb's lower molecular weight allows some of the tracer to remain in the extracellular matrix of the injection site. However, given the large size of PRV virions (~ 180nm), the spread of CTb within the injection site is greater than for PRV and provides a generous estimate of the actual confined spread of PRV (O'Donnell et al., 1997; Chen et al., 1999).

A number of factors contribute to the speed of PRV transport, including viral quantity, concentration and afferent synaptic density and survival time (Card et al., 1993; Card et al., 1999). Therefore, proper titration and multiple time-points must be examined for each particular circuit to ensure accurate interpretations. We found that 480 nl of $1.7 \times 10^9$ pfu/ml of PRV yielded adequate transport to known primary afferents while maintaining focal injection sites. This produced an injection size, speed and anatomical pattern of retrograde labeling similar to that seen with PRV injections in other circuits (Aston-Jones et al., 2001). Comparison of PRV labeling pattern at 36 hrs post-injection to that of co-injected CTb also revealed a very similar pattern of retrograde labeling (see Figures 2.2 and 2.3)

As with any neuronal tracer, the possibility of uptake by fibers of passage exists. However, Chen et al. (1999) found that PRV is taken up by fibers of passage to a smaller extent than other tracers. In addition, The SCN has also never been reported to be a direct VTA afferent, when tracers were used that are more susceptible to uptake by fibers of
passage, such a horseradish peroxidase (HRP; Phillipson, 1979). The possibility of uptake by damaged fibers was also tempered by our use of a highly-controlled pressure injection system (Picospritzer) and slow infusion rate.

We find in our present studies that bilateral ibotenate lesions of the cd-LS results in increased PRV labeling in the contralateral SCN. These results were surprising at first, but might be explained by the observation that PRV infection is highly sensitive to terminal field density (Card et al., 1999). Both the MPON and cd-LS are major afferents of the VTA, however, only the MPON seems to be a major node in the SCN-VTA circuit. It is possible that the cd-LS and MPON have overlapping terminal fields within the VTA. One could speculate that lesion of cd-LS terminal field in the VTA increased PRV virion availability for MPON retrograde transport to the SCN. Additional experiments are needed to clarify the exact mechanism by which this occurred.

**Topographic aspects of the SCN-VTA circuit**

In our present study, regardless of the VTA sub-region injected with PRV (e.g. rostral/caudal, medial/lateral), all cases yielded labeling of the SCN. However, given our experimental design parameters, this does not necessarily preclude differential VTA topography in the SCN-MPON projection. For example, it has been reported that rostral VTA receives inputs from caudal VTA, but not the converse (Ikemoto et al., 2003). It is possible that rostral VTA does not receive a major input from the SCN/MPON, but rather participates in this circuit through its connection with the caudal VTA. Such fine resolution would require additional experiments, such as shorter interval PRV time-courses, and most specified rostral/caudal VTA injection targets.
We consistently observed that labeling occurs first in the “shell” sub-region of the SCN, with subsequent spread to the “core” SCN. This finding is consistent with previous findings (Aston-Jones et al., 2001), and with other studies that found that the core projects densely to the shell, but not vice versa (Leak et al., 1999). Numerous studies have shown that the core and shell have distinct characteristics, although not necessarily mutually exclusive in function (Moore, 1996a, 1997; Yan et al., 1999; Hamada et al., 2001; de la Iglesia et al., 2004). Our present results indicate that information conveyed to the VTA likely arises from the SCN shell or from the core via its projections through the shell.

**Role of the MPON in the SCN-VTA circuit**

The MPON is a heterogeneous and sexually dimorphic structure that has been implicated in a number of functions, including sex behavior (for review: Hull et al., 2002). Lesioning the MPON on one hemisphere, and the VTA on the other reduces preference for a receptive female and eliminates copulation (Brackett and Edwards, 1984; Edwards and Einhorn, 1986). It has been suggested that the MPON works in conjunction with the VTA to integrate motivational/appetitive aspects with somatomotor responses to generate the full behavioral repertoire (Everitt, 1990; Hull, 1995). Given the circadian rhythmicity of mating behavior (Harlan et al., 1980; Stefanick, 1983), it is possible that the SCN-MPON-VTA temporally coordinates motivational aspects of mating behavior.

The MPON is also generally thought to be one of the few sleep-promoting areas in the brain. This is partly based upon recording studies which have shown that the majority of MPON neurons are sleep-active (Kaitin, 1984). Also, lesions of the MPON reduces sleep (Asala et al., 1990; John and Kumar, 1998). Fos expression in the MPON
is also greatest after long durations of both spontaneous and forced wakefulness (Pompeiano et al., 1994; Cirelli et al., 1995), perhaps reflecting the increased homeostatic pressure to sleep.

Stimulation of the SCN induces both inhibitory and excitatory post-synaptic effects on MPON neuronal activity (Sun et al., 2000), possibly reflecting the heterogenic neurochemistry of MPON neurons (Simerly et al., 1986). The projection from the MPON to the VTA is however, likely GABAergic as electrical stimulation of the MPON results in short latency inhibition (< 10ms) of VTA neurons (Maeda and Mogenson, 1980). Given that a large proportion of MPON neurons are sleep-active, which predominately occurs during the rest phase, it is possible that VTA neurons are tonically inhibited by MPON during the rest phase. During the active phase, VTA neurons are possibly released from this MPON inhibition and allowed to fire with their signature high frequencies.

Conclusions
Because of the SCN’s limited terminal field, it has been hypothesized that the MPON may be a relay nucleus to major arousal systems (Deurveilher and Semba, 2005). Here we show a synaptically linked SCN-MPON-VTA circuit with PRV and lesion studies. Future studies are needed to examine the role of the SCN-MPON-VTA circuit in physiological and behavior processes. However, given the current knowledge about each individual nucleus, it is possible that this circuit is involved in the circadian regulation of sleep/wake and sexual motivation.
CHAPTER THREE

NOVEL NEURONS IN VENTRAL TEGMENTAL AREA FIRE SELECTIVELY DURING THE ACTIVE CIRCADIAN PERIOD

ABSTRACT

The ventral tegmental area (VTA) contains dopamine (DA) and γ-aminobutyric acid (GABA) neurons involved in motivation and behavioral state. These phenomena are also influenced by circadian factors. We used single-unit extracellular recording and juxtacellular labeling techniques in anesthetized rats to examine the impulse activity of neurochemically identified VTA neurons during the rest (light) and active (dark) periods of the circadian cycle in these nocturnal animals. We found multiple neuronal subpopulations including "novel neurons" that selectively fired during the active period under both diurnal and circadian housing conditions. These novel neurons could be electrophysiologically categorized into two groups, novel wide-spike and novel thin-spike neurons. Further characterization of novel wide-spike neurons found they were consistently non-dopaminergic and non-GABAergic (TH -, GAD -). Physiologically, they were fast firing (18.8 ± 1.2 spikes/sec), low bursting neurons (6.2 ± 0.03% of spikes in bursts) with spike durations ≥ 2 ms, but slightly shorter than classic DA-like neurons. They were also consistently non-responsive to footpad stimulation. Pharmacologically, they were inhibited by the D2 agonist quinpirole, although not as robustly as classic DA-like neurons. Quinpirole's effect could be reversed by the D2 antagonist eticlopride, but the reversal took longer than in classic DA-like neurons. The novel thin-spike neurons...
were neurochemically more heterogeneous, and were located more ventrally than thin-
spike neurons found during the rest period. These findings reveal previously unknown
populations of VTA neurons whose activities are sensitive to the circadian period, and
whose functions may be in the temporal regulation of arousal and motivational processes.

INTRODUCTION

Circadian rhythms are adaptations in biochemical, physiological and behavioral processes
that follow a period of about 24 hours. These rhythms allow the organism to be
predictive of, rather than reactive to, its environment. Interestingly, many of the animals
used in biomedical research are nocturnal animals (e.g. rats, mice), but most scientists
perform their experiments in a circadian period in which the animal would normally be
sleeping.

The ventral tegmental area (VTA) is well known to regulate motivational aspects
of both natural and pathological reward processes, including drug addiction (Schultz,
2002; Wise, 2002; Kauer, 2004). These same processes are influenced by circadian
rhythms (Erickson et al., 1998; Stinus et al., 1998). Behavioral measures of addiction in
animal models, such as behavioral sensitization (Andretic et al., 1999; Gaytan et al.,
1999; Abarca et al., 2002), conditioned place preference (Abarca et al., 2002) and drug
self-administration (Baird and Gauvin, 2000), are modulated by circadian factors.
Several lines of animals with mutant circadian clock genes also show altered
responsiveness to drug reward (Abarca et al., 2002; Liu et al., 2005; McClung et al.,
2005; Spanagel et al., 2005). One of these, the mouse clock mutant, exhibits increased
preference for cocaine that is concomitant with increased VTA dopamine neuron
excitability (McClung et al., 2005). Activity of VTA dopamine and GABA neurons also varies with behavioral state, with activity lowest during slow-wave sleep, higher during active wake, and highest during paradoxical sleep (Lee et al., 2001; Maloney et al., 2002).

Based on this evidence, we hypothesized that VTA neuronal impulse activity would vary with the circadian cycle, and may be most active during the active circadian period when the VTA's role in motivational processes would be most important. To address this hypothesis, we recorded VTA neurons during active or rest circadian periods in nocturnal rats housed under light/dark and dark/dark conditions. All recordings were performed under halothane anesthesia to ensure that any changes in impulse activity were endogenous to the animal, and not confounded by varying arousal levels. Our results showed that there exist multiple subpopulations of VTA neurons. In particular, we discovered novel subpopulations that selectively fired during the active circadian period. These neurons were distinct from neurons previously described in the VTA in their neurochemical, electrophysiological, and pharmacological profiles. The circadian fluctuations in VTA neuronal function may influence the temporal expression of a wide variety of behaviors the VTA is known to regulate.

MATERIALS AND METHODS

Animals
One hundred six adult male Sprague Dawley rats (275-425 gm; Charles River Laboratories, Wilmington, MA, USA) were used. Rats were housed two per cage in light-tight environmental chambers under controlled conditions (21-23°C). For the
light/dark housing group, animals were housed on a 12 hr/12 hr (7am or 7pm lights on) light/dark cycle for at least 21 days before recording experiments. For the dark/dark housing group, animals were first entrained to a 12 hr/12 hr (3am or 3pm lights on) light/dark cycle for 14 days. This accounted for phase advancement during the dark/dark housing such that recording sessions occurred under normal working hours. Animals were then housed under continuously dark (dark/dark) conditions for at least 21 days before recordings began. During Days 15-21 of dark/dark housing, animals were single housed with photobeam detectors (San Diego Instruments, San Diego, CA) that continuously logged their locomotor activity in 15 minute bins. Periodicity of locomotor activity was found to advance 1.5-2.0 hrs during the week, confirming free-running circadian rhythms. Recording sessions typically lasted approximately 6 hrs and always took place within the 12 hour temporal confines of each respective diurnal/circadian phase (6 hrs within ZT/CT 2-10 or ZT/CT 14-22). For recordings during a dark phase, animals were transferred from their housing cages to the recording area in light-tight transfer cages. During the induction phase of anesthesia, black electrical tape was placed over the animals’ eyes to insure they were not exposed to photic input once room lights were re-illuminated. All protocols and procedures were approved by the University of Pennsylvania’s Institutional Animal Care and Use Committee.

Surgery

Animals were initially anesthetized with 5% halothane (Halocarbon, Riveredge, NJ, USA) in medical grade air administered through a facemask. A tracheotomy was performed, and 2% halothane was delivered through a tracheal cannula via spontaneous
respiration. The jugular vein was cannulated for drug delivery. Animals were placed in a stereotaxic frame, and body temperature was maintained at 36-37° C using a thermistor-controlled electric heating pad. During recording experiments halothane concentrations were kept at 1.25%. For VTA recordings, a hole was unilaterally drilled above the VTA and the dura carefully retracted. For recording pipettes oriented vertically (0°), the holes were drilled 0.8 mm lateral to midline and 5.3-5.5 mm caudal to bregma. To facilitate recording of medially located VTA neurons without disruption of the sagittal sinus, some recording pipettes were angled 10° from the vertical and inserted through a hole 2.0 mm lateral to midline, and 5.3-5.5 mm caudal to bregma.

**Ventral Tegmental Area Recordings**

A glass micropipette (6-10 MΩ) filled with 2% pontamine sky blue (PSB; BDH Chemicals Ltd. Poole, England) solution in 0.5 M sodium acetate was used. For experiments with juxtacellular labeling, a glass micropipette (25-35 MΩ) filled with 1.5% biotinamide (Invitrogen, Carlsbad, CA, USA) in 0.5 M sodium acetate was used. Signals were amplified and filtered (0.5-5 kHz bandpass) using an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA) in bridge mode and then further amplified using a CWE amplifier (Ardmore, PA, USA). Data were acquired and stored on a computer using a hardware/software interface system (CED 1401, Spike2; Cambridge Electronic Design, Cambridge, UK). VTA neurons were recorded for 3-5 min to establish a mean baseline firing rate. Total recording time for each particular neuron did not typically extend more than 15 min to ensure maximum sampling during the recording session. Single-unit recordings were ensured by a 100 µm minimum electrode travel.
between two isolated cells and by Spike2 template-based spike sorting. VTA recordings were obtained from each animal using a sampling grid that extended 0.4-1.2 mm from midline, 7.0-9.0 mm from brain surface, and 4.8-6.2 mm caudal to bregma.

