Abstract

Cataplexy is a hallmark of narcolepsy characterized by the sudden onset of muscle weakness or paralysis during wakefulness. It can occur spontaneously but is typically triggered by positive emotions like laughter. Although cataplexy was identified over 130 years ago, its neural mechanism remains unclear. Here, we show that a newly identified GABA circuit within the central nucleus of the amygdala (CeA) may play a causal role in promoting cataplexy. We found that, in narcoleptic mice, chemogenetic activation of GABA cells within the CeA triggered a 253% increase in the number of cataplexy attacks without affecting their duration. We also show that GABA cell activation only promotes cataplexy attacks associated with emotionally rewarding stimuli such as wheel running, without impacting spontaneous attacks. Our results indicate that the CeA plays a pivotal role in controlling cataplexy onset, and that emotionally rewarding stimuli may trigger cataplexy by activating GABA cells in the CeA.
Acknowledgments

My successful completion of this project would not have been possible without the help and support of many individuals. First and foremost, I would like to sincerely thank my supervisor, Dr. John Peever, for giving me the opportunity to pursue graduate studies in his lab. John, you have been a superb mentor who has cultivated my passion for sleep research. You have challenged me intellectually and contributed immensely to my academic and professional development, enabling me to become the scientist I am today.

I would like to thank my supervisory committee, Dr. Richard Horner and Dr. Sheena Josselyn, whose exceptional guidance of and enthusiasm for my work augmented my experience as a graduate student. I would also like to thank Dr. Horner for directly supporting my research through the Sleep and Biological Rhythms Toronto program. Finally, I would like to thank Dr. Kaori Takehara-Nisiuchi and Dr. Richard Stephenson for participating in my oral examination.

I am particularly grateful to my colleagues in the Peever Lab who have provided me with unequalled support and insight and have created a wonderful environment in which to work. Dr. Jimmy Fraigne, Dr. Jennifer Lapierre, and Dr. Nicole Yee, your unparalleled scientific skill and expertise, and your contributions to the lab as whole and my work specifically, have been tremendously helpful and greatly appreciated. Zoltan Torontali, your knowledge and energy have made my lab experience truly memorable and your friendship is valued beyond measure. Sharshi Bulner and Daniel Li, I sincerely thank you both for your help, understanding, and good humor through all we have experienced and accomplished. Simon Lui, Dillon McKenna, Sara Pintwala, and Gabrielle Thibault-Messier, I am grateful for your encouragement and assistance; it has been a pleasure to work alongside you. I also wish to acknowledge the fantastic team of undergraduate students whose hard work has contributed in various ways to my research and would especially like to recognize Victoria Chuen, Shirin Mollaeva, Hinal Patel, Amanda Stojcevski, Aren Thomasian, and Wendy Xie. Thank you as well to all my friends on the third floor.

I would like to thank my sisters, Lindsay and Kimberly, for their unwavering support and pride in my accomplishments, and my parents, Howard and Emily, whose unique perspectives and continuous guidance have colored my experiences in graduate school and beyond.

I would like to express a special thank you to my dear Hayley and the rest of the Eidelman family. Hayley, you have stood by me and supported me through more than I can express and your interest, encouragement, friendship, and love have helped me in every step of my research career. I could not have done this without you.

This work was supported by the Canadian Institutes of Health Research, the Sleep and Biological Rhythms Toronto program, and the Natural Sciences and Engineering Research Council of Canada. Conference attendance was made possible in part by the Canadian Sleep Society, the School of Graduate Studies, and the Department of Cell and Systems Biology.
# Table of Contents

Acknowledgments .................................................................................................................. iii  
Table of Contents .................................................................................................................. iv  
List of Tables ......................................................................................................................... vii  
List of Figures ....................................................................................................................... viii  
List of Appendices ............................................................................................................... ix  
Chapter 1 – Introduction ...................................................................................................... 1  
  1.1 Narcolepsy ...................................................................................................................... 1  
    1.1.1 Overview .................................................................................................................. 1  
    1.1.2 Etiology .................................................................................................................. 2  
    1.1.3 Diagnosis ............................................................................................................... 4  
    1.1.4 Treatment .............................................................................................................. 6  
  1.2 Cataplexy ......................................................................................................................... 7  
    1.2.1 Clinical Manifestation ............................................................................................ 8  
    1.2.2 REM Sleep Intrusion Hypothesis .......................................................................... 12  
    1.2.3 GABA Mechanisms of Cataplexy ....................................................................... 14  
    1.2.4 Other Mechanisms of Cataplexy ....................................................................... 16  
  1.3 The Amygdala ................................................................................................................ 18  
    1.3.1 Anatomy and Connectivity ................................................................................. 18  
    1.3.2 Functions ............................................................................................................. 22  
    1.3.3 The Amygdala and Cataplexy .......................................................................... 23  
  1.4 Current Model of Cataplexy .......................................................................................... 26  
  1.5 Methodological Background ......................................................................................... 28  
    1.5.1 DREADDs ............................................................................................................ 28
4.6 Conclusions

