Do Neurotransmitters Switch During the Sleep/Wake Cycle?

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Amy Guzdar

Committee in Charge:
Professor Nicholas C. Spitzer, Chair
Professor Darwin K. Berg
Professor Ella Tour

2013
The Thesis of Amy Guzdar is approved and it is acceptable in quality and form for publication on microfilm and electronically:

----------------------------------------
Chair

University of California, San Diego

2013
DEDICATION

This master’s thesis is dedicated to:

Yezdi Guzdar, Perveen Guzdar, Erin Press, Freny Katrak, and Erik Kumas

who have all consistently encouraged me to follow my goals and aspirations, no matter how varied they may be. Thank you for supporting me in all the choices I make.
TABLE OF CONTENTS

Signature Page..................................................................................................................................................iii
Dedication.......................................................................................................................................................iv
Table of Contents...........................................................................................................................................v
List of Figures..................................................................................................................................................vi
List of Tables..................................................................................................................................................vii
Acknowledgments..........................................................................................................................................viii
Abstract of the Thesis......................................................................................................................................ix
Introduction.....................................................................................................................................................1
Materials and Methods....................................................................................................................................10
Results............................................................................................................................................................15
Discussion.......................................................................................................................................................28
Supplemental Figures......................................................................................................................................36
References.......................................................................................................................................................39
LIST OF FIGURES

Figure 1: Homeostatic neurotransmitter switching..................................................3
Figure 2: 24 hr activity graphs..................................................................................16
Figure 3: GAD65 in situ hybridization.....................................................................19
Figure 4: GAD65 in situ hybridization.....................................................................20
Figure 5: GAD65+ cell populations between sleep and wake..................................22
Figure 6: CST-14 in situ hybridization...................................................................24
Figure 7: CST-14 in situ hybridization...................................................................25
Figure 8: CST-14+ cell populations between sleep and wake.................................27
Figure 9: Summary of experimental results.............................................................29
Figure 10: Sleep and homeostatic transmitter switching........................................30
Figure 11: Transmitter switching within a sleep-implicated cell population...............32
Figure S1: 24 hr activity graphs.............................................................................36
Figure S2: 24 hr activity graphs.............................................................................37
Figure S3: 24 hr activity graphs.............................................................................38
LIST OF TABLES

Table 1: Summary of experimental conditions .........................................................10
Table 2: Percentage time spent active during light/dark phases ..................................17
Table 3: Stereological estimates of GAD65+ cell populations ..................................21
Table 4: Stereological estimates of CST-14+ cell populations ..................................26
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my committee chair, Nicholas Spitzer, for his advice and accessibility during the course of my project. He was always available for questions and his enthusiasm for science was a great source of inspiration throughout my project and will continue to inspire me in my future career. In addition, his patience with countless drafts and edits to this manuscript were greatly appreciated.

I would also like to thank my mentor, Claudia Alvarez-Baron, for teaching me all the techniques that I have used this past year. She offered advice and support in dealing with all situations, and was therefore a great source of guidance throughout my project.

Lastly, I would like to thank my committee members, Ella Tour and Darwin Berg, for their advice and comments on the completion of this manuscript. Their fresh perspective proved invaluable.
ABSTRACT OF THE THESIS

Do Neurotransmitters Switch During the Sleep/Wake Cycle?

By

Amy Guzdar

Master of Science in Biology

University of California, San Diego, 2013

Professor Nicholas C. Spitzer, Chair

For years it was thought that a neuron releases only a single neurotransmitter for the entirety of its life. Recent research, however, has provided evidence that neurons can change the neurotransmitters they synthesize based on activity within a circuit. This phenomenon, referred to as “activity-dependent neurotransmitter respecification,” is thought to be a homeostatic process that prevents over-excitation or over-inhibition within a circuit. I searched for population-level transmitter respecification in response to sleep/wake cycles. Sprague-Dawley rats were housed in a 12 hr light : 12 hr dark cycle. They were sacrificed after most time spent awake or most time spent asleep to look for differences in the number of neurons expressing specific transmitters between the two time points. In situ hybridization was performed on separate but adjacent sections to label cells that express the mRNA
encoding glutamic acid decarboxylase (GAD65 – an enzyme involved in the synthesis of GABA), or cortistatin (CST-14 – a neuropeptide expressed in cortical GABAergic interneurons involved in sleep). Cell populations in the primary motor cortex were compared between end of sleep and end of wake using stereology. No significant differences were observed in the CST-14+ population between sleep and wake. The GAD65+ population was 20-30% larger ($p < 0.05$) in animals sacrificed at the end of wake than those sacrificed at the end of sleep. My findings are consistent with transmitter switching, but a complementary change in another transmitter population must be observed in order to attribute them to neurotransmitter respecification.
INTRODUCTION

Neurotransmitter respecification

The brain was once thought to be a continuous reticular tissue; however, in the early 1900s Santiago Ramón y Cajal discovered that it is composed of individual cells (Cajal, 1911). In order for these discrete entities – called neurons – to work together to create movement, thoughts, sensation, and love, there has to be a reliable basis for communication among them. The basis of this communication is chemical synaptic transmission. Chemical signals called neurotransmitters are released by one neuron and detected by another, causing excitation or inhibition of the receiving neuron. This process occurs throughout neuronal networks in order to transmit signals all over the brain, and makes up the vast majority of signal transmission. It is increasingly important to learn more about this signaling mechanism, as it is implicated in every thought we have or action we perform. In addition, various pathologies of the central nervous system are the result of problems in neurotransmitter synthesis or release. For example, Parkinson’s disease is the result of decreased dopamine in the basal ganglia (Obeso et al., 2010). Myasthenia gravis is a disease in which skeletal muscles do not work appropriately due to a decreased cholinergic receptor population at the neuromuscular junction (Vincent, 2002). Many mood disorders such as depression, seasonal affective disorder, and schizophrenia have been linked to altered levels of neurotransmitters (Stahl et al., 2003; Johansson et al., 2001, Horn et al., 1971). In order to help treat these problems, it is essential to learn more about plasticity in transmitter metabolism.