*Electrophysiological Categorization of VTA Neurons*

**Classic VTA Neurons**

A cell was categorized as a “classic wide-spike” neuron when it met the following criteria classically used to identify DA neurons: (1) wide, triphasic (filtered recordings) waveform > 2.0 ms in duration as measured by its full waveform, and (2) slow spontaneous firing rate <10 spike/sec (Yim and Mogenson, 1980; Grace and Bunney, 1983; Chiodo, 1988). Recent work indicates that not all neurons that fulfill these characteristics are TH (+) (Ungless et al., 2004). These authors distinguished DA from non-DA neurons by comparing spike durations taken from the beginning of the spike to the peak of the negative trough, rather than the full waveform. In that study, neurons with such initial spike durations ≥ 1.1 ms were TH (+), and neurons that were < 1.1 ms in duration tended to be TH (-). Therefore, we divided the classic wide-spike neurons into two subclasses. Neurons with < 1.1ms initial spike durations were termed “classic wide-spike < 1.1ms” cells, and neurons with ≥ 1.1ms initial spike durations were termed “classic DA-like” cells.

A cell was categorized as a “classic thin-spike” neuron when its electrophysiological characteristics met the following criteria classically used to identify possible VTA GABAergic neurons: (1) thin, biphasic (filtered recordings) waveform < 2.0 ms in duration as measured by its full waveform, and (2) located dorsal to at least one
recorded classic wide-spike neuron (Yim and Mogenson, 1980; Johnson and North, 1992a; Steffensen et al., 1998).

**Novel VTA Neurons**

A cell was categorized as a “novel wide-spike” neuron when its electrophysiological characteristics met the following criteria: (1) wide, triphasic (filtered recordings) waveform ≥ 2.0 ms in duration as measured by its full waveform, (2) mean firing rate > 10 spikes/sec, and (3) topographically located either ventral to least one recorded classic thin-spike neuron or amongst classic wide-spike neurons.

A cell was categorized as a “novel thin-spike” neuron when its electrophysiological characteristics met the following criteria: (1) thin, biphasic (filtered recordings) waveform < 2.0 ms in duration as measured by its full waveform, and (2) located ventral to at least one recorded classic wide-spike neuron.

**Intravenous Drug Administration**

Quinpirole and eticlopride (100 μg/kg; Sigma, St. Louis, MO, USA), were prepared in 0.9% sterile saline and administrated through a jugular cannula. Doses of 100 μg/kg quinpirole (quin) or eticlopride (etic) were used because of their efficacies in modulating VTA DA neuron firing rates while still maintaining autoreceptor specificity (Jeziorski and White, 1989; Gao et al., 1998; Marinelli et al., 2003).
Footpad Stimulation

Footpad stimulation was presented through a pair of needle electrodes implanted subcutaneously in the medial surface of the rear footpad. Electrical stimulation (10 mA, 0.5 Hz, 0.5 msec duration pulses) was administered using Spike2 and a stimulus isolator (ISO-Flex, Alomone Labs, Jerusalem, Israel). Peristimulus time histograms (PSTHs) of footpad stimulation responses were generated for 50 consecutive stimuli. PSTHs were used to determine excitatory and inhibitory epochs as previously described (Chiang and Aston-Jones, 1993; Georges and Aston-Jones, 2002). Neurons were then classified into one of three categories: “Excited” neurons exhibited an excitation epoch in response to footpad stimulation. “Inhibited” neurons exhibited an inhibition epoch. “Nonresponsive” neurons exhibited neither an excitation nor an inhibition epoch. Results were expressed as relative percentages of response type for each cell group. The frequency of each response category was entered in a 2x2 contingency table and statistically compared across all cell types using the Fisher Exact Test.

Antidromic Stimulation

Electrical stimulation was performed with bipolar, concentric electrodes (FHC, Bowdoin, ME) placed in either the medial prefrontal cortex (mPFC; +2.7mm from bregma, 0.6mm from midline, 5.0mm from cortical surface) or nucleus accumbens (NAc; +1.2mm from bregma, 1.5mm from midline, 7.3mm from cortical surface). Driven impulses were considered antidromic if they met the following criteria: (1) constant latency at 10 or 5mA, 0.5ms, 0.5Hz stimulation, (2) ability to follow double stimulation pulse of ≤ 10ms ISI and (3) collision of driven spikes by spontaneous impulses.
**Juxtacellular Labeling**

Juxtacellular labeling involved entraining the impulse activity of a well-isolated single neuron to current pulses applied through the biotinamide-filled recording electrode. Entrainment techniques were similar to those previously described (Pinault, 1996; Schreihofer and Guyenet, 1997). In brief, current pulses (50% duty cycle, 250 ms, 0.5-10 nA) were applied through the recording electrode, while continuously monitoring the cell’s activity. Upon entrainment of the cell to current pulses, stimulation typically continued for 1-5 minutes.

**Immunohistochemistry**

At the end of recording experiments with juxtacellular labeling, animals were perfused with 4% paraformaldehyde in 0.1M phosphate buffer. Frontal sections of brain were cut at 40 μm intervals on a cryostat. Biotinamide-labeled cells were revealed using streptavidin Alexa Fluor 488 nm (Invitrogen). The tissue was subsequently processed for glutamic acid decarboxylase-67 (GAD67) immunoreactivity by incubating with a polyclonal rabbit antibody (Chemicon, Temecula, CA, USA), followed by a Alexa Fluor 594 nm-conjugated donkey anti-rabbit antibody (Invitrogen). Tyrosine hydroxylase (TH) immunoreactivity was revealed by incubating with a polyclonal mouse antibody (DiaSorin, Stillwater, MN, USA), followed by an Alexa Fluor 350 nm-conjugated goat anti-mouse antibody (Invitrogen).
**Histology**

At the end of recording experiments in which a PSB filled pipette was used -20 μA of continuous current was applied for 12 min to histologically mark the recording site. To mark electrical stimulation sites, +10 μA was passed through the stimulating electrode for 1 min. Brains were removed and snap-frozen in a -70°C solution of methyl butane. Brains were cut on a cryostat and counterstained with Neutral Red (Fisher, Fairlawn, NJ, USA).

**Bursting Analysis**

For midbrain DA neurons, burst onset is typically defined as at least two spikes with an inter-spike interval (ISI) < 80ms (Grace and Bunney, 1983, 1984). However, this criterion is not satisfactory for rapidly firing cells, e.g., those whose mean ISIs are close to or less than 80 msec. An alternative method was employed, in which the cell’s bursting activity is not directly proportional to firing rate. This method was based on the burst analysis scheme described by Kaneoke and Vitek (1996). In brief, a discharge density histogram (DDH) was constructed for each neuron and used to determine burst threshold. The DDH was calculated by first sub-dividing the spike train into bin sizes equal to the mean ISI of the spike train. The number (i.e. density) of spikes that occurred during each bin was counted, and a frequency histogram was created based upon this discharge density. Burst threshold was then calculated by comparing the actual distribution of the DDH to the predicated DDH if the spike train followed the null hypothesis that no burst periods occur (e.g., if it followed a Poisson process). The burst threshold was the first DDH bin (representing at least 2 spikes per mean ISI bin) with a higher occurrence of
events than expected for a Poisson process. The number of spikes with an ISI equal to or less than the burst threshold was then divided by the total number of spikes to generate the percentage of spikes in bursts (% SIB). Results were expressed as the median ± SEM SIBs, and were statistically analyzed by the Kruskal-Wallis test, followed by Dunn’s post-hoc test.

As noted above, VTA recordings were performed from both animals housed under light/dark conditions and dark/dark conditions. However, the majority of novel cell characteristics were gathered from light/dark animals and dark/dark animals were primarily used to confirm our diurnal effects. Therefore, unless noted, presented results are from recordings of light/dark animals.

RESULTS

Novel VTA neurons fire selectively during the active circadian phase.

VTA neurons were recorded from animals during their rest or active circadian phase. Multiple neuronal cell types were found, including cells that matched classic electrophysiological categories described in prior studies (see Materials and Methods and Figure 3.1). Neurons with wide action potentials (≥ 2 ms duration) and slow firing rates (< 10 Hz) with intermittent bursting activity resembled cells previously identified as dopaminergic (Wang, 1981; Grace and Onn, 1989). As in previous work (Ungless et al., 2004), we also found we could subdivide our classic wide-spike neurons into those with initial spike durations < 1.1 ms or ≥ 1.1 ms, the latter presumably being TH (+) (see Materials and Methods). These neurons were categorized as “classic wide-spike < 1.1
ms" or "classic DA-like", respectively (Figure 3.1). In addition, we recorded neurons with thin (< 2 ms) action potentials and fast firing rates (> 10 Hz) with phasic on/off activity. These cells were found in clusters dorsal to classic DA neurons, resembling previously described GABAergic neurons (Steffensen et al., 1998), and were termed "classic thin-spike" neurons (see Materials and Methods and Figure 3.1).

During our recordings, particularly in the active phase in both diurnal and circadian housing conditions, neurons were found that did not resemble neurons previously reported in VTA and did not fit the criteria for either classic wide-spike or classic thin-spike cells, and therefore were termed "novel neurons" (Figure 3.1). These novel neurons made up a significantly larger portion of VTA neurons recorded in the dark than in the light phase (dark phase 14.1%, vs. light phase 1.5%; p < 0.001, Fisher exact test) (Figures 3.2A). Examination of the novel neuron population alone revealed that novel neurons were almost exclusively found during the dark phase, with a peak prevalence at ZT 16-18 (dark phase 93.8% vs light phase 6.2%; p < 0.001; Figure 3.2B). This selective activity is a circadian effect as novel neurons were also selectively found during the subjective night under dark/dark conditions, although there was dampening effect (active period 61.1% vs. rest period 38.9%; p < 0.05; Figure 3.2B). As shown in Figure 3.2C, novel neurons were found throughout VTA DA neuron-rich areas.
Figure 3.1 Electrophysiological categories of VTA neurons. Multiple electrophysiological cell types exist in the VTA. The flowchart outlines relationships among cell categories.
Figure 3.2 Novel VTA neurons fire selectively during the active phase under both diurnal and circadian housing conditions. (A) Relative proportions of cell types found in light or dark phase of the diurnal cycle. Novel neurons make up 14.1% of all VTA neurons recorded during the light phase, as compared to only 1.5% during the dark phase. (B) Relative proportion of total novel neurons found at each ZT/CT range. Each point represents different novel neurons found within a particular ZT/CT range. Under diurnal conditions, novel neurons tend to appear most frequently during the middle of the dark phase (ZT 16 - 18). Novel neurons were also selectively found during the subjective night under circadian conditions, although the effect was slightly blunted. The dotted vertical line denotes lights off at ZT 12 or offset of locomotor activity at CT 12. (C) Novel neurons are found throughout DA neuron-rich areas of the VTA. (C, left, middle) Plots of novel cells that were either themselves juxtacellularly labeled or histologically localized with respect to another labeled cell (adapted from Swanson, 2004). (C, right) Bright-field photomicrographs of TH immunohistochemistry corresponding to areas shown in left and middle panels.
The cell/track measure was determined for all neuronal subtypes found in light and dark phases. The cell/track measure is the number of spontaneously active neurons found per electrode track, and has been used extensively as an indirect measure of population activity (Bunney and Grace, 1978; White and Wang, 1983; Floresco et al., 2001). When all recorded VTA neurons were taken into account, there was a nearly significant increase in the overall cell/track ratio in the dark phase vs. light phase (dark phase 3.5 ± 0.3, n = 9 animals, 231 neurons; light phase 2.7 ± 0.3, n = 8 animals, 117 neurons; p = 0.06, t-test). Concomitantly, the novel neurons' cell/track measure significantly increased during the dark phase (dark phase 0.36 ± 0.36, n = 9 animals, 231 neurons; light phase 0.03 ± 0.16, n = 8 animals, 117 neurons; p < 0.001, t-test). In contrast, the cell/track measure of all other neuronal subtypes did not change between light and dark phases (p value range 0.41-0.89, t-test). As the novel neuron population was the only subtype to increase its cell/track measure, it seems likely to be the strongest contributing factor to the overall increase in cell/track in the dark phase. This overall increase probably does not quite reach significance because the novel neuron population is comparatively small.

**Novel VTA neurons can be subdivided into two electrophysiological classes.**

Novel neurons were divided into two categories (see Materials and Methods and Figure 3.1). The “novel wide-spike” neurons had a wide, triphasic waveform shape similar to classically defined DA neurons. However, they had an average firing rate more than four times faster than classic wide-spike neurons in either light or dark phases. Figure 3.3A-B plots the spike duration of all wide (≥ 2 ms) spike neurons as a function of firing rate.
There were many more fast-firing wide-spike (novel) neurons in the dark phase than in the light phase (p < 0.001, Fisher exact test).