References

Appendix 1 – Behavioral State Analysis Macro
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Diagnostic criteria for narcolepsy</td>
<td>5</td>
</tr>
<tr>
<td>Table 2</td>
<td>Behavioral state architecture in $orexin^{-/-}$ and $orexin^{-/-}$, VGAT-Cre mice</td>
<td>48</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Electrophysiological representation of cataplexy and wakefulness in a narcoleptic human</td>
<td>10</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Common cataplexy triggers in narcoleptic humans</td>
<td>11</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Schematic depicting the major afferent and efferent pathways of the amygdala</td>
<td>21</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Summary of evidence implicating the amygdala in cataplexy</td>
<td>25</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Hypothesized circuit underlying cataplexy</td>
<td>27</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Cre-dependent hM3Dq constructs permit manipulation of specific cell populations</td>
<td>30</td>
</tr>
<tr>
<td>Figure 7</td>
<td>CeA activation exacerbates cataplexy in narcoleptic mice</td>
<td>42</td>
</tr>
<tr>
<td>Figure 8</td>
<td>The behavioral arrests triggered by CeA activation are cataplexy</td>
<td>43</td>
</tr>
<tr>
<td>Figure 9</td>
<td>CeA activation does not alter sleep-wake architecture in wild-type mice</td>
<td>45</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Orexin^{-/-} mice that express Cre in GABA neurons exhibit typical cataplexy</td>
<td>49</td>
</tr>
<tr>
<td>Figure 11</td>
<td>hM3Dq receptors are expressed in GABA cells of the CeA</td>
<td>51</td>
</tr>
<tr>
<td>Figure 12</td>
<td>GABA cells in the CeA promote cataplexy</td>
<td>54</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Activating GABA CeA cells does not influence sleep-wake behavior</td>
<td>55</td>
</tr>
<tr>
<td>Figure 14</td>
<td>GABA cells in the CeA mediate cataplexy associated with rewarding stimuli</td>
<td>58</td>
</tr>
<tr>
<td>Figure 15</td>
<td>The amygdala acts as a “relay centre” between the cortex and the pons that enables positive emotions to elicit cataplexy</td>
<td>63</td>
</tr>
</tbody>
</table>
List of Appendices

Appendix 1  Behavioral State Analysis Macro  82
Chapter 1 – Introduction

1.1 Narcolepsy

Narcolepsy is a serious sleep disorder that affects over 3 million people worldwide (Overeem et al., 2001; Longstreth et al., 2007). It was first described by Dr. Carl Friedrich Otto Westphal in 1877, who treated a patient suffering from daytime hypersomnolence and frequent bouts of muscle weakness or paralysis during wakefulness (Westphal, 1877; Schenck et al., 2007). Dr. Jean-Baptiste-Édouard Gélineau, who encountered an individual experiencing similar symptoms, gave the disorder its name three years later, deriving the term “narcolepsy” from the Greek words for “drowsiness” and “to seize” (Gélineau, 1880; Schenck et al., 2007). These etymological roots highlight the core problem in narcolepsy: a failure by the brain to effectively regulate sleep-wake architecture, or a “disease of state boundary control” (Broughton et al., 1986; Fromherz and Mignot, 2004; Thorpy and Dauvilliers, 2015). This breakdown in control manifests in the symptomatology of narcolepsy, four of which are classically known as the “narcoleptic tetrad”: excessive daytime sleepiness (EDS), cataplexy, hypnagogic hallucinations, and sleep paralysis (Yoss and Daly, 1957; Aldrich, 1993; Overeem et al., 2011).

1.1.1 Overview

EDS, which refers to inescapable feelings of tiredness and/or sleep attacks, and cataplexy, the sudden and involuntary loss of muscle tone during wakefulness despite preserved consciousness, are considered the “core” symptoms of narcolepsy and represent the major complaints of narcoleptic individuals (Overeem et al., 2001; Liblau et al., 2015). These are often accompanied by hypnagogic (or hypnopompic) hallucinations (vivid dreamlike events) and sleep paralysis (muscle atonia despite preserved consciousness), both of which occur during transitions between wakefulness and sleep (Liblau et al., 2015). Many of these symptoms resemble features of rapid eye movement (REM) sleep, which has led to the hypothesis that cataplexy and other symptoms of narcolepsy represent intrusions of REM sleep into wakefulness (Dauvilliers et al., 2014; Fraigne et al., 2015). More recently, disturbed nocturnal sleep has been added as a fifth
symptom of narcolepsy, further underscoring the apparent dysregulation of arousal state that characterizes the disorder (Overeem et al., 2001).