For years, neuroscientists believed that neurons – incapable of regenerating in most areas of the brain – released a single neurotransmitter for the life of that neuron. Neurons were thought to release only classical transmitters including but not limited to: glutamate (the primary excitatory transmitter in the brain), GABA (the primary inhibitory transmitter),
acetylcholine and the catecholamines. This principle, popularized by Henry Dale and John Eccles in the early 1950s, has been discovered to be false. It is now well established that neurons are capable of releasing a classical transmitter in conjunction with a modulatory peptide at synapses. These modulatory peptides are referred to as neuropeptides, and while classical neurotransmitters typically exert fast excitation or inhibition of a postsynaptic cell, neuropeptides are capable of causing a vast array of effects on their targets. Acting through G-protein-coupled receptors, neuropeptides can excite or inhibit a cell, change gene expression, local blood flow, or cell morphology (van den Pol, 2012). This co-release of a classical transmitter with a modulatory neuropeptide occurs in a variety of brain structures. GABAergic interneurons of the cortex alone are capable of releasing multiple neuropeptides, including neuropeptide Y, somatostatin and others (Tatemoto et al., 1982; Patel et al., 1978; van den Pol, 2012). The result of these various types of co-release is inhibition of the postsynaptic cell by way of GABA, and modulation of various cellular properties by the neuropeptide. In addition to classical co-transmission with neuropeptides, it has even been discovered that two classical transmitters can be co-released from a single neuron. This is observed in the developing tadpole spinal cord, where single neurons are capable of co-releasing glutamate and acetylcholine from the same synaptic vesicles (Li et al., 2004). It has also been reported that glycine and GABA are co-released from interneurons in the spinal cord (Jonas et al., 1998).

In recent years, a novel aspect of transmitter release has been explored: transmitter respecification. This is a process by which some neurons are capable of switching the neurotransmitters that they synthesize and release based on activity patterns within a given circuit. Over-excitation in a circuit can lead to seizures, while over-inhibition can lead to complete silencing of a circuit. Therefore, transmitter respecification is thought to be homeostatic: high activity patterns in a circuit potentially lead neurons that typically release
excitatory transmitters to synthesize and release inhibitory transmitters. Similarly, low-activity
patterns in a circuit lead neurons that produce inhibitory transmitters to produce excitatory
ones. The current evidence indicates that this is the result of different activity patterns
activating different calcium-dependent transcription factors, which in turn regulate the
expression of particular transmitters (Spitzer et al., 2005).

Figure 1: Different activity patterns can homeostatically lead to the synthesis of excitatory of inhibitory
transmitters (Spitzer et al., 2005).

For years, examples of transmitter switching were reported in the literature, but these
studies only described a loss of expression of a neurotransmitter without a gain of expression
of another transmitter. Therefore, these examples were not interpreted as an actual transmitter
“switch,” but rather an activity-dependent loss of expression (Spitzer, 2012). For example,
primates with an eyelid sutured to prevent vision exhibit a decreased number of GABAergic neurons relative to control conditions. When the sutures were removed, the number of GABAergic neurons returned to its original level (Hendry et al., 1986). This finding, along with others, indicated that there is certainly plasticity in transmitter expression, but it was still unknown whether this phenomenon occurs in response to natural sensory stimuli.

To observe whether transmitter switching can occur in the adult mammalian brain in response to natural stimuli, experiments were conducted on adult rats exposed to different light/dark cycles, or photoperiods (Dulcis et al., 2013). When rats were housed on a control 12 hr light: 12 hr dark cycle, the paraventricular nucleus of the hypothalamus, a region that receives input from the retina via the suprachiasmatic nucleus, contained both dopaminergic and somatostatin-positive neurons (Buijs et al., 1993). When light exposure was switched to 5 hr light: 19 hr dark, the number of dopaminergic neurons increased by nearly 50% from the control, with a corresponding decrease in the number of somatostatin-positive neurons. When the light cycle was switched to 19 hr dark: 5 hr light, the number of somatostatin-positive neurons increased by roughly 45% from the control, with a corresponding decrease in the number of dopaminergic neurons (Dulcis et al., 2013). Unlike the results of previous experiments on transmitter specification, this experiment showed an activity-dependent loss of function of a particular transmitter coupled with an activity-dependent gain of function of another transmitter – both at the protein and the mRNA level. With the appropriate controls (ruling out circadian variations, neurogenesis, or apoptosis), this study demonstrated that transmitter switching at a population level was a real phenomenon that can occur in the adult mammalian brain (Dulcis et al., 2013). It is, therefore, important to explore where else in the brain, and in response to what kind of activity, this switch occurs.
Sleep

Though a great deal of research has been conducted on sleep, it still maintains its place as one of the great mysteries of the brain. Many ideas have been proposed to explain what occurs in the brain to stimulate sleeping and waking, but the results remain equivocal. There is, however, a consensus in the neurobiology community that sleep is regulated by two components. One is circadian: that is the regulation through natural light/dark cycles; the other is homeostatic: regulated by the amount of time we have previously spent awake (Borbély, 1982). With these two components working together, some “flip-flop switch” in the brain causes a switch in state from wake to sleep. This is generally thought of as a whole-brain state: that is, the entire brain is either asleep or awake. Pathways from the hypothalamus and midbrain (generally considered "sleep-control centers") project all over the brain, resulting in one of these two distinct states of consciousness (Saper et al., 2005). Research has suggested, however, that sleep can occur in distinct local circuits based on prior activity of that brain region (Krueger et al., 1993). Though this concept had first been proposed nearly 20 years ago, it has only received substantial recognition in the last decade, due to the empirical evidence from an expanding number of publications.