**Figure 3.3** Novel wide-spike neurons are fast firing with wide spike durations. Novel wide-spike neurons are characterized by their wide (> 2ms) triphasic, fast-firing (> 10 spikes/sec) activity. Spike duration is plotted as a function of firing rate for all wide (> 2ms) spike neurons found in either (A) light or (B) dark phases. In some cases, points are visually obscured because of direct overlap with another point. Red dotted line indicates firing rate level at 10 spikes/sec. Many more fast-firing wide-spike neurons are found during the dark phase than in the light phase (p < 0.001, Fisher exact).
The second class of novel VTA cells was termed "novel thin-spike" neurons. Like classic thin-spike neurons, these cells had a thin biphasic waveform shape, and were fast firing. However, these novel thin-spike neurons (n = 36) were located more ventrally than classic thin-spike cells (novel neurons 8.17 ± 0.04 mm below cortical surface, n = 26 animals, 36 neurons; classic neurons 7.47 ± 0.03 mm n = 17 animals, 87 neurons; p < 0.0001, t-test). Figure 3.4A-B plots the dorsal/ventral coordinates of all thin-spike neurons (< 2 ms spike duration). There are many more ventrally located thin-spike (novel) neurons in the dark phase than in the light phase (p < 0.01, Fisher exact).

Figure 3.4 Novel thin-spike neurons are ventrally located and have thin spike durations. Novel thin-spike neurons are characterized by their thin (< 2 ms) biphasic waveform, and are topographically located ventral to a classic wide-spike neuron in the same electrode track. All thin-spike neurons found in either the (A) light or (B) dark phases are plotted as functions of their medial/lateral coordinates (distance from midline) and dorsal/ventral coordinates (distance from cortical surface). Red dotted line indicates average depth of first (most dorsal) classic wide-spike neuron recorded in each animal. In some cases, points are visually obscured because of direct overlap with another point. Many more thin-spike neurons are found ventral to classic wide-spike neurons during the dark phase than in light phase (p < 0.01, Fisher exact).
Novel neurons do not consistently express TH or GAD67.

Because the waveforms of novel wide-spike cells were similar to those of classic DA waveforms, we used the juxtacellular labeling method (Pinault, 1996) to test whether these were DA neurons that fired faster during the dark phase. First, to confirm the juxtacellular method in our hands, a subset of electrophysiologically categorized classic DA neurons were labeled and processed for TH immunoreactivity (Figure 3.5). As expected, all 8 labeled classic DA-like neurons in the light phase were also TH (+) (Table 3.1).

Figure 3.5 Classic DA-like neurons from the light phase are TH (+). Representative example of a juxtacellulary labeled classic DA-like neuron from the light phase that is TH (+). (A, top) Digitized spike waveform showing the typical wide duration, tri-phasic shape of a classic DA-like neuron. (A, bottom) The same classic DA-like neuron fires in a regular pattern with intermittent bursts. (B) Fluorescent photomicrograph showing juxtacellular labeling of the same classic DA-like cell with biotinamide revealed with streptavidin Alexa Fluor 488nm. (C) The same section as Panel B processed for TH immunoreactivity and revealed with Alexa Fluor 594nm. (D) A merged photomicrograph of Panels B and C showing that the biotinamide-filled neuron is TH (+). Scale bar = 30μm.
Table 3.1 TH immunoreactivity of juxtacellularly labeled classic DA-like neurons. Juxtacellular labeling confirms that classic DA-like neurons in the light phase have a TH (+) neurochemical identity (8 of 8). However, a substantial population of classic DA-like neurons in the dark phase was TH (-) (2 of 5).

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We then juxtacellularly labeled a subset of novel wide-spike neurons and processed them for TH and GAD67 immunoreactivity. Results showed that the majority of novel wide-spike neurons (9 out of 11) stained for neither TH nor GAD (Table 3.2; representative staining for novel wide-spike neuron, Figure 3.6A-D). Quantification of cell soma sizes revealed that novel wide-spike neurons do not differ in size from classic wide-spike neurons (182.4 ± 19.8 μm² vs. 201.9 ± 19.8 μm², respectively).
Figure 3.6 Novel wide-spike neurons express neither TH nor GAD. Representative example of a juxtacellularly labeled novel wide-spike neuron that is neither TH (+) nor GAD (+). (A) A biotinamide-filled novel wide-spike neuron revealed with streptavidin Alexa Fluor 488 nm. (B) The same section subsequently processed for GAD67 immunoreactivity and revealed with Alexa Fluor 594 nm. (C) The section was then processed for TH immunoreactivity and revealed with Alexa Fluor 350 nm. (D) The merged photomicrograph shows that the biotinamide-filled neuron contains neither TH nor GAD67. Scale bar = 30µm.

Because novel thin-spike neurons were physiologically similar to classic thin-spike neurons, we initially hypothesized these were ventrally located GABA neurons that fire selectively during the dark phase. Results revealed that these novel thin-spike cells did not have a consistent neurochemical phenotype (Table 2). Interestingly, overall, 6 of 16 thin-spike neurons were TH (+) (4 novel thin-spike, 2 classic thin-spike), and these thin spike TH (+) cells were found in both dark and light phases, and dorsal and ventral to classic wide-spike neurons. These results indicate that a subset of DA neurons are fast-firing and have thin spikes. In addition, 2 of 5 labeled classic DA-like neurons in the active period were TH (-). This indicates that previously established criteria for classic DA and GABA cells are not entirely satisfactory, particularly in the dark phase.
Table 3.2 Neurochemical identity of electrophysiologically defined groups. Juxtacellular labeling shows that the majority of novel wide spike neurons are TH (-), GAD (-) (9 of 11). Both classic GABA and novel thin-spike neurons had varying neurochemical identities with a substantial population of TH (+) thin-spike neurons (classic and novel; 6 of 16).

Novel wide-spike neurons are fast-firing, low-bursting neurons.

Figure 3.7A compares the firing rate of novel wide-spike neurons to that of classic DA-like and classic thin-spike neurons in light and dark phases. Novel wide-spike neurons fired significantly faster than classic DA-like neurons in both light and dark phases (novel wide-spike 18.8 ± 1.2 Hz, n = 27 animals, 45 neurons; classic DA-like light phase 4.3 ± 0.2 Hz, n = 10 animals, 131 neurons; classic DA-like dark phase 4.3 ± 0.2 Hz, n = 12 animals, 132 neurons; p < 0.001 ANOVA, Neuman Keuls post-test). Their firing rates were also faster than classic thin-spike neurons in the light phase (14.7 ± 1.2Hz, n = 14 animals, 68 neurons, p < 0.01), and comparable to classic thin-spike neurons in the dark phase (18.7 ± 1.5 Hz, n = 20 animals, 84 neurons).

We observed that classic DA-like neurons fired in a regular single-spike pattern with intermittent bursts of spikes (Figures 3.5A bottom and 3.7C), consistent with
previous observations (Grace and Bunney, 1984; Grenhoff et al., 1988). Burst firing results in a supralinear increase in synaptic DA release (Gonon, 1988; Wightman and Zimmerman, 1990) and is thought to act as a reward learning signal (Montague et al., 1996; Hollerman and Schultz, 1998). Because of the functional significance of burst firing, we investigated possible bursting activity in the novel wide-spike neurons recorded during the dark phase. The onset of a burst in midbrain dopamine neurons is traditionally defined as the occurrence of two spikes with an interspike interval < 80 ms (Grace and Bunney, 1983, 1984). However, the fast firing rate of novel wide-spike neurons (mean interspike interval = 53 ms) required an alternative method that can quantify bursting in both the classic DA-like and novel wide-spike neurons. For this, we used a discharge density histogram (DDH) analysis (see Materials and Methods). To validate this method for this study, we compared burst results by the DDH method for classic DA-like neurons to burst results obtained by the 80 ms criterion. We found that for classic DA-like neurons, the % spikes in burst (SIB) values resulting from the 80 ms and DDH methods did not significantly differ from each other (80 ms method, median % SIB = 18.4 ± 1.1; DDH method, median % SIB = 16.5 ± 1.5, n = 14 animals; 165 neurons; p = 0.99, Mann Whitney U test).

Figure 3.7B shows the median % SIB for classic DA-like and novel wide-spike neurons as analyzed by the DDH method. Classic DA-like neurons bursting activity did not differ between dark vs. light phases (Classic DA-like light phase SIB =15.1 ± 1.6%, n = 18 animals, 127 neurons; classic DA-like dark phase SIB = 15.3 ± 1.5%, n = 21 animals, 194 neurons). Novel wide-spike neurons were surprisingly less bursty (SIB = 6.2 ± 0.03%, n = 27 animals, 45 neurons) than classic DA-like neurons in either phase (p
< 0.001, classic DA-like dark phase; p < 0.01, classic DA-like light phase). Firing pattern differences between classic DA-like and novel wide-spike neurons were evident when comparing their respective inter-spike interval histograms (ISIHs). Classic DA-like neurons typically showed distinct burst and non-burst spike populations resulting in a bimodal distribution in their ISIH (Figure 3.6C). In contrast, ISIHs of novel wide-spike neurons tended to be unimodal and skewed to the left (Figure 3.7D), consistent with a fast frequency spike train of uniform inter-spike intervals. Therefore, the fast firing activity of the novel wide-spike neurons is not due to high bursting activity, but rather to high tonic discharge.
Figure 3.7 Novel wide-spike neurons are fast-firing but low-bursting neurons. (A) Mean firing rates ± SEM are plotted for each cell type. Novel wide-spike neurons fire faster than both light and dark phase classic DA-like neurons (* p < 0.001 ANOVA, Neuman Keuls post-hoc), and faster than light phase classic GABA neurons (# p < 0.01). (B) Median % Spikes in bursts ± SEM are plotted for each cell type. Classic DA-like neurons do not differ between light and dark phases. However, novel wide-spike neurons are significantly less bursty than classic DA-like cells during either the light phase (# p < 0.01) or dark phase (*p < 0.001). (C) Inter-spike interval histogram (ISIH, 5 msec bins) of a representative classic DA-like neuron shows a bimodal distribution of both short and long inter-spike intervals. (D) ISIH of a representative novel wide-spike neuron shows a unimodal distribution of short inter-spike intervals.
We investigated whether anesthesia duration was a confounding variable on our recordings. We varied the total time under anesthesia by as much as 4 hours during either the dark or light phases, and found no significant differences in mean firing rates (2-4 hrs of anesthesia yielded $4.3 \pm 0.4$ spikes/sec, $n = 4$ animals, 36 neurons; 6-8 hrs of anesthesia yielded $3.6 \pm 0.2$ spikes/sec, $n = 10$ animals, 78 neurons; $p = 0.21$, t-test) or bursting activity (2-4hrs of anesthesia = $18.9 \pm 3.3$ % SIB, $n = 4$ animals, 36 neurons; 6-8hrs of anesthesia = $19.1 \pm 1.7$ % SIB, $n = 10$ animals, 78 neurons; $p = 0.96$, t-test) of classic DA-like neurons. In addition, anesthesia duration was not correlated with the firing properties of novel neurons. The frequency of appearance of novel neurons peaked in the middle of the dark phase (ZT 16-18, see Figure 3.2B), with fewer appearing under either shorter or longer periods of anesthesia. There were also no significant differences in the mean firing rates of novel neurons recorded at varying zeitgeber times within the active period ($p$ values 0.68-0.94, ANOVA).

**Novel wide-spike neurons have shorter action potential durations and are non-responsive to footpad stimulation.**

We also compared the spike durations of novel wide-spike neurons to those of classic DA-like neurons (Figure 3.8A-B). Although by definition both populations had spike durations ≥ 2 ms, novel wide-spike neurons had shorter spike durations than classic DA-like neurons (Classic DA-like dark phase $3.28 \pm 0.05$ ms, $n = 12$ animals, 132 neurons; Novel wide-spike $2.60 \pm 0.06$ ms, $n = 27$ animals, 45 neurons; $p < 0.0001$, t-test).