Narcolepsy is closely linked to the loss of orexin-producing neurons in the lateral hypothalamus (Peyron et al., 2000; Thannickal et al., 2000), and the disorder most commonly presents in mid-adolescence or early adulthood (Dauvilliers et al., 2003). The current treatments available for narcolepsy focus primarily on managing symptoms, which persist for life and remain debilitating for those who suffer them (Overeem et al., 2001). Narcoleptic individuals report that their condition substantially limits their performance and progress in work and school, impacts their ability to drive, decreases the quality of their social interactions, and reduces their enjoyment of recreational activities (Broughton et al., 1984). Because narcolepsy has such a pervasive negative influence on patients’ lives, establishing the neurobiological basis of its symptoms is a crucial step towards preventing disease onset in vulnerable individuals or more effectively managing symptoms after they have appeared. Therefore, this thesis project investigated the neural pathway that triggers cataplexy, a major complaint of narcoleptic individuals that appears in approximately 70% of cases (Overeem et al., 2001).

1.1.2 Etiology

The pathophysiological basis of narcolepsy remained a mystery for over 100 years after being described by Westphal and Gélineau. The first clue towards the neurobiological cause of narcolepsy came in 1998 with the discovery of a novel neuropeptide produced in the lateral hypothalamus (LH). This compound was discovered independently by two different groups and so had been given two different names: Sakurai et al., who were studying hypothalamic regulation of feeding behavior, termed the substance “orexin” (from the Greek orexis, “appetite”) (Sakurai et al., 1998), while de Lecea et al. named the peptide “hypocretin” due to its structural similarity to the hormone secretin (de Lecea et al., 1998) (for consistency, this peptide will be referred to as orexin throughout this thesis). Orexin is synthesized exclusively by a population of neurons in the LH (Peyron et al., 1998) and comes in two forms, orexin A and B, which arise from alternative splicing of a common precursor known as prepro-orexin (de Lecea et al., 1998; Sakurai et al., 1998). The orexin peptides bind specifically to two G protein-coupled receptors,
OX₃R and OX₂R, which activate neurons via coupling to the G_q signaling pathway (Sakurai et al., 1998).

Soon after the identification of orexin, additional discoveries were made to suggest that the pathophysiological mechanism responsible for narcolepsy was in fact the loss of an intact orexin system. For instance, the gene responsible for narcolepsy in canine models, canarc-1, was found to be a mutant of OX₂R in which the mRNA transcript is improperly spliced, ultimately rendering the receptor constructed non-functional (Lin et al., 1999). Furthermore, disruption of the orexin system in mice by deleting the genes for the orexin peptides (Chemelli et al., 1999) or receptors (Willie et al., 2003), or by destroying orexinergic neurons postnatally with a slow-acting neurotoxin (Hara et al., 2001), produced phenotypes strikingly similar to narcolepsy in humans. Finally, post-mortem examination of brain tissue derived from human narcoleptics showed a distinct lack of orexin, but not other, neurons in the hypothalamus (Peyron et al., 2000; Thannickal et al., 2000), and orexin levels in the cerebrospinal fluid of living narcoleptic patients is lower than normal or undetectable (Nishino et al., 2000a).

Although these observations strongly suggest the proximal cause of human narcolepsy is the specific loss orexinergic neurons, the reason for this loss is not well understood. A current theory is that narcolepsy has an autoimmune origin, as the majority of narcolepsy patients possess specific genetic markers, such as HLA-DR2 and HLA-DQB1*0602, whose protein products are involved in antigen presentation (Mignot et al., 1994). Furthermore, the narcolepsy incidence among individuals possessing these markers tends to increase after flu epidemics (e.g., H1N1) or widespread vaccinations that activate the immune system (Burgess and Scammell, 2012; Ahmed et al., 2015). Despite these intriguing trends, a molecular mechanism linking the immune system to the degradation of orexin neurons has yet to be identified.