This idea that sleep is a property of local circuits is linked to the homeostatic component of sleep and is reflected in non-REM sleep (NREM). The amount of time spent in NREM sleep is directly proportional to the amount of time spent awake (Borbély, 1982). This idea is supported by many studies that utilize electroencephalography (EEG), recordings of brain electrical activity, to demonstrate that sleep intensity differs between cortical regions. In humans, NREM EEG power is higher in the left somatosensory cortex than in the right somatosensory cortex after right-hand stimulation (Kattler et al., 1994). Unilateral whisker stimulation in rats leads to increased NREM sleep in the somatosensory cortex contralateral to the site of stimulation (Vyazovskiy et al., 2000). Transcranial magnetic stimulation-induced
activity in the motor cortex of humans increases NREM activity in that region (Huber et al., 2007).

Thus the motor cortex of the brain is capable of demonstrating this plasticity in NREM sleep. In fact, humans and rodents alike show a frontal dominance in NREM sleep intensity, possibly indicating that the motor cortex contributes to this pattern (Werth et al., 1996; Vyazovksiy et al., 2002). When rats are trained on a unilateral reaching task that is known to increase synaptic strength in the motor cortex, an increase in NREM sleep is seen in the corresponding motor cortex. Remarkably, this increase in NREM sleep is not seen in the motor cortex ipsilateral to the trained paw, indicating that the increase in NREM waves is the result of a homeostatic mechanism that tracks how much a particular cortical region was utilized during the day (Hanlon et al., 2009). In another experiment, researchers immobilized the left arm of human subjects for 12 hr during the day. A corresponding decrease in NREM sleep was seen in right motor cortex, but not in the left, during the night following immobilization (Huber et al., 2006). This allows one to reach the same conclusion that NREM sleep is a property of homeostasis of these local circuits. In addition, since all voluntary muscle movement originates in the motor cortex, these results are consistent with the idea that it is a region that would typically show a great sleep need.

Because GABAergic interneurons contribute substantially to cortical rhythms and oscillations, it is likely that these neurons are implicated in the slow waves seen during NREM sleep recorded by EEG (Galarreta et al., 2002). Indeed, a great deal of research supports this. When cortical GABA levels were measured using microdialysis in cats that display healthy sleep, GABA levels were discovered to be 39% greater during NREM than during wakefulness. It was also observed that there is a linear increase in GABA levels during prolonged wakefulness. This results in a conclusion that inhibition builds up during the day, ultimately leading to sleep (Vanini et al. 2012). When using magnetic resonance spectroscopy
to measure transmitter levels in insomniacs, a correlation was observed between cortical GABA levels and the time spent awake after sleep onset. The more time a subject spent awake throughout the night, the lower the GABA levels compared to healthy control subjects (Winkelman et al., 2008). In a similar study using magnetic resonance spectroscopy on narcoleptics, increased cortical GABA levels were seen in subjects with narcolepsy compared to control subjects (Kim et al., 2008). These results suggest that there is a narrow window of GABA levels that are necessary for sleep to ensue, and perhaps narcoleptic and insomniac patients display too much or too little GABA for healthy sleep, respectively.

It is likely that particular subpopulations of GABAergic interneurons that are implicated in sleep are responsible for these changes in GABA levels. For example, a specific population of GABAergic neurons that express neuronal nitric oxide synthase, the enzyme that synthesizes nitric oxide, have been shown to increase c-fos expression during sleep, suggesting that this population is active during sleep conditions (Gerashchenko et al., 2008). Another such subpopulation is the GABAergic interneurons that express the neuropeptide cortistatin. Similar in structure to somatostatin, but functionally distinct, cortistatin (CST) is only found in GABAergic interneurons of the cortex and hippocampus (deLecea et al., 1996). When injected into rat brain ventricles, it facilitates an increase in slow-wave sleep, possibly by antagonizing the effects of acetylcholine on cortical excitability (Bourgin et al., 2007). Northern blots at different time points during the day demonstrate that mRNA levels of CST steadily increase with time spent awake, and dissipate with time spent asleep (Bourgin et al., 2007). Though not much is known about the role of CST in sleep, given the evidence that does exist, it is likely that the population of CST+ cells plays a role in facilitating sleep.
Project

My project attempts to search for transmitter switching during the sleep/wake cycle in the adult rat brain. As transmitter switching has been shown in response to natural stimuli such as light cycles, it would be of interest to see if it occurs in response to sleep/wake states (Dulcis et al., 2013). The homeostatic component of sleep is particularly interesting due to the link between transmitter switching and homeostasis. Since rats are nocturnal, the high activity of the brain during the dark phase may lead to homeostatic transmitter switching that causes inactivity, or sleep, during the light phase.

I searched for changes in the number of neurons that express GABA in the motor cortex during the sleep/wake cycle in adult male rats. As noted earlier, a higher extracellular concentration of GABA in the somatosensory cortex of cats is found with increasing time spent awake (Vanini et al., 2012). Similarly, it has been shown that CST-14 mRNA in the cortex of rats accumulates with time spent awake (Bourgin et al., 2007). Are these changes in transmitter levels the result of one fixed population of neurons synthesizing and releasing more transmitter, or is it due to an increase in the number of neurons that release these transmitters as a result of activity-dependent transmitter respecification?