Additionally, we examined the response of novel wide-spike neurons to single-pulse footpad stimulation. Figure 3.9A-D shows response proportions for novel wide-
spike and classic DA-like neurons recorded during the dark phase. Classic DA-like neurons (n = 14 animals, 62 neurons) were inhibited (51.6%, n = 32 neurons), excited (8.1%, n = 5 neurons) or non-responsive (40.3%, n = 25 neurons) to footpad stimulation (Figure 3.9A). The average latency to response was 64.3 ± 8.9 ms with an average duration of 176.9 ± 22.1 ms. Figure 3.9B shows a PSTH and raster sweep of a typical classic DA-like neuron inhibited by footpad stimulation. Novel wide-spike neurons (n = 6 animals, 8 neurons) were mostly non-responsive (85.7%, n = 7 neurons) with only one neuron inhibited (14.3%) by footpad stimulation (Figure 3.9C). Figure 3.9D shows a PSTH and raster sweep of a typical novel wide-spike neuron that was non-responsive to footpad stimulation. The proportion of non-responsive novel wide-spike neurons was greater than that for non-responsive classic DA-like neurons (p < 0.05, Fisher’s exact).
Figure 3.8 Novel wide-spike neurons have shorter spike durations than classic DA-like neurons. (A) Averaged waveforms of each neuron type (15 waveforms each) reveal novel wide-spike neurons to have shorter tri-phasic waveforms than classic DA-like neurons. (B) A spike duration frequency histogram shows that classic DA-like and novel wide-spike neurons significantly differ in mean spike durations (* p < 0.001 ANOVA, Neuman keuls post-hoc), albeit with population overlap.
We explored potential projection targets of novel wide-spike neurons using antidromic stimulation. Bipolar, concentric, stimulating electrodes were placed in the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc), known targets of VTA neurons (Beckstead et al., 1979; Swanson, 1982). None of the tested novel wide-spike neurons were antidromically driven from either the mPFC (n = 15 neurons; 5 or 10 mA,
0.5 Hz, 0.5ms duration) or NAc (n = 13 neurons). In contrast, similar stimulation antidromically activated classic DA-like neurons recorded in the light phase (NAc stimulation, 6 of 74 neurons; mPFC stimulation 7 of 74 neurons).

Novel wide-spike neurons are inhibited by D2 receptor agonists.

We investigated the pharmacological profile of classic DA-like and novel wide-spike neurons with intravenous administration of D2 receptor drugs. The activity of DA neurons is well known to be inhibited by D2 autoreceptor agonists (Aghajanian and Bunney, 1977; White and Wang, 1984). Figure 3.10A shows the effect of a D2 receptor agonist and antagonist (quinpirole and eticlopride, respectively) on the impulse activity of classic DA-like (n = 15 animals, 15 neurons) and novel wide-spike neurons (n = 10 animals, 10 neurons). The rate of quinpirole-induced inhibition is similar in the two populations, although the magnitude was slightly but significantly less in the novel wide-spike neurons (p < 0.01, ANOVA). Subsequent administration of eticlopride to the classic DA-like neurons restores firing rates to baseline within 1 min, and to supra-baseline levels thereafter (2-4.5 min after eticlopride administration, mean change = 131.3% of baseline). The baseline overshoot was presumably due to eticlopride's antagonism of both the evoked quinpirole effect and basal D2 autoreceptor inhibition. Administration of eticlopride also restored novel wide-spike neurons to baseline rates. However, this restoration took much longer (~ 3.5 min), and did not show the supra-baseline overshoot seen in the classic DA-like neurons (p < 0.001). Figures 3.10B and 3.10C show the response of representative neurons of each cell type to quinpirole and eticlopride. Although the relative response magnitude is similar (Figure 3.10A), the
absolute response magnitude is much greater in the novel wide-spike neurons because of their faster baseline firing rate. The mean change in the firing rate in novel wide-spike neurons was 11.5 Hz, whereas the mean change in classic DA neurons was only 5.4 Hz (at 1 min after quinpirole administration).

Figure 3.10 Novel wide-spike neurons are responsive to D2 receptor drugs.
Novel wide-spike neurons are inhibited by D2 receptor stimulation. (A) Effect of i.v. administration of quinpirole on classic DA-like and novel wide-spike neuronal activity. Mean % of baseline firing rates ± SEM (10 sec bins) are plotted as a function of time for each cell type. Quinpirole strongly inhibits the activity of classic DA-like neurons. Subsequent administration of eticlopride reverses this inhibition to supra-baseline levels. Quinpirole also inhibits the activity of novel wide-spike neurons, although not as robustly (# p < 0.01, ANOVA). Eticlopride reverses the inhibition in novel wide-spike neurons, but this effect takes longer than in classic DA-like neurons and does not overshoot baseline levels (* p < 0.001, ANOVA). (B) Firing rate (10 sec bins) of representative classic DA neuron in response to quinpirole and eticlopride. (C) Firing rate of representative novel wide-spike neuron in response to quinpirole and eticlopride. Note that the absolute response to drug is greater for the novel wide-spike neuron compared to the classic DA-like neuron because of its faster baseline firing rate.
DISCUSSION

Our study revealed novel VTA neurons with unique physiological, neurochemical, and pharmacological profiles. These novel neurons selectively fired during the active circadian phase, and were separated into different electrophysiological categories; novel wide-spike and novel thin-spike neurons.

Neurochemical characteristics of novel wide-spike neurons

Novel wide-spike neurons were predominantly TH (-), GAD (-). They were tonically fast-firing, low-bursting neurons with slightly shorter spike durations than classic DA-like neurons. Their firing activity was largely unresponsive to footpad stimulation but sensitive to changes in dopaminergic neurotransmission.

The VTA contains dopaminergic and GABAergic neurons (Dahlstrom and Fuxe, 1964; Hökfelt et al., 1984; Mugnaini and Oertel, 1985; Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000). We obtained robust TH staining in numerous VTA and substantia nigra neurons matching previous reports of locations of dopaminergic cells (Swanson, 1982; Hökfelt et al., 1984; German and Manaye, 1993), indicating that our antibody and staining technique were effective. GAD67 is a reliable endogenous marker for GABAergic neurons (Mugnaini and Oertel, 1985) (Esclapez et al., 1993). As with TH, we obtained strong neuronal staining for GAD67 throughout the VTA resembling results from previous studies (Mugnaini and Oertel, 1985; Esclapez et al., 1993).
contain other neuropeptides/neurotransmitters, but few have examined peptide neurons in VTA for whether they contain TH. Many TH neurons in VTA also contain the neuropeptides cholecystokinin and neurotensin (Seroogy and Fallon, 1989; Jayaraman et al., 1990; Bayer et al., 1991). Bayer et al. (1991) reported that all neurons that contain neurotensin also contain TH, indicating that novel neurons in our study are probably not neurotensinergic. It is not known whether the same is true for cholecystokinin (CCK), because CCK staining results are only reported as a proportion of dopamine neurons. Interestingly, a circadian rhythm for CCK concentration has been reported in NAc (Schade et al., 1995), whose CCK afferents arise from the VTA (Seroogy and Fallon, 1989). Another possible neurotransmitter for the novel neurons is glutamate.

Glutaminase colocalization in TH neurons was reported by Kaneko et al (1990). Since then, vGLUT3 (Fremeau et al., 2002) and vGLUT2 mRNA (Dal Bo et al., 2004; Kawano et al., 2006; Nair-Roberts et al., 2006; Yamaguchi et al., 2007) have been reported in VTA dopaminergic neurons. In addition, glutamatergic synapses of VTA terminals have been observed both in culture (Chuhma et al., 2004) and in slice (Sulzer et al., 1998). In particular, a substantial portion of vGLUT2 (+) neurons were TH (-) (Kawano et al., 2006; Yamaguchi et al., 2007) or GAD (-). (Yamaguchi et al., 2007). Our novel neurons may make up a proportion of these glutamatergic neurons.

**Physiological characteristics of novel wide-spike neurons**

We described a population of VTA neurons during the active circadian phase that are fast-firing, but low-bursting, with action potential waveforms ≥ 2ms. It has been previously shown that midbrain DA neurons are sensitive to changes in glucose levels.
(Sailer and Chiodo, 1980), specifically that i.v. glucose administration inhibits the firing rate of putative DA neurons. It could be argued that the changes we observed were a consequence of decreased glucose levels or anesthesia during our multi-hour recording sessions. To address these issues, recording sessions began at various zeitgeber times to allow for varying time spent in the home cage (and presumably varying glucose level), and total anesthesia time. We found no differences in the firing activity of classic or novel neurons as a function of time under anesthesia, or away from the home cage. Although we cannot disregard the possibility that the firing properties of novel neurons differ somewhat in the unanesthetized animal, their activity is nevertheless sensitive to the circadian period. In addition, anesthesia cannot account for the novel neurons’ unique neurochemical identity, and is unlikely to account for their unique response to footpad stimulation and D2 receptor drugs.

It is noteworthy that we and others (Yim and Mogenson, 1980; Georges and Aston-Jones, 2003; Koeltzow and White, 2003) did not find fast-firing wide-spike neurons in the VTA during the light phase in anesthetized animals. This indicates that these neurons are either silent during the light and selectively fire during the dark phase, or that they fire at a slower rate (i.e. < 10Hz) during the light phase and increase their firing rate during the dark phase. Evidence from cell/track measurements indicates that the former possibility is more likely. There is a significant increase in the cell/track of the novel neuron population (p < 0.001), and a nearly significant increase in the overall cell/track (p = 0.06) in the dark phase. If the novel neurons were slower firing but active during the light phase, we would predict a decrease in the cell/track of at least one other cell subtype in the dark phase. However, no other neuronal subtype cell/track

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measurement decreases or significantly increases (p = 0.41-0.89). This finding indicates that novel wide-spike neurons are probably silent during the light phase and selectively fire during the dark phase.

**Electrophysiological markers of VTA neurons**

We also found a mismatch between observed electrophysiological and expected neurochemical properties of classic VTA neurons. We found that 2 of 5 (40%) juxtacellularly labeled cells with electrophysiological properties of classic DA neurons (i.e. classic DA-like) in the active period were TH (-). This relative proportion resembles others’ observations in VTA slice preparations which demonstrated a similar mismatch (Johnson and North, 1992a; Jones and Kauer, 1999). Additionally, we found that 37.5% (6 of 16) of our fast-firing, thin-spike neurons (i.e. presumed GABA cells based on previous criteria) were actually TH (+). A comprehensive characterization of VTA GABA neurons is detailed in Steffensen et al. (1998); however, there was no report of the proportion of recorded neurons that also express GABA immunoreactivity. It is possible that not all examined neurons were GABAergic, as our findings suggest. Furthermore, Margolis et al. (2006) found that TH content could not be predicted by size, shape, input resistance, I_h size or spontaneous firing rate. Our present results, along with the above mentioned studies, highlight the difficulty in determining the neurochemical content of VTA neurons without direct histochemical techniques.

Although the established criteria for VTA DA and GABA neurons are imperfect, we consistently found that classic DA-like neurons in the rest phase were TH (+) and novel wide-spike neurons were TH (-), GAD (-). That classic DA-like neurons in the rest
phase were TH (+) demonstrates the validity of using this classification to compare them to novel wide-spike neurons.

It is also important to differentiate between the criteria used to define the novel wide-spike neurons and the subsequent characteristics found based on these criteria. The novel wide-spike neurons were defined by their wide spike-duration (> 2 ms) and fast-firing rate (> 10 spikes/sec). Based on these criteria, we found that they were low bursting, TH (-) GAD (-), non-responsive to footpad stimulation, inhibited by D2 agonists, and selectively fired during the active phase. Alternatively, if VTA neurons were defined by D2 agonist inhibition or TH (+) immunoreactivity, the resulting group characteristics would be much less homogenous. Therefore, we feel our definition of the novel wide-spike population is an adequate metric for describing a unique population of VTA neurons.

**Pharmacological characteristics of novel-wide spike neurons**

We found that novel wide-spike (presumably non-DA) neurons were consistently inhibited by the D2 receptor agonist, quinpirole; this effect was readily reversed by the D2 receptor antagonist, eticlopride. Others have observed inhibition of presumed non-DA neurons by local application of DA or D2 agonists (Yim and Mogenson, 1980; Cameron et al., 1997; Kiyatkin and Rebec, 1998; Margolis et al., 2006). Although our result is consistent with the possibility that novel wide-spike neurons contain D2 receptors, it is not possible to make such a conclusion from the present results with systemic drug application. Nevertheless, novel wide-spike neurons are sensitive to changes in DAergic neurotransmission. The lack of overshoot in impulse activity after
D2 antagonist administration, however, may indicate that novel wide-spike neurons do not receive basal dopaminergic input under our experimental conditions, in contrast to classic DA neurons.

Recent evidence has implicated mesocorticolimbic DA in the regulation of behavioral arousal (Nishino et al., 1998; Wisor et al., 2001; Isaac and Berridge, 2003). Given that our novel neuron population is sensitive to changes in DA neurotransmission, it is possible that novel neurons interact with DA circuitry in regulating behavioral state.

Functional implications

A striking feature of novel neuron activity is its dependence on the circadian phase. Novel neurons selectively fire during the active circadian period in animals housed under continuously dark conditions indicating that they exhibit an endogenous circadian rhythm. Although the functional impact of the novel neurons is not known, it is possible to they are associated with the circadian regulation of arousal.