In healthy individuals, orexin neurons project diffusely throughout the brain, innervating not only other areas within the hypothalamus but also the locus coeruleus (LC), thalamus, periaqueductal grey, raphe nuclei, amygdala, cortical areas, and more (Peyron et al., 1998). Orexinergic neurons produce other neurotransmitters as well, such as glutamate (Torrealba et al., 2003), and have been implicated in many functions including stimulating appetite (Sakurai et al., 1999), promoting motor behavior (Hagan et al., 1999), and, importantly in the context of narcolepsy, maintaining arousal and wakefulness (Chemelli et al., 1999; Hagan et al., 1999).
While orexin signaling is higher in wakefulness than in sleep, it is maximal in contexts that often trigger cataplexy in narcoleptic humans or animals such as positive emotion, social interaction, food seeking, exploratory behaviors, and other goal-oriented actions (Lee et al., 2005; Mileykovskiy et al., 2005; Blouin et al., 2013). Moreover, orexin cells project to and strongly excite brainstem areas such as the LC, a region essential for supporting muscle tone during wakefulness (Mileykovskiy et al., 2000; Peyron et al., 2000). Therefore, since narcolepsy is characterized by motor dysfunctions such as cataplexy that do not occur in healthy individuals, orexin may also play a role in coupling arousal with skeletal muscle tone (Mileykovskiy et al., 2005; Liblau et al., 2015). The orexin system, then, is anatomically and functionally well-positioned to prevent narcolepsy and particularly cataplexy, although the details surrounding how orexin normally facilitates arousal and muscle tone are still under study.

1.1.3 Diagnosis

Narcolepsy is generally diagnosed on the basis of EDS and disordered REM sleep phenomena that are not better explained by another factor, as well as cataplexy and/or orexin deficiency (American Academy of Sleep Medicine, 2014). The specific diagnostic criteria for narcolepsy have been revised several times as its symptoms and underlying pathophysiology has become better understood. Initially, narcolepsy had been divided into two subtypes based on whether or not cataplexy was exhibited, but once orexin cell loss had been identified as the cause of narcolepsy, the cataplexy-based categorization became difficult to use in practice since some narcoleptic patients with orexin deficiency do not display cataplexy (Andlauer et al., 2012). Therefore, the most recent edition of the International Classification of Sleep Disorders has redefined the subtypes of narcolepsy to address orexin deficiency rather than the presence of cataplexy: type 1 narcolepsy (with orexin deficiency) and type 2 narcolepsy (without orexin deficiency) (American Academy of Sleep Medicine, 2014) (Table 1). However, orexin deficiency is hard to measure directly since orexin assays are not readily available and require a sample of cerebrospinal fluid (Sateia, 2014). For this reason, and because most narcoleptic patients with orexin deficiency do have cataplexy, the presence of cataplexy remains an important inclusion criterion for type 1 narcolepsy and the main symptom that differentiates it from type 2 narcolepsy (Nishino et al., 2000a; Sateia, 2014).
### Table 1. Diagnostic criteria for narcolepsy

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<thead>
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<th>Type 1 Narcolepsy</th>
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<td>• The patient has daily periods of irrepresible need to sleep or daytime lapses into sleep, occurring for at least 3 months.</td>
<td>• The patient has daily periods of irrepresible need to sleep or daytime lapses into sleep, occurring for at least 3 months.</td>
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<td>• The presence of one or both of the following:</td>
<td>• A mean sleep latency of ≤8 minutes and 2 or more SOREMPs on a MSLT performed according to standard techniques. A SOREMP on the preceding nocturnal PSG may replace one of the SOREMPs on the MSLT.</td>
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<tr>
<td>1. Cataplexy and a mean sleep latency of up to 8 minutes and 2 or more SOREMPs on an MSLT performed according to standard techniques. A SOREMP on the preceding nocturnal PSG may replace one of the SOREMPs on the MSLT.</td>
<td>• Cataplexy is absent.</td>
</tr>
<tr>
<td>2. CSF orexin-A concentration, measured by immunoreactivity, is either up to 110 pg/mL or &lt;1/3 of mean values obtained in normal subjects with the same standardized assay.</td>
<td>• Either CSF orexin-A concentration has not been measured or CSF hypocretin-1 concentration measured by immunoreactivity is &gt;110 pg/mL or &gt;1/3 of mean values obtained in normal subjects with the same standardized assay.</td>
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<td>• The hypersonolence and/or MSLT findings are not better explained by other causes such as insufficient sleep, obstructive sleep apnea, delayed sleep phase disorder, or the effect of medication or substances or their withdrawal.</td>
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**Abbreviations:** CSF, cerebrospinal fluid; MSL, multiple sleep latency test; PSG, polysomnogram; SOREMPs, sleep-onset REM periods. Criteria based on the *International Classification of Sleep Disorders* (3rd ed.)
1.1.4 Treatment