Since it has been shown that the motor cortex displays plasticity in sleep homeostasis, I thought this would be an appropriate place to look for activity-dependent transmitter switching (Hanlon et al., 2009; Huber et al., 2006; Huber et al., 2007). Due to the lifestyle of these rats, it is likely that the motor cortex is a highly utilized cortical region. Living in the vivarium, these animals do not have many varying visual, auditory, gustatory or somatosensory cues. In addition, half of the rats were provided with running wheels to create more distinct circadian phases (Welsh et al., 1988). Using in situ hybridization (ISH) on rat brain sections, I labeled cells that express glutamic acid decarboxylase 65 (GAD65), the enzyme that synthesizes GABA and a widely used marker of GABAergic cells. I also used
ISH to label cortistatin-positive cells. I looked for changes in the number of cells that express GAD and cortistatin at the end of sleep and at the end of wake in adult rats using stereological analysis. Given the search for the homeostatic component of sleep, I anticipated observing higher levels of GAD65 at the end of wake than at the end of sleep.
MATERIALS AND METHODS

Animals

All animal procedures were performed in compliance with the UCSD Institutional Animal Care and Use Committee (IACUC). Sixteen 300g Harlan Sprague-Dawley male rats were housed in an animal facility on a 12 hr:12 hr light/dark cycle, with lights on at 7:00 a.m. and lights off at 7:00 p.m. Food and water were provided ad libitum. After three days of acclimation, eight of the rats were provided running wheels in their cage to be used voluntarily, and eight were left without running wheels.

Table 1: Summary of the different conditions of animals involved in the experiments.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time sacrificed</th>
<th>Number of animals</th>
<th>Running wheel</th>
<th>End of sleep (EOS) or end of wake (EOW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7:00 am</td>
<td>4</td>
<td>Yes</td>
<td>EOW</td>
</tr>
<tr>
<td>2</td>
<td>7:00 am</td>
<td>4</td>
<td>No</td>
<td>EOW</td>
</tr>
<tr>
<td>3</td>
<td>7:00 pm</td>
<td>4</td>
<td>Yes</td>
<td>EOS</td>
</tr>
<tr>
<td>4</td>
<td>7:00 pm</td>
<td>4</td>
<td>No</td>
<td>EOS</td>
</tr>
</tbody>
</table>

One week after running wheels were provided, all rats were sacrificed at either 7:00 am or 7:00 pm (see Table 1). Rats were sacrificed by decapitation and the brains were rapidly dissected and frozen in isopentane at -80° C until sectioning.

Behavioral Assessment

To determine and track whether animals were in a sleep or wake state while housed in the animal facility, Swann video surveillance cameras were set up in the animal housing.
facility next to each cage for the entire week of housing. Infrared light allowed monitoring at night, while the lights were off, as well as during the day. At the end of the week, videos were downloaded and analyzed using J-Watcher™, a downloadable software program that monitors animal movement. Animal locomotion was tracked as a proxy for sleep/wake conditions. Using this program, activity graphs were created to illustrate sleep/wake patterns in the rats for the day prior to sacrifice.

**Cryostat Sectioning**

The whole brain was mounted on a sectioning plate, embedded in optimum cut temperature compound (OCT), and cut coronally through the motor cortex at 30 µm using a cryostat. Every sixth section was collected as part of a set, generating 6 different sets of tissue throughout the motor cortex. Sections were directly mounted on Fisherbrand® Superfrost® Plus microscope slides and allowed to dry for 2 hr at room temperature. Following drying, mounted sections were stored at -20°C until further processing.

**Probe Preparation**

CST-14 cDNA (kindly provided by Luis deLecea’s lab at Stanford University) and GAD65 cDNA (kindly provided by Claudia Alvarez-Baron) were transformed into Invitrogen One Shot® TOP10 chemically competent *E. coli*. cDNA was added to a vial of One Shot® cells, and then transformed via heat shock for 30 s at 42°C. 50 µL of transformed cells were spread on a pre-warmed selective plate containing 100 µg/mL ampicillin and incubated overnight at 37°C. Plasmids were then purified from the colonies and sequenced. DNA was digested and linearized using restriction enzymes at 37°C overnight. DNA was phenol extracted, ethanol precipitated, transcribed and the RNA probe was labeled with digoxigenin (DIG) using transcription buffer, DIG-RNA labeling mix, RNAsin, and polymerase for 2 h at
37°C. RNA was precipitated using ethanol, and incubated at -80°C overnight. The pellet was then air dried, and thoroughly resuspended in nuclease-free water. The resultant probes were stored at -80°C until use.

**In Situ Hybridization (ISH)**

Sections were frozen on slides and stored at -20°C until in situ hybridization was performed. All reagents and containers used on the first day of the procedure were treated with DEPC and were sterile to avoid contamination with RNases. Sections were warmed to room temperature at 50°C for 15 min and then immediately fixed in 4% paraformaldehyde in PBS for 20 min. Following fixation, sections were treated with 1 µg/2.5 mL Proteinase K in a buffer of Tris, EDTA, and water for 13 min, and subsequently fixed again in 4% paraformaldehyde for 15 min. Sections were acetylated by incubation in a solution of 0.1 M triethanolamine with 0.25% acetic anhydride for 10 min and transferred into a prehybridization solution (50% DI formamide, 25% 20x SSC, stock RNA, heparin, 1% Denhardt’s solution, 0.1% Tween, 0.5 M EDTA) at 65°C for 1 h. Following this, sections were left at 65°C for 17 h in the hybridization solution with the appropriate RNA probe (CST-14 or GAD65). The probe concentration in hybridization buffer (1-2 µg/mL) was determined empirically. Sections were washed in a series of SSC solutions (NaCl and sodium citrate) to remove excess probe. First, they were washed in 2X SSC for 15 min at 65°C, then 30 min in 2X SSC at 37°C. Lastly, they were washed in 0.2X SSC twice for 30 min each at 65°C. Sections were permeabilized and blocked with normal sheep serum and PBS with 0.1% Triton, followed by incubation overnight at 4°C in 1:2000 Roche anti-digoxigenin antibody. Sections were washed in 1X TBST (a solution of NaCl, KCl, Tris Base, and Triton) four times for 15 min each and washed in NTMT (a solution of Tris base, NaCl, and MgCl₂ and Triton) three times for 5 min each. Finally, sections were developed in Roche nitro-blue.
tetrazolium chloride (NBT)/ 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) for 5 h (GAD65 probe) or 15 h (CST-14 probe). Following ISH, sections were coverslipped in Southern Biotech Fluoromount-G™ Mounting Media and stored at room temperature until imaging.