The possible contribution of non-DAergic, non-GABAergic elements in the VTA to arousal has largely been unexplored, but VTA projections to the hippocampus may be involved. The projection from the VTA to the hippocampus is 82-94% non-dopaminergic (Swanson, 1982; Gasbarri et al., 1994). Hippocampal activity has long been functionally associated with arousal states, e.g. theta waves during exploratory behavior and REM sleep (Vanderwolf, 1969; Buzsaki, 2002), and sharp-wave ripple oscillations during slow wave sleep (Buzsaki, 1986; Kudrimoti et al., 1999; Molle et al., 2006). One might speculate that a possible projection of novel neurons to hippocampus could provide a means for regulation of cognitive functions associated with the hippocampus (e.g.,...
memory) by arousal and the circadian cycle. Other possible circuits whereby our novel
VTA neurons could influence arousal may also exist. Our results imply that there are
multiple functional subgroups in the VTA, and that the VTA regulation of circadian
processes may involve both DA and non-DA elements.
CHAPTER FOUR

DIURNAL AND CIRCADIAN FLUCTUATIONS IN VENTRAL TEGMENTAL AREA DOPAMINE-LIKE AND GABA-LIKE NEURON IMPULSE ACTIVITY

ABSTRACT

The ventral tegmental area (VTA) is a heterogeneous, midbrain structure that has been extensively studied for its interfacing role in reward and arousal. Like most biological phenomena, reward and arousal are also influenced by circadian factors. Here we used extracellular, single-unit recordings of VTA neurons in anesthetized rats to examine the influence of the daily cycle on VTA dopamine-like (DA-like) and γ-aminobutyric acid-like (GABA-like) neurons. We found that GABA-like neurons increased their firing activity during the late dark phase of the diurnal (light/dark) cycle; however, the effect was abolished under circadian (dark/dark) housing conditions. Overall, there was not a diurnal rhythm in DA-like neuron firing rates, although some subtle variations did exist at different zeitgeber times. Using antidromic stimulation and topographical mapping, we found that distinct populations of DA-like neurons fire at different phases of the diurnal cycle. During the light phase, spontaneously firing DA-like neurons were more frequently found in dorsal and anterior VTA. These anteriorly located neurons contained both mesoprefrontal (PFC projecting) and mesoaccumbal (NAC projecting) neurons. During the dark phase, spontaneously firing DA-like neurons were more frequently found in ventral and posterior VTA. These posteriorly located neurons contained mesoaccumbal, but not mesoprefrontal neurons. Furthermore, no spontaneously firing
mesoprefrontal neurons were ever found in any part of VTA, suggestive that mesoprefrontal neurons are generally silent during the dark diurnal phase. Under circadian conditions, the dorsal/ventral segregation persisted, although the anterior/posterior segregation showed an inverted effect (i.e. more subjective dark DA-like neurons found in anterior VTA). There were no topographical differences in GABA-like neurons in either diurnal or circadian conditions. Taken together, these results suggest that multiple topographically and functionally segregated populations of VTA neurons exist, with varying responses to circadian influences.

INTRODUCTION

The Earth’s pervasive 24 hr light/dark cycle has resulted in a fundamental rhythmicity in nearly all living organisms. This rhythmicity depends on synchronization of endogenous circadian oscillators (i.e. entrainment), but also direct influences of environmental factors (i.e. masking), such as light. Entrainment and masking are dissociable phenomena, as animals without circadian rhythms can still show 24 hr periodicity in behaviors when housed under light/dark conditions (Redlin and Mrosovsky, 1999; Mrosovsky, 2001).

The ventral tegmental area (VTA) is well known to regulate motivational aspects of both natural and pathological reward processes, including drug addiction (Schultz, 2002; Wise, 2002; Kauer, 2004). These same processes are influenced by circadian rhythms (Erickson et al., 1998; Stinus et al., 1998). Behavioral measures of addiction in animal models, such as behavioral sensitization (Andretic et al., 1999; Gaytan et al., 1999; Abarca et al., 2002), conditioned place preference (Abarca et al., 2002) and drug self-administration (Baird and Gauvin, 2000), are modulated by circadian factors.
Several lines of animals with mutant circadian clock genes also show altered responsiveness to drug reward (Abarca et al., 2002; Liu et al., 2005; McClung et al., 2005; Spanagel et al., 2005). One of these, the mouse clock mutant, exhibits increased preference for cocaine that is concomitant with increased VTA dopamine neuron excitability (McClung et al., 2005). Activity of VTA dopamine and GABA neurons also varies with behavioral state, with activity lowest during slow-wave sleep, higher during active wake, and highest during paradoxical sleep (Lee et al., 2001; Maloney et al., 2002).

MATERIALS AND METHODS

Animals

Sixty-six adult male Sprague Dawley rats (275-425 gm; Charles River Laboratories, Wilmington, MA, USA) were used. Rats were housed two per cage in light-tight environmental chambers under controlled conditions (21-23°C). All protocols and procedures were approved by the University of Pennsylvania’s Institutional Animal Care and Use Committee.

Light/Dark Group: Animals were divided into a “light phase” group (12h/12h light/dark; 9am lights on) or a “dark phase” group (12h/12h dark/light; 9am lights off) for at least 21 days before recording experiments. Recording sessions typically lasted approximately 6 hrs and always took place within the 12 hour temporal confines of each respective diurnal phase (6 hrs within ZT2-10 or ZT14-22). For recordings during the dark phase, animals were transferred from their housing cages to the recording area in light-tight transfer cages. During the induction phase of anesthesia, black electrical tape was placed over the
animals’ eyes to insure they were not exposed to photic input once room lights were re-illuminated.

**Dark/Dark Group:** Animals were first entrained to a 12 hr/12 hr (3am or 3pm lights on; “rest-phase” and “active-phase” groups, respectively) light/dark cycle for 14 days. This accounted for phase advancement during the dark/dark housing such that recording sessions occurred under normal working hours. Animals were then housed under continuously dark (dark/dark) conditions for at least 21 days before recordings began. During days 15-21 of dark/dark housing, animals were single housed with photobeam detectors surrounding their home cages (San Diego Instruments, San Diego, CA). These photobeam detectors continuously logged their locomotor activity in 15 minute bins. Periodicity of locomotor activity was found to advance 1.5-2.0 hrs during the week, confirming free-running circadian rhythms. All animals’ eyes were covered with black electrical tape during surgical and recordings session as described above. Recording sessions also had similar temporal parameters as “light/dark” animals with CT 0 and CT 12 defined as the offset and onset, respectively, of locomotor activity.

**Surgery**

Animals were initially anesthetized with 5% halothane (Halocarbon, Riveredge, NJ, USA) in medical grade air administered through a facemask. A tracheotomy was performed, and 2% halothane was delivered through a tracheal cannula via spontaneous respiration. The jugular vein was cannulated for drug delivery. Animals were placed in a stereotaxic frame, and body temperature was maintained at 36-37°C using a thermistor-controlled electric heating pad. During recording experiments halothane concentrations...
were kept at 1.25%. For VTA recordings, a hole was unilaterally drilled above the VTA and the dura carefully retracted. For recording pipettes oriented vertically (0°), the holes were drilled 0.8 mm lateral to midline and 5.3-5.5 mm caudal to bregma. To facilitate recording of medially located VTA neurons without disruption of the sagittal sinus, some recording pipettes were angled 10° from the vertical and inserted through a hole 2.0 mm lateral to midline, and 5.3-5.5 mm caudal to bregma.

**Ventral Tegmental Area Recordings**

A glass micropipette (6-10 MΩ) filled with 2% pontamine sky blue (PSB; BDH Chemicals Ltd. Poole, England) solution or 1.5% biotinamide (Invitrogen, Carlsbad, CA, USA) in 0.5 M sodium acetate was used. Signals were amplified and filtered (0.5-5 kHz bandpass) using an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA) in bridge mode and then further amplified using a CWE amplifier (Ardmore, PA, USA). Data were acquired and stored on a computer using a hardware/software interface system (CED 1401, Spike2; Cambridge Electronic Design, Cambridge, UK). VTA neurons were recorded for 3-5 min to establish a mean baseline firing rate. Total recording time for each particular neuron did not typically extend more than 15 min to ensure sampling from a maximum number of neurons during the recording session. At least 100 μm of electrode travel was required between two isolated cells to ensure recordings were from separate single-units. VTA recordings were obtained from each animal using a sampling grid that extended 0.4-1.2 mm from midline, 7.0-9.0 mm from brain surface, and 4.8-6.2 mm caudal to bregma. An average of 8.6 ± 0.5 tracks within the VTA was taken during each recording session.
Mapping topographical locations of recorded neurons

The relative topographical location of each recorded neuron was documented (e.g. Neuron B found 100 μm ventral to Neuron A). An iontophoretic deposit of Pontamine Sky Blue or Juxtacellular labeling was performed on the last recorded neuron. The histological location of this neuron was mapped using a Rat Brain Atlas (Swanson, 2004) to generate a histological “anchor” (See Figure 4.1). All other neurons’ topographical locations were determined relative to these coordinates.

![Histological Reference Coordinate](image)

**Figure 4.1 Strategy for topographical mapping of recorded VTA neurons.** During recordings, relative stereotaxic coordinates were noted. A juxtacellularly labeled neuron or Pontamine Sky Blue deposit was then used to generate histological reference coordinates. Absolute stereotaxic coordinates were created for all other neurons recorded with the same electrode with respect to the reference coordinates. X-Y-Z axis ranges indicate extent of VTA sampling grid.

**Antidromic Stimulation**

Electrical stimulation was performed with bipolar, concentric electrodes (FHC, Bowdoin, ME) placed in either the medial prefrontal cortex (PFC; electrode oriented vertically (0°) at +2.7mm from bregma, 0.6mm from midline, 4.5mm from cortical surface) or nucleus
accumbens (NAC; electrode oriented at 20° above contralateral hemisphere to allow access to the ipsilateral hemisphere at +1.2mm from bregma, 1.5mm from midline, 7.3mm from ipsilateral cortical surface (8.0mm from contralateral)). Only spontaneously firing neurons were tested for antidromic activation. Driven impulses were considered antidromic if they met the following criteria: (1) constant latency at 10 or 5mA, 0.5ms, 0.5Hz stimulation, (2) driving by paired stimulus pulses at least 100Hz, and (3) collision of driven spikes by spontaneous impulses.

Electrophysiological Categorization of VTA Neurons

Classic DA-like Neurons: A cell was categorized as a “classic DA-like” neuron when it met the following criteria classically used to identify DA neurons: (1) wide, triphasic (filtered recordings) waveform ≥ 2.0 ms in duration as measured by its full waveform, and ≥ 1.1 ms in duration as measured from the beginning of the spike to the peak of the negative trough and (2) slow spontaneous firing rate < 10 spike/sec (Yim and Mogenson, 1980; Grace and Bunney, 1983; Chiodo, 1988; Ungless et al., 2004).

Classic GABA-like Neurons: A cell was categorized as a “classic GABA-like” neuron when its electrophysiological characteristics met the following criteria classically used to identify possible VTA GABAergic neurons: (1) thin, biphasic (filtered recordings) waveform < 2.0 ms in duration as measured by its full waveform, and (2) located dorsal to at least one recorded classic wide-spike neuron (Yim and Mogenson, 1980; Johnson and North, 1992a; Steffensen et al., 1998).
**Firing Pattern Categorization**

Recorded neurons were placed into one of three categories, "Regular", "Irregular" or "Poisson"; A method based on the firing pattern scheme described by Kaneoke and Vitek (1996) and Levy et al. (2001). Figure 4.2 outlines the steps used to categorize cell firing pattern. A “discharge density histogram” was first generated by calculating the number of spikes that occur during a bin size equal to the mean inter-spike-interval. The number of spikes that occur during each time bin was counted to create a frequency histogram of the “discharge density”. The discharge density histogram was then statistically compared to a predicated density histogram if the spike train followed a Poisson process. Then based on its variance, the cell was categorized as Regular, Irregular or Poisson. In addition to this categorization, the coefficient of variation (standard deviation / mean inter-spike intervals) was also calculated as an accompanying measure of firing regularity.

1. Generate a discharge density histogram for each spike

   \[
   \frac{1}{\text{mean firing rate}} = \text{Mean ISI}
   \]

   ![Discharge Density Histogram](image)

   \(\chi^2\) goodness of fit:
   
   Compare to predicted discharge density histogram if present spike train followed a Poisson firing pattern

2. Cells placed into one of three categories

   - **Poisson Pattern**
     
     Mean = 1, Variance = 1

   - **Regular Pattern**
     
     Mean = 1, Variance < 1

   - **Irregular Pattern**
     
     Mean = 1, Variance > 1

   Equal probability of finding one spike
   
   High probability of finding exactly one spike during a time interval as not finding one spike during a time interval
   
   High probability of finding either none or more than one spike during a time interval

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**Figure 4.2 Method for categorizing VTA neuronal firing patterns.** VTA neurons were classified as exhibiting "Regular", "Irregular", or "Poisson" firing patterns based upon their discharge density histogram.

*Bursting Analysis*

The onset of the burst was defined as the occurrence of two spikes with an inter-spike interval < 80ms (Grace and Bunney, 1983). The percentage of spikes in bursts (% SIB) was calculated by dividing the number of spikes occurring in bursts by the total number of spikes occurring in the same period of time. Burst threshold was also calculated by the Discharge Density Method (Kaneoke and Vitek, 1996) as in Chapter Three. However, because both methods produced comparable outputs, for clarity, only the resulting % SIB using the 80ms method is presented here.