There is currently no cure for narcolepsy, but there are many treatments available to help patients manage their symptoms. In many cases, improving “sleep hygiene” behaviorally by imposing a structured nap schedule can help individuals alleviate their sleepiness (Overeem et al., 2001). More often, however, sleepiness is controlled medicinally through the use of stimulants and wake-promoting agents such as amphetamines, methylphenidate, or modafinil, which elevate levels of arousal-promoting monoamines in the brain (Overeem et al., 2001; Leschziner, 2014). Modafinil (and its enantiomer armodafinil) in particular is favored because it is not addictive and has few serious side effects in contrast to the more classical stimulants (De la Herran-Arita and Garcia-Garcia, 2013).

While certain stimulant drugs have some efficacy in combating cataplexy in addition to EDS, specific anticatatonic medications are often prescribed, and these often help reduce hypnagogic hallucinations and sleep paralysis as well (Overeem et al., 2001). Tricyclic antidepressants (e.g., clomipramine), selective serotonin reuptake inhibitors (SSRIs) (e.g., fluoxetine), and serotonin/noradrenaline reuptake inhibitors (SNRIs) (e.g., venlaxafine) elevate monoamine levels in the brain and are thought to improve cataplexy by inhibiting REM sleep (Overeem et al., 2001; De la Herran-Arita and Garcia-Garcia, 2013; Leschziner, 2014). Monoamine oxidase inhibitors (MAOIs) such as selegeline hydrochloride accomplish the same but have fallen out of favor due to their harsh side effects (Dauvilliers et al., 2014). Sodium oxybate (or gamma-hydroxybutyrate, GHB) is a hypnotic that functions as a GABA<sub>B</sub> receptor agonist and also modulates dopamine signaling (Dauvilliers et al., 2014). It has gained traction as a holistic treatment for narcolepsy as it targets both EDS and cataplexy in addition to fragmented nocturnal sleep (De la Herran-Arita and Garcia-Garcia, 2013), and is regarded as a good alternative to classical stimulants and tricyclic antidepressants due to relatively mild side effects (Overeem et al., 2001; Dauvilliers et al., 2014). However, it is not always the preferred medication as it must be taken twice throughout the night due to its short half-life, and its higher cost combined with its potential for abuse limit its availability in some populations (Leschziner, 2014).

More recently, attention has been focused on novel therapies geared towards preventing disease progression rather than simply targeting symptoms. For instance, there has been
experimentation with intravenous immunoglobulin or plasmapheresis soon after narcolepsy onset aimed at counteracting autoimmune destruction of orexin neurons, although results surrounding the effectiveness of these treatments have been inconsistent (Plazzi et al., 2008; Valko et al., 2008; Dauvilliers et al., 2014). Enhancing histamine release via inverse agonists of the H3 receptor is another area currently under investigation and recent work indicates this strategy is useful for treating narcoleptic mice and humans (Lin et al., 2008). Finally, orexin peptide replacement therapy, orexin gene therapy, and synthetic orexin receptor agonists have also been the subject of much research. Although intravenous or intraventricular administration of the orexin peptide or transduction of existing neurons with the orexin gene show promise in animal models of narcolepsy, there are difficulties with translating this approach to humans (John et al., 2000; Mieda et al., 2004; Liu et al., 2008).

1.2 Cataplexy

The hypothesis that narcolepsy represents a loss of state boundary control was first put forth by Broughton et al. in 1986 based on several observations (Broughton et al., 1986). Electroencephalographic (EEG) recordings of individuals performing a vigilance task showed that whereas healthy subjects maintained a constant state of wakefulness throughout, narcoleptics continuously fluctuated between wakefulness and light sleep (Valley and Broughton, 1983). Additionally, narcoleptics who were administered the multiple sleep latency test entered non-REM (NREM) sleep with substantially decreased latency or even bypassed NREM sleep entirely and entered REM sleep directly instead (Broughton et al., 1986). However, while narcolepsy as a whole may involve inappropriate transitions between all arousal states, emphasis has been placed on the notion that dissociated REM sleep specifically underlies most symptoms of narcolepsy, including cataplexy (Dauvilliers et al., 2007; Siegel, 2011; Dauvilliers et al., 2014). As the focus of this thesis is on cataplexy, the following section will examine the neurobiology of this symptom in detail, including discussion of its clinical features, the hypothesized involvement of REM sleep circuitry, and the neuroanatomical and neurochemical substrates that mediate it.
1.2.1 Clinical Manifestation