**Stereology**

Cells positive for CST-14 or GAD65 were counted with brightfield illumination on a Zeiss Axioskop 2 using StereoInvestigator (MBF Bioscience). Twelve coronal sections through the primary motor cortex (M1), each spaced 6 sections apart, were used to quantify the cell populations. Contours for M1 were drawn using a 2.5x air objective. Based on the Paxinos and Watson atlas “The Rat Brain In Stereotaxic Coordinates,” the appropriate contours for the motor cortex were determined by measuring laterally 2 mm from the midline and measured 500 µm wide by the length of the cortex (generally around 2 mm) to ensure that all cortical layers were included (Paxinos et al., 2007). Because the contour that was chosen generally encompassed roughly half or more of the primary motor cortex, it is likely that motor neurons that innervate the hindlimbs and forelimbs were included in the contour (Neafsey et al., 1986). Cells were scored at high magnification using a 63x oil objective. For CST-14, a grid size of 100 µm x 100 µm was used, with a counting frame of 100 µm x 100 µm. The disector height was 10 µm; the guard zones were 2 µm on top and the remainder of the tissue thickness on the bottom. For GAD65, a grid size of 250 µm x 250 µm was used, with a counting frame of 100 µm x 100 µm. The disector height was 10 µm; the guard zones measured 2 µm on top and the remainder of the tissue thickness on bottom. Total M1 cell populations for GAD65 and CST-14 were determined with StereoInvestigator software based on section thickness measured at sites with cells. In order for the cell population estimate to
be counted as a result, the Gundersen coefficient of error \((m=1)\), the error with which StereoInvestigator estimates the cell population, had to be less than 10%.

**Statistical Analyses**

Paired t-tests were used to determine significance between cell population numbers (GAD65 or CST-14) in animals sacrificed at the end of sleep versus animals sacrificed at the end of wake. This sort of statistical analyses was performed because rats were housed and sacrificed in batches of four (one of each condition – see Table 1). In each batch, the animals were littermates and were exposed to all the same conditions. Therefore, using paired t-tests allowed animals to be compared to others within the same batch.
RESULTS

Assessing the sleep/wake cycle

Rats were maintained on a 12 hr light: 12 hr dark cycle that was expected to lead to sleep during the light phase and activity during the dark phase, since rats are nocturnal animals. Activity was monitored with infrared video cameras as a proxy for sleep. Videos of the rats in the housing facility were analyzed the day prior to sacrifice to ensure that activity/inactivity cycles were synchronized with the light/dark cycle. Activity graphs were created for each of the 16 rats that were used in the experiments, based on the movement of the animal during the course of the day. Examples of activity graphs of rats from each of the four conditions are shown below (Figure 2), and the rest are in Supplemental Figures.

Animals with a running wheel showed more distinct sleep/wake cycles than those without (Table 2), as previously reported (Welsh et al., 1988). Both animals with and without a running wheel displayed increased spontaneous activity during the later part of the light phase (7 a.m. to 7 p.m.), which matches previous findings (Borbély et al., 1978). Intermittent phases of inactivity and activity are characteristic of the wake and sleep states, respectively (Franken et al., 1995). No rats displayed abnormal sleep/wake patterns; the majority of them tended to “mask” (cover their heads and curl into a ball) when the lights came on, and then slowly became inactive thereafter. Similarly, when the lights turned off, the rats became active quickly, running on their wheels (if they had one) or burrowing in their cage.
Figure 2: Activity graphs over a 24-hr period for rats from different experimental conditions. Color indicates activity; white indicates inactivity. **A.** Rat sacrificed at the end of sleep without access to a running wheel. **B.** Rat sacrificed at the end of wake without access to a running wheel. **C.** Rat sacrificed at the end of sleep with access to a running wheel. **D.** Rat sacrificed at the end of wake with access to a running wheel.
Table 2: Mean percentage of time spent active during dark and light phases. Rats with a running wheel spent more time active during the dark phase and less time active during the light phase, than those without a running wheel.

<table>
<thead>
<tr>
<th></th>
<th>Mean percentage time spent active during dark phase</th>
<th>Mean percentage time spent active during light phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Running wheel</strong></td>
<td>71.96%</td>
<td>16.15%</td>
</tr>
<tr>
<td><strong>No running wheel</strong></td>
<td>57.45%</td>
<td>21.65%</td>
</tr>
</tbody>
</table>
Scoring the GAD65+ cell population

Rat brain sections were processed for in situ hybridization for GAD65 mRNA to identify the number of GABAergic neurons in the motor cortex under different experimental conditions. GAD65+ cells were counted on sections from animals sacrificed either at the end of sleep or the end of wake. I was blind to all conditions (end of sleep or end of wake, running wheel or no running wheel) when counting.