*Population Activity (Cell/track)*

The cell/track measure is the number of spontaneously active neurons found per electrode track, and has been used extensively as an indirect measure of population activity (Bunney and Grace, 1978; White and Wang, 1983; Floresco et al., 2001). All results are presented as Mean ± SEM unless otherwise noted.

**RESULTS**

**Diurnal fluctuations in VTA neuronal firing activity**

In addition to selective firing of “novel VTA neurons” (see Chapter Three) during the dark diurnal phase, “classic VTA neurons” exhibited subtle changes in firing activity between light and dark phases. Figure 4.3A’ shows that electrophysiologically identified DA-like neurons (see Materials and Methods) exhibited firing rate changes within the
light phase with their peak at ZT 4-6, and trough at ZT 8-10 (ZT 4-6, 5.1 ± 0.4 spikes/sec, n = 7 animals, 38 neurons; ZT 8-10, 3.5 ± 0.3 spikes/sec, n = 5 animals, 32 neurons; p < 0.01, ANOVA, Newman-Keuls post-test). The trough at ZT 8-10 was followed by an increase in firing activity during middle dark phase, ZT 16-18 (ZT 16-18, 4.6 ± 0.4 spikes/sec, n = 11 animals, 52 neurons; p < 0.05, ANOVA, Newman-Keuls post-test).

However, if all DA-like neurons are combined with respect to either light or dark phases, no overall differences were observed in firing rates. In addition, no differences were observed in bursting activity, coefficient of variation, number of recorded neurons per electrode track, or firing patterns when data were combined by diurnal phase (Figure 4.3B-E and Table 4.1), or by 2 hr ZT binning (data not shown).
Figure 4.3 Diurnal fluctuations in classic DA-like neuronal firing activity. A, Overall firing rates of classic DA-like neurons did not differ between light and dark phases. A', However, firing differences occurred within the light phase (ZT 4-6 vs. ZT 8-10; * p < 0.01, ANOVA, Newman-Keuls post-test), and between late light phase and middle dark phase (ZT 8-10 vs. ZT 16-18, # p < 0.05). Each point represents mean firing activity of different neurons recorded during each respective ZT range. B-E, No overall differences were detected in (B) % of spikes in burst, (C) coefficient of variation, (D) cell per electrode track, and (E) firing pattern. ZT, zeitgeber time.

Figure 4.4A shows that VTA GABA-like neurons fired faster during the dark phase (light phase 14.7 ± 1.2 spikes/sec, n = 14 animals, 68 neurons; dark phase 18.7 ± 1.5 spikes/sec, n = 23 animals, 84 neurons; p < 0.05, t-test). This increase was due to a
peak in firing activity at ZT 18-20 (25.0 ± 3.1 spikes/sec, n = 10 animals, 18 neurons).

No overall differences were observed in coefficient of variation, cell/track, or firing patterns (Figure 4.4B-D).

Figure 4.4. Diurnal fluctuations in classic GABA-like firing activity.  A, Classic GABA-like neurons fire faster during the dark phase (* p < 0.05, t-test) with (A') a peak during ZT 18-20 (* p < 0.05 compared to all other time-points, ANOVA, Newman-Keuls post-test).  B-D, No overall differences were detected in (B) coefficient of variation, (C) number of recorded cells per electrode track, and (E) firing patterns.  ZT, zeitgeber time.

Diurnal fluctuations in topographically and functionally segregated VTA DA-like neuronal populations

The typical length of our recording sessions (4-6 hrs) afforded us the opportunity to sample from a large of extent of the VTA (see Figure 4.1 and Materials and Methods).
Using a histological reference point (either a juxtacellarly labeled neuron or Pontamine Sky Blue deposit mapped to a Rat Brain Atlas, Swanson (2004)), we calculated stereotaxic coordinates for all other recorded neurons sampled with the same electrode. We then calculated mean topographical location (i.e. Anterior/Posterior, Dorsal/Ventral, and Medial/Lateral) for spontaneously firing DA-like neurons from either light or dark phases. We found that during the dark phase, DA-like neurons were more posterior (dark phase -5.52 ± 0.02mm from Bregma, n = 20 animals, 257 neurons; light phase -5.45 ± 0.03mm, n = 10 animals, 128 neurons; p < 0.05, Kolmogorov-Smirnov) and more ventral (dark phase 8.20 ± 0.02mm from cortical surface, n = 20 animals, 257 neurons; light phase 8.02 ± 0.03mm, n = 10 animals, 128 neurons; p = 0.02, Kolmogorov-Smirnov).

Figure 4.5A-B plots proportion of spontaneously firing DA-like neurons found at each anterior/posterior and dorsal/ventral location within the VTA. Figure 4.5C is a smoothed subtraction plot (dark phase – light phase coordinates) showing that during the light phase, proportionally more spontaneously firing neurons were found in anterior-dorsal VTA. During the dark phase, proportionally more spontaneously active neurons were found in posterior-ventral VTA. However, a ventrally located neuron was not necessarily also a posteriorly located neuron, as anterior/posterior coordinates did not correlate with dorsal/ventral coordinates (light AP vs. DV, rs = 0.03; dark AP vs DV, rs = -0.06; spearman correlation). Interestingly, the lowest firing rates of DA-like neurons found in light phase (ZT 8-10, 3.5 ± 0.3 spikes/sec, see also Figure 4.3A') were also the most anterior population of neurons (5.35 ± 0.05mm) from either phase. In addition, the highest firing rates of DA-like neurons found in the dark phase (ZT 16-18, 4.6 ± 0.4
spikes/sec, see also Figure 4.3A) were also the most posterior population of neurons (5.61 ± 0.02mm) from either phase.

![Diagram](image)

**Figure 4.5** Topographical mapping of recorded VTA DA-like neurons in light and dark phases. A-B, Normalized "sagittal-like" plots of spontaneously firing DA-like neurons in (A) light and (B) dark phases. Dotted line through graph indicates line of best fit. DA-like neurons were found more ventral and posterior during the dark phase. C, Subtraction (dark – light) surface plot of recorded DA-like neuron topographical locations. Distance-Weighted Least Squares algorithm was used to smooth the surface plot.

Unlike most other monoaminergic neurons, VTA neurons show little axon collateralization, (Fallon and Loughlin, 1982; Albanese and Minciacchi, 1983). We speculated that perhaps the topographical differences we observed were due to VTA neurons with different projection targets firing at different phases of the daily cycle. Therefore, we used antidromic stimulation to examine the proportion of mesoprefrontal (PFC; prefrontal cortex projecting) and mesoaccumbal (NAC; nucleus accumbens)
projecting) VTA neurons that were spontaneously firing between light and dark phases. Figure 4.6A shows that during the light phase, spontaneously firing DA-like neurons were antidromically driven from both PFC (9.46 %; 7 of 74 tested neurons) and NAC (8.11%; 6 of 74 tested neurons). However, during the dark phase, no neuron was ever antidromically driven from PFC (0.00%; 0 of 82 tested neurons; p < 0.0001 vs. light phase PFC driven, Fisher Exact). This absence of spontaneously firing mesoprefrontal neurons during the dark phase seemed to be compensated by an increased number of spontaneously firing mesoaccumbal neurons (14.1%; 11 of 78 tested neurons). Figure 4.6B shows topographical locations of VTA mesoaccumbal and mesoprefrontal neurons in light and dark phases. Mesoaccumbal DA-like neurons in dark phase were more posterior (mesoaccumbal dark phase 5.61 ± 0.05mm vs. mesoprefrontal light phase 5.16 ± 0.12mm; p < 0.01, Kolmogorov-Smirnov) and lateral (mesoaccumbal dark phase 0.77 ± 0.04mm vs. mesoprefrontal light phase 0.56 ± 0.04mm; p < 0.01) than mesoprefrontal neurons in the light phase. Generally speaking, antidromically driven neurons in dark phase were more posterior than antidromically driven neurons in light phase (dark phase 5.61 ± 0.05mm, n = 11 mesoaccumbal neurons, vs. light phase 5.23 ± .09mm, n = 6 mesoaccumbal and 7 mesoprefrontal neurons; p < 0.01, Kolmogorov-Smirnov). This anterior/posterior difference is similar to the overall topographical result depicted in Figure 4.5.
Circadian fluctuations are largely absent in VTA neuronal impulse activity

To examine whether our diurnal effects were also endogenous circadian fluctuations, we recorded VTA neurons in animals housed under continuously dark (dark/dark) conditions. Similar measures were taken from rest (subjective day) or active phase (subjective night) VTA recordings. Figure 4.7 shows firing activity of DA-like neurons. There were no longer significant changes in firing rate within rest phase, and between late rest phase and middle active phase, as previously observed under light/dark conditions. All other measures also did not differ between rest and active phases.
Figure 4.7 Classic DA-like neuronal firing activity in animals housed under dark/dark conditions. No overall differences were detected between rest and active phases in (A-A') firing rate, (B) % of spikes in burst, (C) coefficient of variation, (D) firing pattern, or (E) number of recorded cells per electrode track. CT, circadian time.

Figure 4.8 shows firing activity of GABA-like neurons in animals housed under dark/dark conditions. No longer present were firing activity increases during dark phase as previously observed under light/dark conditions. There were also no changes in GABA-like neuron firing patterns between rest and active phases. However, there was an increased in the proportion of "Irregular" neurons in dark/dark animals as compared to light/dark animals regardless of which circadian phase the neurons were recorded ("Irregular" light vs. rest, p < 0.01, Fisher Exact; "Irregular" dark vs. active, p < 0.05; see

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also Table 3.1). This increase in “Irregularity” in dark/dark animals is likely due to an
increase in phasic on/off activity as shown in Figure 4.8E.

Figure 4.8 Classic GABA-like neuronal firing activity in animals housed under dark/dark
conditions. A, There was no longer an increase in firing rate during active phase as previously
observed during dark phase (light/dark housing conditions). B-C, No overall differences were
detected in (B) coefficient of variation or (C) number of recorded cells per electrode track. D, No
differences in neuronal firing patterns were detected between rest and active phases, although
there were overall more “irregularly” firing neurons in the dark/dark group as compared to the
light/dark group. This increase in Irregular neurons seemed to be due to an increase in (E)
phasic on/off firing pattern in dark/dark recorded GABA-like neurons.
Circadian fluctuations in VTA topography differ from those observed under diurnal conditions

As shown in Figure 4.9, we found that VTA DA-like neurons during active phase were more ventral, (active phase 8.21 ± 0.05mm, n = 10 animals, 70 neurons; rest phase 8.00 ± 0.05mm, n = 12 animals, 83 neurons; p = 0.02, Kolmogorov-Smirnov) and anterior (active phase 5.45 ± 0.03mm; rest phase 5.58 ± 0.03mm; p = 0.001) than DA-like neurons found in rest phase. Although the dorsal-ventral finding is consistent with results obtained under diurnal conditions, the anterior-posterior finding is inverted (i.e. more anterior in active phase vs. more posterior in dark phase). However, as with our diurnal findings, a ventrally located neuron was not necessarily also an anteriorly located neuron; anterior/posterior coordinates did not correlate with dorsal/ventral coordinates (rest AP vs. DV, rs = -0.13; active AP vs DV, rs = -0.15; spearman correlation). In addition, there were differences in topography between dark/dark and light/dark animals regardless of which circadian phase the neurons were recorded. DA-like neurons recorded from the dark/dark were found more medial than light/dark neurons (active vs. dark, p < 0.01, Fisher Exact; “Irregular” dark vs. active, p < 0.05; see also Table 4.2)
Figure 4.9 Topographical mapping of DA-like neurons in the rest and active circadian phases. A-B, "Sagittal-like" plots of spontaneously firing DA-like neurons in (A) rest and (B) active phases. In agreement with diurnal effects, neurons were more ventral during active phase. However, neurons were found more anterior during the active phase, opposite of the effect found during the dark diurnal phase (i.e., more posterior during dark phase).
Table 4.1 Summary of classic DA-like and GABA-like neuronal firing activity under diurnal and circadian housing conditions. Results expressed as Mean ± SEM (number of animals, number of neurons). “% of spikes in burst” was not calculated for classic GABA-like neurons as they do not exhibit bursting activity (Yim and Mogenson, 1980; Steffensen et al., 1998)