Cataplexy refers to the abrupt, involuntary loss of muscle tone bilaterally during wakefulness despite preserved consciousness (Fig. 1). It is a pathognomonic symptom of narcolepsy and represents part of the diagnostic criteria of the disease (Dauvilliers et al., 2003; Dauvilliers et al., 2014). Narcolepsy manifests most often in the mid-teens or mid-30s, and although most patients present first with complaints of EDS (often accompanied by sleep paralysis and/or hypnagogic hallucination), three-quarters eventually develop cataplexy as well (Guilleminault et al., 1974; Overeem et al., 2001). Cataplexy sometimes presents at the same time as EDS, but it more often appears years later, and tends to improve with age (Dauvilliers et al., 2003; Mattarozzi et al., 2008).

Cataplexy can affect all striated muscles, although those that control eye movements and respiration tend to be affected least, while those in the face, neck, and legs tend to be affected most (Overeem et al., 2001; Dauvilliers et al., 2014). Individual attacks can involve a partial loss of muscle tone leading to slurred speech, head drooping, and weakness in the extremities, or complete muscle paralysis that causes full postural collapse (Dauvilliers et al., 2014). Unfortunately, partial cataplexy is often ignored by patients and overlooked by physicians, which may contribute to a delay in the diagnosis of narcolepsy until cataplexy attacks become more severe years later (Dauvilliers et al., 2014). Cataplexy episodes typically last for seconds but may persist for minutes, and may occur several times per month or year or multiple times a day (Gelb et al., 1994). Rarely, some narcoleptic individuals experience an attack that persists for several hours, known as status cataplecticus, which most often occurs after withdrawal from anticataplectic medication (Dauvilliers et al., 2003). Despite its abrupt onset, cataplexy rarely causes injury to narcoleptics since they often sense an attack before it begins and either resist its onset or orient themselves into a safer position before losing muscle tone (Overeem et al., 2011; Dauvilliers et al., 2014). Finally, there are autonomic and spinal reflex changes associated with cataplexy, such as decreased heart rate, brief periods of apnea, absent sympathetic skin responses, and suppression of the H-reflex (Dauvilliers et al., 2014).

Cataplexy may occur spontaneously but most often is precipitated by an emotional trigger. A questionnaire study conducted by Overeem et al. in a population of Dutch narcoleptic patients identified excited laughter as the most common trigger, followed by making a sharp-minded
remark, telling a joke or anticipating its punch line, and being tickled (Overeem et al., 2011) (Fig. 2). The observation that the most common triggers are specifically humorous in nature suggests that cataplexy may be an extreme version of “feeling weak with laughter”, which results from a suppression of the H-reflex (Overeem et al., 1999). Although the emotions that precipitate cataplexy are typically positive, negative emotions such as anger, fear, or pain have been documented as triggers for some cataplexy attacks as well (Overeem et al., 2011). Since strong emotions so often produce cataplexy, it is hypothesized that brain areas involved in processing emotions, such as the amygdala, are part of the neural pathway underlying cataplexy (Dauvilliers et al., 2014; Fraigne et al., 2015). Therefore, the major focus of this thesis is on the role the amygdala, and particularly its central nucleus (CeA), plays in triggering cataplexy attacks.
Figure 1. Electrophysiological representation of cataplexy and wakefulness in a narcoleptic human. In narcoleptic humans, cataplexy (left) is characterized by the abrupt, involuntary loss of skeletal muscle tone during wakefulness. A, Electroencephalographic recordings during cataplexy are typically rich in theta frequencies (4-8 Hz) and may resemble brain activity during REM sleep, whereas wakefulness (right) is characterized by a mix of different frequencies. B-E, Electromyographic recordings demonstrate dramatic decreases in muscle tone during cataplexy in jaw, shoulder, calf, and thigh muscles that sharply contrast the basal level of muscle tone during wakefulness. F, A unique feature of cataplexy is the loss of the H-reflex, which otherwise only occurs during REM sleep. Modified from Guilleminault et al, 1974.
Figure 2. **Common cataplexy triggers in narcoleptic humans.** A group of narcoleptic individuals who experience cataplexy ($n=109$) were surveyed regarding behaviors and emotions that preceded their cataplexy attacks. The chart above depicts the percentage of individuals who responded they always, often, sometimes, or never experienced cataplexy following each of the triggers listed. Note that the most common triggers of cataplexy are associated with positive emotions (green), with excited laughter eliciting cataplexy in over 90% of respondents, while neutral (yellow) and negative (red) triggers occur less often. *Modified from Overeem et al, 2011.*
1.2.2 REM Sleep Intrusion Hypothesis