The GAD65 mRNA probe was highly specific, causing little background staining. Because I was searching for transmitter switching and not changes in the level of GAD65 expression, all cell bodies clearly labeled for GAD65 (regardless of brightness) were counted. There were, however, some stains that could not be well differentiated from background staining or axon terminals, and these were not counted (Figure 3).
Figure 3: Images of in situ hybridization of GAD65 in the motor cortex of rats that did not have access to a running wheel. All cells that displayed a clear GAD65 label, regardless of brightness, were counted (squares). However, there were some stains that were difficult to differentiate between potential background, an axon terminal, or a labeled cell. In those instances, the stain was not counted (circles). A. Animal sacrificed at the end of wake. Cells magnified to 63x in lower right corner. Black arrowhead indicates a clearly labeled cell that was counted in stereological analysis. Unfilled arrowhead indicates potential background, and was not counted in stereological analysis. B. Animal sacrificed at the end of sleep.
Figure 4: Images of in situ hybridization of GAD65 in the motor cortex of rats that utilized a running wheel. 

A. Animal sacrificed at the end of wake. 
B. Animal sacrificed at the end of sleep.
Stereological estimates of the GAD65+ cell population

Cell population numbers estimated by StereoInvestigator (MBF Bioscience), a stereology cell counting system, are presented in Table 3. All estimated cell populations had a Gundersen coefficient of error (m=1) of less than 10%.

Table 3: Mean estimated cell populations of GAD65+ cells for each condition as calculated by StereoInvestigator.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean estimated population of GAD65+ cells in left M1</th>
<th>Mean estimated population of GAD65+ cells in right M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of wake (no running wheel)</td>
<td>46,562</td>
<td>43,082</td>
</tr>
<tr>
<td>End of sleep (no running wheel)</td>
<td>35,792</td>
<td>36,079</td>
</tr>
<tr>
<td>End of wake (with running wheel)</td>
<td>36,924</td>
<td>36,734</td>
</tr>
<tr>
<td>End of sleep (with running wheel)</td>
<td>34,997</td>
<td>34,235</td>
</tr>
</tbody>
</table>

Graphical representations of these data are presented in Figure 5. The differences between end of sleep and end of wake conditions in animals that had access to a running wheel are not significant. However, the differences between conditions in animals that did not have access to a running wheel are pronounced. Animals sacrificed at the end of wake had 30% more GAD65+ cells in the left M1 and 20% more GAD65+ cells in the right M1 than animals sacrificed at the end of sleep. Using a paired t-test, I found the differences between end of wake and end of sleep conditions in animals that did not have access to a running wheel to be statistically significant ($p < 0.05$).
Figure 5: Graphs depicting the mean numbers of GAD65+ cells (±SEM) in the left and right motor cortex of rats. No significant differences were observed between hemispheres. A. GAD65+ cell numbers in rats that did not have access to a running wheel. Significant differences ($p < 0.05$) were observed between animals sacrificed at the end of sleep and animals sacrificed at the end of wake. B. GAD65+ cell numbers in rats that utilized a running wheel. No significant differences between conditions were observed.
Scoring the CST-14+ cell population

Rat brain sections were processed for in situ hybridization for CST-14 mRNA in order to identify the number of cells that express CST-14 in the motor cortex of rats under different experimental conditions. CST-14+ cells were counted on sections from animals sacrificed at the end of sleep or the end of wake. As with the GAD65 counting, I was blind to all conditions (end of sleep or end of wake, and with or without running wheel).

The CST-14 mRNA probe was less specific than the GAD65 probe. There was a great deal of background, and therefore only cells that were clearly and brightly labeled were counted (Figure 6) (Spier et al., 2000).
Figure 6: Images of in situ hybridization of CST-14 in the motor cortex of rats that did not have access to a running wheel. Only cells that were clearly and brightly labeled (squares) were counted to avoid counting stains that could be merely background (circles). A. Animal sacrificed at the end of wake. Cells magnified to 63x in lower right corner. Black arrowhead indicates clearly labeled cell that was counted in stereological analysis. Unfilled arrowhead indicates potential background and was not counted in stereological analysis. B. Animal sacrificed at the end of sleep.
Figure 7: Images of in situ hybridization of CST-14 in the motor cortex of rats that utilized a running wheel. A. Animal sacrificed at the end of wake. B. Animal sacrificed at the end of sleep.
Stereological estimates of the CST-14+ cell population

Cell population numbers were determined by StereoInvestigator software (Table 4 and Figure 8). All estimated cell populations had a Gundersen coefficient of error (m=1) of less than 10%.

Table 4: Mean estimated cell populations of CST-14+ cells for each condition as calculated by StereoInvestigator.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean estimated population of CST-14+ cells in the left M1</th>
<th>Mean estimated population of CST-14+ cells in the right M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of wake (no running wheel)</td>
<td>3,077</td>
<td>3,185</td>
</tr>
<tr>
<td>End of sleep (no running wheel)</td>
<td>2,397</td>
<td>2,434</td>
</tr>
<tr>
<td>End of wake (with running wheel)</td>
<td>3,278</td>
<td>3,210</td>
</tr>
<tr>
<td>End of sleep (with running wheel)</td>
<td>2,878</td>
<td>2,870</td>
</tr>
</tbody>
</table>

No statistically significant differences were observed in the CST-14 cell population between the end of sleep and end of wake. There appears to be a trend toward a greater number of cells expressing CST-14 at the end of wake than the end of sleep, but it is not significant.
Figure 8: Graphs depicting the mean numbers of CST-14+ cells (±SEM) in the left and right motor cortex of rats. No significant differences were observed between hemispheres or between conditions. 