<table>
<thead>
<tr>
<th></th>
<th>Firing Rate (Hz)</th>
<th>% Spikes in burst</th>
<th>Coefficient of variation</th>
<th>Cell/Track</th>
<th>Firing Pattern (% of total cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classic DA-like</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Regular</td>
</tr>
<tr>
<td>Light</td>
<td>4.01 ± 0.18 (18,156)</td>
<td>19.50 ± 1.20 (18,156)</td>
<td>0.80 ± 0.03 (18,156)</td>
<td>1.28 ± 0.15 (9,55)</td>
<td>73.33 (18,156)</td>
</tr>
<tr>
<td>Dark</td>
<td>4.23 ± 0.15 (21,225)</td>
<td>19.45 ± 0.96 (21,225)</td>
<td>0.78 ± 0.02 (21,225)</td>
<td>1.46 ± 0.14 (8,96)</td>
<td>75.78 (21,225)</td>
</tr>
<tr>
<td>Rest</td>
<td>4.12 ± 0.24 (13,79)</td>
<td>21.97 ± 1.60 (13,79)</td>
<td>1.00 ± 0.08 (13,79)</td>
<td>1.52 ± 0.11 (13,79)</td>
<td>70.89 (13,79)</td>
</tr>
<tr>
<td>Active</td>
<td>4.29 ± 0.23 (14,82)</td>
<td>21.64 ± 1.59 (14,82)</td>
<td>0.80 ± 0.03 (14,82)</td>
<td>1.53 ± 0.10 (14,82)</td>
<td>76.84 (14,82)</td>
</tr>
</tbody>
</table>

|                |                  |                   |                          |            | Regular | Irregular | Poisson |
| **Classic GABA-like** |                |                   |                          |            |         |           |         |
| Light          | 14.70 ± 1.20 (14,68) | N/A               | 1.64 ± 0.11 (14,68) | 1.09 ± 0.16 (9,55) | 64.10 (14,68) | 7.69 (14,68) | 28.21 (14,68) |
| Dark           | 18.66 ± 1.54 (23,84) | N/A               | 1.36 ± 0.08 (23,84) | 1.27 ± 0.17 (8,96) | 69.23 (23,84) | 5.77 (23,84) | 25.00 (23,84) |
| Rest           | 14.47 ± 1.39 (11,48) | N/A               | 1.68 ± 0.13 (11,48) | 1.65 ± 0.12 (11,48) | 37.04 (11,48) | 61.11 (11,48) | 1.85 (11,48) |
| Active         | 12.60 ± 1.51 (10,54) | N/A               | 1.68 ± 0.12 (10,54) | 1.54 ± 0.13 (10,54) | 56.25 (10,54) | 43.75 (10,54) | 0.00 (10,54) |

Table 4.2 Summary of topographical coordinates for classic DA-like neurons under diurnal and circadian housing conditions. Results expressed as Mean ± SEM (number of animals, number of neurons). Anterior/Posterior coordinates expressed as distance posterior from Bregma. Dorsal/Ventral coordinates expressed as distance from cortical surface. Medial/Lateral coordinates expressed as distance from midline.

<table>
<thead>
<tr>
<th></th>
<th>A/P (mm)</th>
<th>D/V (mm)</th>
<th>M/L (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classic DA-like</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>5.45 ± 0.03 (10,128)</td>
<td>8.02 ± 0.03 (10,128)</td>
<td>0.88 ± 0.01 (10,128)</td>
</tr>
<tr>
<td>Dark</td>
<td>5.52 ± 0.02 (20,257)</td>
<td>8.20 ± 0.02 (20,257)</td>
<td>0.84 ± 0.01 (20,257)</td>
</tr>
<tr>
<td>Rest</td>
<td>5.58 ± 0.24 (13,83)</td>
<td>8.00 ± 0.05 (13,83)</td>
<td>0.62 ± 0.02 (13,83)</td>
</tr>
<tr>
<td>Active</td>
<td>5.48 ± 0.03 (12,70)</td>
<td>8.16 ± 0.05 (12,70)</td>
<td>0.62 ± 0.01 (12,70)</td>
</tr>
</tbody>
</table>
DISCUSSION

In the present study, we recorded from VTA neurons in anesthetized rats housed under either light/dark or dark/dark conditions to examine possible diurnal/circadian fluctuations in VTA impulse activity. We found that impulse activity could vary in overall firing tone or selectively activation of neuronal subpopulations. These effects could also be differentiated by whether they persisted under free-running circadian conditions, or required the daily light/dark cycle. VTA GABA-like neurons exhibited a diurnal but not circadian rhythm in overall firing tone, with general increased firing activity during the dark phase. The daily cycle did not change overall DA-like firing rates, but did selectively activate different subpopulations of VTA neurons. These subpopulations varied in two topographical axes, dorsal/ventral and anterior/posterior. Under both diurnal and circadian conditions, more spontaneously firing DA-like neurons were found in dorsal VTA during rest phase and more ventral VTA during the active phase. Under diurnal conditions, more spontaneously firing DA-like neurons in the light phase were found in anterior VTA, which contained both mesoprefrontal and mesoaccumbal neurons. More spontaneously firing DA-like were found in the posterior VTA, which contained mesoaccumbal but not mesoprefrontal neurons. Mesoprefrontal neurons appeared to be generally silent during the dark phase. This particular anterior/posterior segregation seemed to be a masking effect of light, as the effect was inverted under circadian conditions (i.e. more posterior during rest phase and vice versa).
Electrophysiological markers of VTA DA and GABA neurons

The electrophysiological criteria for VTA neurons is based upon seminal intra- and extracellular studies on substantia nigra pars compacta neurons (Guyenet and Aghajanian, 1978; Grace and Bunney, 1983; Lacey et al., 1989). Similar characteristics were found in VTA neurons (Yim and Mogenson, 1980; Johnson and North, 1992a; Steffensen et al., 1998), and therefore the same criteria were ascribed for them. However, more recent evidence (Cameron et al., 1997; Margolis et al., 2003; Ungless et al., 2004) suggests not all neurons that satisfy the electrophysiological criteria for VTA DA or GABA neurons necessarily express their defining proteins (i.e. tyrosine hydroxylase and γ-aminobutyric decarboxylase, respectively). Juxtacellular labeling of putative VTA DA and GABA neurons in our lab also confirm these findings (see Chapter Three). Unfortunately, there is presently not a perfect set of electrophysiological criteria to describe the majority of VTA DA and GABA neurons (Margolis et al., 2006). Therefore, we termed our recorded neurons DA- and GABA-like to acknowledge the possible discrepancy in presumed neurochemical identities. However, we categorized neurons by a set criterion of electrophysiological characteristics, and these defined populations nevertheless alter their impulse activity with respect to circadian phase.

Characteristics of VTA GABA-like neuronal activity

We find VTA GABA-like neurons increase their activity during the dark diurnal phase, when the animal would normally be active. This result may seem counterintuitive at first, given the hypnotic effects of GABA receptor agonists (e.g. barbiturates and benzodiazepines). However, recent evidence support the notion that VTA GABA activity
is associated with cortical arousal as firing activity increases concomitantly with increased \( \gamma \) activity during paradoxical sleep (Lee et al., 2001) and immediately preceding contingent brain stimulation reward (Steffensen et al., 2001).

In addition, we found GABA-like neurons exhibited increased irregularity in animals housed under dark/dark conditions as compared to light/dark animals, regardless of circadian phase. Our definition of “irregular” neurons stipulates an increased probability in finding none or more than one spike per mean ISI bin (see figure 4.2). This definition is consistent with phasic on/off activity (0.5-2.0 sec on/off periods; see Figure 4.8 and Lee et al., 2001) often observed in GABA-like neurons from anesthetized rats. Lee et al. (2001) found that this phasic activity is directly proportional with anesthesia depth. Although alveolar concentrations of halothane were not measured, it is possible animals housed under long-term dark/dark (e.g. 3 weeks as in our study) exhibit altered sensitivity to halothane anesthesia. However, we did not find overt differences in VTA DA-like neuron activity between light/dark and dark/dark animals (Figures 4.3 and 4.7), suggestive that possible changes in anesthesia sensitivity is only reflected in the GABA-like population.

“Silent” mesoprefrontal DA-like neurons during dark diurnal phase

Using antidromic stimulation of PFC, spontaneously firing mesoprefrontal VTA DA-like neurons were never found during the dark diurnal phase. Our results confirm studies reporting diurnal fluctuations in dopamine, its metabolite homovanillic acid (HVA) and the dopamine transporter (DAT); all three with their lowest levels during dark phase (de Saint Hilaire et al., 2000; Sleipness et al., 2007). Furthermore, the diurnal fluctuation in
PFC DAT depends on an intact SCN (Sleipness et al., 2007). VTA mesoprefrontal neurons have been intensely studied for their role in cognitive and affective functions (O'Donnell and Grace, 1998; Tzschentke, 2001; Schultz, 2002; Seamans and Yang, 2004). Phasic increases in mesoprefrontal firing are theorized to code for "salience" or "prediction error" (Le Moal and Simon, 1991; Schultz et al., 1998), so it was surprising that our results suggest mesoprefrontal neurons are generally silent during the diurnal phase animals would normally be active. However, this basal quiescence may be advantageous if viewed in the context of "signal-to-noise". If under basal conditions mesoprefrontal neurons are silent, then phasic firing increases may be more efficacious during behaviorally relevant situations. Interestingly, in the de-afferented slice preparation, proportionally more mesoprefrontal neurons are spontaneously firing than mesoaccumbal neurons (personal communication, Dr. Elyssa Margolis). These results suggest mesoprefrontal DA neurons are actively inhibited under basal conditions. Further experiments are needed to examine the mechanism of this possibility.

**Anterior/posterior topography in VTA DA-like neuronal activity**

Unlike VTA GABA-like neurons, the major change in DA-like neuron activity was not in overall firing tone, but rather in selective activity of different populations of neurons. The lack of correlation between dorsal/ventral and anterior/posterior coordinates suggests that at least two topographically segregated populations of neurons shifted between dark/active and light/rest periods. Our finding that antidromically activated neurons shifted in anterior/posterior, but not dorsal/ventral axes, is also consistent with this conclusion. Previous studies have shown the efficacy of behavioral responses depends on
whether the experimental design targeted the anterior or posterior VTA. In particular, intracranial nicotine (Ikemoto et al., 2006), ethanol (Rodd et al., 2004) and a potent μ-receptor agonist (Zangen et al., 2002) are all more rewarding when injected into posterior VTA. Concurrently, behavioral sensitization (Gaytan et al., 1999; Sleipness et al., 2005) and drug-seeking behavior to psychostimulants is highest during dark diurnal phases (Fitch and Roberts, 1993; Roberts and Andrews, 1997; Roberts et al., 2002). It is possible that under "natural" conditions, posterior VTA, including NAC projecting neurons are more active during dark diurnal phase, and in turn, are a more proximal contribution to the reinforcing effects of abused drugs. When experimenters pharmacologically manipulate the posterior VTA system during light phases (when most behavioral experiments are performed), it "artificially" activates the more drug sensitive, posterior system which normally is not active during light phase.

Diurnal vs. circadian fluctuations in VTA impulse activity

We found the only diurnal variation that persisted under circadian conditions was dorsal/ventral segregation of DA-like neurons (i.e. DA-like were found more ventral in active phase). Anatomically, it has generally been shown that ventral VTA projects to more dorsal forebrain and dorsal VTA projects to more ventral forebrain (Oades and Halliday, 1987). In particular, PFC receives afferents from dorsal VTA, and cingulate cortex, which is dorsal to PFC, receives afferents from ventral VTA (Swanson, 1982). This characteristic presents the possibility that a population of ventrally located, active phase VTA neurons project to cingulate cortex. Furthermore, amygdala projecting VTA tend to originate from ventral VTA. Although we did not test for antidromic activation,
the amygdala could also be a potential projection target of our ventrally located, active phase VTA neurons.

In general, we found minimal circadian effects in VTA impulse activity. It is possible that this may be due to our relatively long-term housing in dark/dark (3-4 weeks). Without a zeitgeber, neurons may continue to individually show circadian oscillations but synchrony between cells may dampen over time, such that our present methods were no longer sufficient to detect a consolidated rhythmic output, as observed in other systems (Welsh et al., 1995; Yamazaki et al., 2000). Indeed, the primary role of light in a circadian timing system is to entrain (maintain the phase relationship) endogenous oscillators to the light/dark cycle.

There was also a strong masking effect of light on anterior/posterior segregation, as the diurnal effect reversed under circadian conditions (i.e., DA-like neurons were found more anterior in dark diurnal phase but more posterior in active circadian phase). These results again confirm the dichotomy in two topographically segregated populations (dorsal/ventral and anterior/posterior) that are differentially regulated by circadian factors. It is unclear if, and how, the SCN-MPON circuit (Chapter Two) is involved in our masking effects. While circadian rhythms and masking are dissociable phenomena, it is not conclusive whether SCN is involved. It has been reported that SCN lesioned hamsters show intact negative masking to light (Redlin and Mrosovsky, 1999), however a more recent report indicates negative masking may indeed compromised in these animals (Li et al., 2005). Furthermore, clock and melanopsin mutants exhibit impaired light masking (Mrosovsky and Hattar, 2003; Redlin et al., 2005). However, because these
proteins are expressed in brain areas other than SCN, it is not known to what extent the SCN is involved in these masking effects.