Although cataplexy is an especially debilitating symptom of narcolepsy, the brain circuits that cause this symptom to occur have not yet been identified. The defining feature of cataplexy is muscle atonia, a phenomenon that otherwise only occurs during REM sleep, and cataplexy, like REM sleep, is typified by theta-rich EEG activity and loss of the H-reflex (Guilleminault et al., 1974; Peever, 2011; Dauvilliers et al., 2014). Furthermore, sleep paralysis is similar to REM sleep atonia, hypnagogic hallucinations may reflect the vivid dreaming associated with REM sleep, and sleep attacks often progress into REM sleep after substantially reduced latency (sleep-onset REM periods, SOREMPs) (Vogel, 1960; Rechtschaffen et al., 1963; Aldrich, 1993; Dauvilliers et al., 2003). Finally, some narcoleptic patients report experiencing hypnagogic hallucinations during cataplexy, while others transition from cataplexy into REM sleep before resuming normal wakefulness (Vetrugno et al., 2010). These observations have led to the hypothesis that many symptoms of narcolepsy, and cataplexy in particular, are a consequence of the inappropriate intrusion of REM sleep atonia into wakefulness (Dauvilliers et al., 2003; Peever, 2011; Fraigne et al., 2015).

From a neurocircuitry standpoint, REM sleep is thought to be generated by a “flip-flop switch” mechanism whereby a series of mutually inhibitory REM-on and REM-off regions in the brainstem tightly control transitions into and out of this state (Lu et al., 2006). A characteristic feature of REM sleep is muscle atonia, which occurs due to active inhibition and disfacilitation of spinal motoneurons (Peever, 2011). A core component of the REM-on nuclei, the subcoeruleus region (SubC, or sublaterodorsal tegmentum), is important for actively inhibiting motoneurons (Boissard et al., 2002; Lu et al., 2006). The SubC contains a large population of glutamatergic cells that send excitatory projections to GABA/glycine neurons in parts of the ventral medial medulla (VMM) such as the ventral gigantocellular reticular nucleus (GIG), which directly inhibit spinal motoneurons and so suppress muscle tone (Lu et al., 2006; Clement et al., 2011; Brooks and Peever, 2012). Disfacilitation simultaneously occurs because spinal motoneurons lose excitatory inputs originating from monoaminergic regions in the brainstem such as the noradrenergic LC (Peever, 2011; Burgess and Peever, 2013; Dauvilliers et al., 2014). During wakefulness, orexin neurons help stabilize REM-off areas such as the ventrolateral periaqueductal grey (vIPAG, which inhibits the SubC), LC, dorsal raphe nucleus (DRN), and
lateral pontine tegmentum (LPT), and in this way are able to indirectly inhibit REM-on areas and support muscle tone (Peyron et al., 1998; Boissard et al., 2003; Lu et al., 2006; Burgess and Peever, 2013). Consequently, the loss of orexin neurons in narcolepsy may weaken the REM-off half of the flip-flop switch, predisposing the REM-on half to being inappropriately recruited and producing phenomena such as cataplexy (Dauvilliers et al., 2014; Fraigne et al., 2015).

There is substantial evidence suggesting physiological similarity between REM sleep and cataplexy. For instance, many brain areas show similar patterns of activity during both these states in humans (Maquet et al., 1996; Hong et al., 2006). In the narcoleptic dog, atonia-promoting neurons in the VMM, as well as cells in the CeA, were shown to have maximal activity during both REM sleep and cataplexy (Siegel et al., 1991; Gulyani et al., 2002). The LC, which helps maintain muscle tone during wakefulness, is virtually silent during both REM sleep and cataplexy (Wu et al., 1999). Elevating monoamine availability by pharmacological means such as amphetamines, tricyclic antidepressants, and MAOIs suppress both REM sleep and cataplexy (Mayer et al., 1995; Dauvilliers et al., 2007; Burgess et al., 2010). Cataplexy and REM sleep are also characterized by a predomination of theta wave activity in the EEG that peaks at \( \sim 7 \) Hz (although in cataplexy, there is more “slow” theta activity in the 4-6 Hz range) (Vassalli et al., 2013).