A. CST-14+ cell numbers in rats that did not have access to a running wheel. 

B. CST-14+ cell numbers in rats that utilized a running wheel.
DISCUSSION

GAD65+ cell population

The goal of this project was to test the hypothesis of activity-dependent neurotransmitter respecification in the motor cortex of the adult rat brain in response to sleep/wake cycles. I observed a 20-30% increase ($p < 0.05$) in the number of GAD65+ cells from the end of sleep to the end of wake (Figure 9A). This finding is a launching point for further experiments that can confirm or reject the hypothesis of transmitter switching.

The change in GAD65+ cell population between conditions could be the result of two or more potential mechanisms. One possibility is a circadian fluctuation of GABA during the course of the day. It has been known for years that many genes are regulated by circadian mechanisms. These “clock genes” cycle through patterns of upregulation and downregulation throughout a 24-hr period (Tononi et al., 1995). Could the change I observed in the size of the GAD65+ cell population between end-of-sleep and end-of-wake conditions simply be the result of circadian regulation? Perhaps the increased number of GAD65+ cells at the end of wake are also GAD65+ at the end of sleep, but have such low amounts of transcript due to circadian downregulation that they are not detected by in situ hybridization (Figure 9B).

In my experiments, the light cycle was set such that the lights were on from 7 a.m. to 7 p.m. (sleep phase), and off from 7 p.m. to 7 a.m. (wake phase). Rats were sacrificed at 7 a.m. (after most time spent awake) and 7 p.m. (after most time spent asleep). A simple modification in my experimental protocol could be used to test this circadian hypothesis. The first group of rats could be sacrificed at 7 a.m., and the second group of rats could be sleep deprived through gentle handling until 7 p.m., when they could be sacrificed. This would eliminate sleep as a variable, and isolate the circadian component. If the same difference between conditions that
Figure 9: A. My experimental results show a 20-30% increase in the number of cells that express GABA at the end of wake from the end of sleep condition. B. This could be the result of circadian downregulation, when the same cells are still producing GABA, but at very low levels that are difficult to detect. C. Alternatively, this could be the result of transmitter switching, whereby the same neurons are now producing a different transmitter.

I observed in my experiments were observed in the circadian control experiment, this would indicate that the changes may be more dependent on the time of day than whether the animal was asleep or awake. However, if the differences in GAD65 cell numbers between time points disappeared, this would be consistent with the hypothesis that the sleep/wake cycle is causing a homeostatic transmitter switch.
As mentioned in the Introduction, a true transmitter “switch” involves the downregulation of one gene product accompanied by the upregulation of another. In order to attribute the changes observed in the GAD65+ cell population between sleep and wake to transmitter switching, corresponding changes in another transmitter must be observed (Figure 9C). The homeostatic nature of sleep led to the hypothesis that transmitter switching occurs between glutamate and GABA in cortical neurons. As the amount of time spent awake increases, the need for sleep accumulates and the number of cells that release GABA becomes larger, ultimately causing a sleep state. Similarly, as the amount of time spent asleep increases, the need for activity accumulates and the number of cells that release glutamate becomes larger, ultimately causing arousal (Figure 10). To look for changes in the number of cells expressing glutamate in the motor cortex, one can perform in situ hybridization for VGluT1 and VGluT2, vesicular glutamate transporters in the cortex. If the number of glutamatergic neurons were greater at the end of sleep than the end of wake, this would provide further evidence for a population-level transmitter switch between glutamate and GABA.

Figure 10: This diagram illustrates the homeostatic nature of sleep and how transmitter switching may contribute to it. As we spend more time awake, we move away from an activity-level set point, causing more GABA to be synthesized and released. This ultimately leads to sleep, bringing us back to our activity set point. As we spend more time asleep, we move away from the activity set point, causing more glutamate to be synthesized and released, ultimately leading to wake and bringing us back to the activity set point.
**Cortistatin+ cell population**

Cortistatin expression was included in my project to focus on a subpopulation of GABAergic neurons that have been implicated in sleep, to determine whether changes were occurring within that subpopulation. My data suggest there is a trend toward a greater number of cells expressing CST-14 transcripts at the end of wake than the end of sleep; however, the standard error is large and the differences between conditions are not significant. If the differences in CST-14 between conditions are negligible, this enables an experiment that could produce more convincing evidence for sleep/wake-based transmitter switching between glutamate and GABA. Because GABAergic interneurons make up a large population of the cortex and are implicated in a vast array of activities in the brain, it would be important to identify this homeostatic transmitter switch during the sleep/wake cycle in a fixed subpopulation of GABAergic neurons that has been implicated in sleep onset.

In order to find out whether this is an appropriate experiment, one would first need to determine whether the cortistatin population is constant during the sleep/wake cycle by increasing the number of animals examined. If the population is indeed stable, then one could search for transmitter switching within the CST-14 population. The same experimental set up could be used (light cycle, time of sacrifice, etc); however, instead of staining for GAD65 and CST-14 on separate sections, one could search for colocalization of CST-14 and GAD65 on the same sections. Presuming that the changes follow those that I have observed, a higher number of neurons coexpressing GAD65+ and CST-14+ would be expected at the end of wake than the end of sleep. Using separate brain sections from the same animals, one can look for changes in the number of colocalized VGluT and CST-14+ between sleep and wake. It would be interesting to observe a corresponding increase in glutamatergic neurons at the end of sleep in this CST-14+ population that is known to be involved in sleep onset (Figure 11). If CST-14+ neurons turn out not to be a fixed population throughout the sleep/wake cycle,
another marker of sleep-implicated cells could be used. For example, cortical GABAergic cells that coexpress nNOS express c-fos during sleep, indicating that they are sleep-active (Gerashchenko et al., 2008).