Our results indicate different VTA neurons exhibit light-dependent or endogenous circadian fluctuations. The diurnal variation observed in VTA GABA-like neurons tended to be changes in overall tone, with faster firing rates exhibited by GABA neurons that did not correlate with their VTA topographical location. In contrast, different topographically segregated populations of DA-like fire at different phases of the daily cycle. These findings suggest different VTA circuits are active between day and night, rather than simple increases and decreases in firing activity of one defined circuit. Our results again confirm that the daily cycle can have a substantial impact on a broad number of nuclei. This is particularly relevant to researchers who typically perform behavioral experiments during the daytime in nocturnal animals, as our results suggest the underlying brain circuitry may be different from that observed during nighttime when these animals are normally awake.
CHAPTER FIVE

GENERAL DISCUSSION

Using anatomical tract-tracing and electrophysiological techniques, I describe a novel output pathway from suprachiasmatic nucleus (SCN) to ventral tegmental area (VTA), a midbrain nucleus with a pivotal role in motivation and arousal. Microinjection of pseudorabies rabies virus (PRV; Bartha strain) in all examined aspects of VTA consistently resulted in trans-synaptic, retrograde labeling of SCN at time-points indicative of an indirect input. Labeling was first observed in SCN “shell” with spread to the rest of SCN at longer time-points. Based upon known afferent/efferent circuitry and labeling at time-points prior to SCN labeling, the caudal-dorsal aspect of lateral septum (cd-LS) and medial preoptic nucleus (MPON) were candidate relay nuclei. Bilateral, ibotenic lesions of cd-LS or unilateral lesions of MPON were made one week prior to PRV injections in VTA. Lesions of MPON, but not cd-LS, resulted in a significant decrease in PRV labeling in SCN. These results indicate synaptically linked neurons project from SCN through MPON, to the VTA.

Given the existing knowledge about SCN, MPON and VTA, it is possible our circuit has regulatory implications in sleep/wake, and sexual motivation/reward. MPON contains temperature sensitive and sleep active neurons (Kaitin, 1984; Scammell et al., 1993), and has been implicated in the hypnogogic effects of increased ambient temperature (Mahapatra et al., 2005; Ray et al., 2005). Moreover, MPON is an important nucleus in the regulation of male sexual behavior (for review: Hull et al. 2002).
reinforcing properties of sex can be experimentally demonstrated in rodents, as male rats will level press to gain access to a female (Beck, 1971). It has been suggested that MPON works in conjunction with VTA to integrate motivational/appetitive aspects with somatomotor responses to generate the full behavioral repertoire (Everitt, 1990; Hull, 1995). Further experiments would be needed to examine if, and how, an SCN-MPON-VTA circuit would impact the circadian regulation of behaviors such as sleep/wake and sexual motivation/reward.

We examined the more proximal effects of a SCN-MPON-VTA circuit by testing whether daily fluctuations in VTA impulse activity exist. Single-unit, extracellular recordings of spontaneously firing VTA neurons were performed in animals housed under diurnal or circadian conditions. Halothane anesthesia was used throughout our recordings to ensure that any observed differences were due to endogenous changes, and not confounded by varying arousal levels. We recorded multiple populations of VTA neurons, including ones that fit classically defined criteria; classic wide-spike (putative DA) and classic thin-spike (putative GABA) neurons. Classic-wide spike neurons could also be sub-divided into classic wide-spike < 1.1ms, and classic DA-like neurons, as recent evidence suggests the criteria for classic DA-like neurons are more accurate for identifying tyrosine-hydroxylase (+) neurons.

We juxtacellulary labeled classic VTA neurons in both light and dark phases and observed two major trends. First, electrophysiologically defined thin-spike neurons (i.e. putative GABA neurons) were not GAD67 (+) in 43.9% of our labeled cells from either light or dark phases. Therefore, the established electrophysiological criteria for VTA GABA neurons are not accurate predictors of GAD67 content. Secondly, the established
criteria for DA-like neurons (i.e. putative DA neurons) were accurate predictors for those neurons found in light phase, but not for dark phase. We calculated cell/track measure for all neuronal sub-populations in an attempt to assess how DA (i.e. TH+) neurons change their firing activity (e.g. firing faster, non-firing, thinner action potentials) such that we can no longer define them by previous criteria. We expected to see a decrease in the cell/track of DA-like neurons, and an increase in the proportion of at least one other neuronal subpopulation. However, we observed no change, or an increase in all subpopulations including DA-like neurons. Alternatively, instead of a large population of TH (+) neurons changing their firing activity, there might be multiple small changes such that we cannot follow all transitions using the cell/track method.

Unfortunately, the mismatch in electrophysiological characteristics and neurochemical content is a weakness in the VTA physiology field itself that was observed by earlier studies, but only recently becoming more widely publicized. The electrophysiological characteristics of VTA neurons were based upon seminal intra- and extracellular studies on substantia nigra dopamine and GABA neurons (Guyenet and Aghajanian, 1978; Grace and Bunney, 1983; Lacey et al., 1989). Because similar characteristics were found in VTA neurons, the same criteria were ascribed to them (Yim and Mogenson, 1980; Johnson and North, 1992a; Steffensen et al., 1998), although Johnson and North (1992) did note that 37.5% of putative DA neurons were TH (-). It was not until 1997 when Cameron et al., explicitly characterized what they termed "tertiary" neurons, that it became more widely publicized that VTA is more heterogeneous than initially thought. These tertiary neurons had a similar action potential shape and frequency as putative DA neurons, exhibited an \( I_h \), and hyperpolarized to 98.
dopamine application. However, only 29% of tertiary neurons were TH (+). Similar mis-matches were found in later studies (Jones and Kauer, 1999; Margolis et al., 2003; Ungless et al., 2004).

Two of these studies suggested additional criteria for more accurately predicting TH content from physiological criteria. Ungless et al (2004) found that TH (+) neurons could be defined by initial spike durations ≥ 1.1 ms and inhibition to footpinch. We incorporated these criteria into our recorded neurons and found that 100% of light phase neurons were TH (+). Unfortunately, the same criteria applied to dark phase neurons resulted in only 60% being TH (+). It is currently unknown why the same criteria are not sufficient for putative DA neurons in the dark phase, but nonetheless, it reinforces the notion that VTA impulse activity in the dark phase is markedly different from that in light phase. Margolis et al., (2003) found all putative DA neurons inhibited by a kappa opioid receptor agonist and projected to prefrontal cortex were TH (+). In order to incorporate these criteria into our study design, we would need to antidromically stimulate PFC, and locally apply a kappa agonist to every recorded neuron. While this is feasible, it likely would have significantly reduced our sample size such that our observed daily fluctuations would likely go undetected. In summary, there are currently no perfect criteria to describe the majority of VTA DA neurons in both light and dark phase. Despite this shortcoming, our defined electrophysiological groups represent populations with varying impulse activity with respect to circadian phase and further characterize the heterogenic regulation and neurochemistry of the VTA.

In particular, our experiments uncovered what we termed “novel neurons”. These neurons selectively fired during active phase under both diurnal and circadian conditions,
Novel neurons could be divided into two electrophysiological groups: novel wide-spike and novel thin-spike neurons. Novel wide-spike neurons were defined by their wide spike duration and fast firing rates. Novel thin-spike neurons were defined by their thin spike duration and ventral location within VTA. Further characterization of novel wide-spike neurons revealed they were non-DAergic, non-GABAergic, low-bursting, non-responsive to footpad stimulation, with slightly thinner spike durations than classic DA-like neurons, and inhibited by a D2 receptor agonist.

We currently do not know what neurotransmitter the bulk of novel wide-spike neurons express. Glutaminase, a precursor to glutamate, was first observed by Kaneko et al., (1990) to co-localize with VTA TH (+) neurons. With the advent of more sensitive histochemical tools and discovery of vesicle glutamate transporters, the co-localization theory is currently being re-evaluated. Recent reports indicate a population of TH (-), GAD (-), glutamatergic neurons exist in VTA, although the exact size of this population is still unclear (Kawano et al., 2006; Yamaguchi et al., 2007). It is possible that a proportion of our novel wide-spike neurons are glutamatergic, and therefore would constitute the first physiological data to describe VTA glutamate neurons.

In addition to circadian fluctuations in novel VTA neurons, we detected daily variations in DA-like and GABA-like neurons. VTA GABA-like neurons exhibited a diurnal but not circadian rhythm in overall firing tone, with general increased firing activity during dark phase. The daily cycle did not change overall DA-like firing rates, but did selectively activate different subpopulations of VTA DA-like neurons. These subpopulations varied in two topographical axes, dorsal/ventral and anterior/posterior. Under both diurnal and circadian conditions, more spontaneously firing DA-like neurons
were found in dorsal VTA during rest phase and more in ventral VTA during active phase. Under diurnal conditions, more spontaneously firing DA-like neurons in light phase were found the in anterior VTA, which contained both mesoprefrontal and mesoaccumbal neurons. More spontaneously firing DA-like were found in posterior VTA, which contained mesoaccumbal but not mesoprefrontal neurons. Mesoprefrontal neurons seem to be generally silent during dark phase. This particular anterior/posterior segregation appears to be a masking effect of light, as the effect was inverted under circadian conditions (i.e. more posterior during rest phase and vice versa).

Figure 5.1 summarizes some of the major findings of this thesis. We have defined a SCN-MPON-VTA circuit and observed multiple types of changes in VTA impulse activity under diurnal and circadian conditions. Do all these different components work together, and if so, how? One hypothesis is that novel neurons are intrinsic neurons of VTA and regulate the activity of the dorsal/ventral shifting DA-like population. This hypothesis is based on the result that novel neurons do not seem to project to the NAC or PFC, two major projection targets of VTA. Furthermore, novel neurons readily respond to changes in dopaminergic neurotransmission and are located in the dorso-ventral center of a dopamine-rich area (Table 5.1). Based on *in vitro* studies, VTA GABA neurons have long been theorized to provide local inhibition of VTA DA neurons (Johnson and North, 1992b); however, no published anatomical studies have confirmed GABA inhibitory synapses on DA neurons. Furthermore, a large proportion of VTA GABA neurons are themselves projection neurons (Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000), and studies have failed to find the expected inverse relationship between GABA and DA neuron activity (Steffensen et al., 2001; Maloney et al., 2002). It is
possible novel neurons are primary local regulatory population of VTA and provide a “switch” for activity of topographically segregated DA-like neurons.

Figure 5.1 Summary of SCN-MPON-VTA circuit under diurnal conditions. Using MPON as a relay nucleus, SCN projects to all examined aspects of VTA. All noted VTA populations in the figure refer to locations of spontaneously firing neurons. During the light phase, GABA-like neurons are active in dorsal VTA. Two populations of DA-like neurons are active, one in dorsal VTA, although ventral to the GABA-like population. Another DA-like population is active in anterior VTA that does not show segregation within dorso-ventral laminae. The anterior population contains both active mesoaccumbal and mesoprefrontal neurons. Very few novel neurons are active during the light phase. During dark phase, GABA-like neurons are still active in the same dorsal aspect of VTA as in light phase. Two different populations of DA-like neurons are active in dark phase, one in ventral VTA, and one posterior VTA. The posterior population contains mesoaccumbal but not mesoprefrontal neurons. Mesoprefrontal neurons are largely silent during dark phase. Most novel neurons are active during the dark phase at a dorso-ventral location similar to DA-like neurons.
During the rest phase (DD conditions), mean firing activity of SCN is at its highest (Groos and Hendriks, 1982). The net effect on MPON neurons is also to increase their activity, as evidenced by the high proportion of sleep-active MPON neurons (Kaitin, 1984). It is possible MPON provides a tonic inhibition (Maeda and Mogenson, 1980) to novel neuron activity. Novel neurons are therefore largely silent during the rest phase, which in turn results in the activity of posteriorly and dorsally located DA-like populations. During the active phase, the MPON inhibitory effect on novel neurons is lifted, allowing them to fire at their signature fast-firing rate. This “switch” then modifies the activity of DA-like neurons such that now an anteriorly and ventrally located DA-like population is spontaneously firing.

Under light/dark conditions, the novel neurons continue to exert similar effects on the dorsal/ventral DA-like population as during circadian conditions. However, the fluctuations in GABA-like neuron activity, mesoprefrontal/mesocortical neurons, and inverted anterior/posterior DA-like population seem to be due to the masking effects of light. One mechanistic possibility for these masking effects may derive from serotonergic (5HT) afferents of the raphe complex. The SCN receives both direct and indirect projections from the raphe complex (Moore and Card, 1985), which are thought
to mediate arousal effects in response to light in the circadian timing system (Morin, 1999). The raphe is also a prominent afferent to VTA (Phillipson, 1979; Geisler and Zahm, 2005). It is possible 5HT contributes to masking in VTA via its regulation of SCN. Alternatively, it could directly regulate novel neuron activity via its direct projection to VTA, as Cameron et al., (1997) reported that “tertiary” neurons depolarize to 5HT application.

In summary, there are two general conclusions from this thesis. First, there exists a synaptic circuit from SCN through MPON, to VTA. This circuit represents a pathway by which the central pacemaker could influence a variety of physiological and behavioral processes. More specifically the SCN-MPON-VTA may be involved in circadian regulation of sleep/wake and sexual motivation. Secondly, our observed differences in VTA impulse activity suggest that different circuits are operational between light and dark phases. To assume neuronal systems merely increase or decrease their “volume” as a function of the daily cycle is to oversimplify a biological system that has evolved over millions of years. Our results are just one example of how a seemingly elementary light/dark cycle can produce such a neurobiologically “different” animal on multiple levels.
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