![Figure 11: A diagram showing potential transmitter switching within a specific cortical population that is known to be involved in sleep (cortistatin+ cells).](image)

**Circadian activity and the effect of the running wheel on differences in cell populations**

Activity analysis was included to ensure that rats did not show abnormal sleep/wake patterns that contradicted the light/dark cycle. All rats displayed the majority of activity in the dark phase and the majority of inactivity in the light phase; however, spontaneous bouts of activity and inactivity in sleep and wake phases, respectively, are normal in the adult Sprague-Dawley rat (Franken et al., 1995; Tobler et al., 1994). Unlike humans, no single period of waking or sleeping is observed in the rodent (Yasenkov et al., 2012).

The addition of a running wheel to the rodent cage has been documented in multiple studies to create well-defined sleep and wake states. Rodents that have a running wheel show increased time asleep during the light phase, and increased time awake during the dark phase compared to rodents that do not have access to a running wheel (Vyazovskiy et al., 2006; Welsh et al., 1988). Because rats do not have a single sleep period and a single wake period
but instead display spontaneous activity and inactivity throughout the day, I added running wheels to the cages of half of the animals. As a result, those that were sacrificed at the end of the light period had spent a longer amount of time asleep, and animals that were sacrificed at the end of the dark period had spent a greater amount of time awake. The activity graphs illustrate that animals with a running wheel spent more time active during the dark phase and inactive during the light phase, compared to the animals that did not have access to the running wheel (Table 2).

Given the effect of the running wheel, it was surprising to find that the difference in cell population number of GAD65+ cells between the end of sleep and end of wake conditions nearly vanished when animals were provided a running wheel (Figure 5B). This occurred to a lesser degree in the CST-14+ population (Figure 8B). Confounding effects of the running wheel provide an explanation. Rodents who have access to a running wheel show an elevated number of GABAergic neurons in the motor cortex that correlate with a decreased anxiety level compared to those who do not exercise (Hill et al., 2010). Though my data do not show an increased GAD65+ cell population in animals that had access to a running wheel compared to those who did not, this effect on GABA levels could contribute to the absence of the same differences between sleep and wake in these animals. Activity on the running wheel could affect a population of GABAergic neurons that masks the sleep/wake changes. This concern provides further support for studying transmitter switching in a specific population of GABAergic cells implicated in sleep (Figure 11).

**Sleep deprivation**

Sleep deprivation emphasizes the homeostatic component of sleep, as an increased duration of time spent awake leads to increased sleep intensity (Borbély et al., 1982). Because of the properties of sleep deprivation and the sleep that follows (known as recovery sleep), it
has strong potential for transmitter switching. If transmitter switching between glutamate and GABA is not found between a normal 24 hr-cycle of sleep and wake, the homeostatic sleep drive may not be as strong as it needs to be to induce changes in transmitter expression. Therefore, it would be of interest to examine differences in the size of the GABAergic and glutamatergic cell populations between animals sacrificed at the end of sleep deprivation and animals sacrificed at the end of recovery sleep. Studies differ in the duration of sleep deprivation that is necessary to observe structural and functional changes in neurons; however, changes in GABAergic receptor populations in the basal forebrain can be seen with as little as three hr of sleep deprivation in rats (Modirrousta et al., 2007).

**Conclusions**

My data suggest that the number of GAD65+ neurons in the motor cortex varies significantly between sleep and wake; however, a great deal more work must be accomplished before any conclusions of transmitter switching can be reached. To start, the possibility of circadian regulation of GAD65 must be ruled out, and a corresponding change in another transmitter must be shown. In addition, it would be helpful to show this transmitter switch in a specific population of GABAergic neurons implicated in sleep to avoid confounding effects, such as those seen in animals with the running wheel.

Learning more about where and how transmitter switching occurs in the brain is key to treating disorders caused by deficits or an overabundance of specific transmitters. In the sleep field alone, narcolepsy and insomnia are characterized by their alterations in transmitter levels (Kim et al., 2008; Winkelman et al., 2008). In addition, a variety of disorders of the central nervous system (including those not related to sleep) are the result of altered transmitter levels. The ability for natural stimuli to induce the synthesis of novel transmitters as potential treatment could be much safer than the effects of using pharmacological agents
that target the central nervous system. It is, therefore, highly important that activity-dependent transmitter respecification be further investigated to uncover more about the mechanism of this novel form of brain plasticity.
Figure S1: Activity graphs over a 24-hr period for rats from different experimental conditions. Color indicates activity; white indicates inactivity. A. Rat sacrificed at the end of sleep without access to a running wheel. B. Rat sacrificed at the end of wake without access to a running wheel. C. Rat sacrificed at the end of sleep with access to a running wheel. D. Rat sacrificed at the end of wake with access to a running wheel.
Figure S2: Activity graphs over a 24-hr period for rats from different experimental conditions. Color indicates activity; white indicates inactivity. A. Rat sacrificed at the end of sleep without access to a running wheel. B. Rat sacrificed at the end of wake without access to a running wheel. C. Rat sacrificed at the end of sleep with access to a running wheel. D. Rat sacrificed at the end of wake with access to a running wheel.
Figure S3: Activity graphs over a 24-hr period for rats from different experimental conditions. Color indicates activity; white indicates inactivity. A. Rat sacrificed at the end of sleep without access to a running wheel. B. Rat sacrificed at the end of wake without access to a running wheel. C. Rat sacrificed at the end of sleep with access to a running wheel. D. Rat sacrificed at the end of wake with access to a running wheel.
REFERENCES


Modirrousta M, Mainville L, Jones BE (2007). Dynamic changes in GABA<sub>A</sub> receptors on basal forebrain cholinergic neurons following sleep deprivation and recovery. *BMC Neurosci.* Web. 8:15