Notably, however, there have been a number of observations that underscore differences between cataplexy and REM sleep. For instance, some neurons in the VMM that are active during REM sleep are silent during cataplexy (Siegel et al., 1991). The SubC is only half as active during cataplexy as it is during REM sleep (Siegel et al., 1992). Wake-on/REM-off serotonin neurons in the DRN have an intermediate level of activity during cataplexy that is similar to that of NREM sleep (Wu et al., 2004). Saporin lesions of REM-off neurons in the vIPAG of narcoleptic mice increase the number of REM sleep bouts without a concomitant increase in cataplexy (Kaur et al., 2009). Also, cataplexy is associated with preserved consciousness, which may be related to sustained histaminergic activity that is absent during REM sleep (John et al., 2004). Finally, REM sleep is homeostatically regulated and has a defined ultradian rhythm, even in narcolepsy, whereas cataplexy can be triggered at any time and is not confined to a specific diurnal pattern (Nishino et al., 2000b).
In summary, it appears that most parallels between cataplexy and REM sleep relate to brain areas or neurotransmitter systems involved in regulating muscle tone. The differences, in contrast, seem to be associated with other parameters, such as where these states originate in the brain or whether environmental awareness is preserved. These mixed observations suggest that cataplexy, despite being similar in many respects to REM sleep, is not the same state per se. Rather, the neural circuits responsible for generating cataplexy and REM sleep are likely to be distinct, but the pathways responsible for certain elements of these states, such as those that produce muscle atonia, may overlap. Thus, the model of cataplexy examined in this thesis is based on the hypothesis that cataplexy shares the same circuits that are specifically responsible for muscle atonia during REM sleep (see Section 1.4).

1.2.3 GABA Mechanisms of Cataplexy

The previous section addressed the hypothesis that cataplexy represents an intrusion of REM sleep into wakefulness. The flip-flop switch model of REM sleep generation is based largely on mutually inhibitory GABA signaling between different regions in the brainstem, and a major source of muscle atonia during REM sleep is the GABA-mediated inhibition of motoneurons (Brooks and Peever, 2008, 2011; Peever, 2011; Brooks and Peever, 2012). Furthermore, REM-off regions of the brainstem are themselves under GABAergic regulation from more rostral areas such as the CeA (Burgess et al., 2013). Although the GABAergic regulation of REM sleep has been well-characterized thus far, little work has been done to date on GABA mechanisms in cataplexy. However, if cataplexy shares neural circuits with REM sleep, GABA is likely to play an important role in cataplexy as well. Therefore, studying GABAergic signaling in the context of cataplexy is not only likely to provide additional insights into the pathophysiology of this symptom, but also permit additional comparisons between cataplexy and REM sleep. This section will focus on GABA mechanisms of cataplexy as the cell population of interest in this study is GABAergic, with reference to REM sleep circuitry as well.

Several GABAergic brain areas important in REM sleep control have also been implicated in cataplexy. For example, GABAergic neurons in the VMM are highly active during both REM sleep and cataplexy (Lai and Siegel, 1988; Siegel et al., 1991). Since excitotoxic lesions of the
VMM in healthy cats produce REM sleep without atonia (Schenkel and Siegel, 1989), inhibition of this region may help rescue muscle tone during cataplexy if it shares the same atonia-generating pathways as REM sleep. The vlPAG is another brainstem area involved in REM sleep and potentially cataplexy. It contains a significant number of GABA cells, some of which are REM-on and may inhibit areas that promote arousal and muscle tone, and others which are REM-off and thought to inhibit other REM-on nuclei, such as the SubC, outside of REM sleep (Lu et al., 2006; Sapin et al., 2009; Luppi et al., 2012; Fraigne et al., 2015). REM-on GABA cells of the vlPAG strongly inhibit the LC, which normally supports muscle tone and is silent during REM sleep and cataplexy (Gervasoni et al., 1998; Wu et al., 1999; Mileykovskiy et al., 2000). Moreover, administration of the GABA_A receptor agonist muscimol increases REM sleep and produces SOREMPs reminiscent of those seen in narcolepsy (Sapin et al., 2009). However, saporin lesions of the vlPAG in narcoleptic mice increase REM sleep without altering cataplexy, but this may be due to the activity-independent nature of the lesioning (i.e., vlPAG cells were ablated regardless of whether they were REM-on or REM-off) and that lesions were not restricted exclusively to GABA cells (Kaur et al., 2009).

Moreover, GABAergic forebrain areas such as the hypothalamus and amygdala may be involved in the control of cataplexy. Recent evidence suggests that melanin-concentrating hormone (MCH) neurons in the hypothalamus, which are active during REM sleep, also co-release GABA onto targets in the brainstem that include the REM-off vlPAG, GABAergic LPT (which inhibits the SubC), and the monoaminergic nuclei (Boissard et al., 2003; Luppi et al., 2011; Jego et al., 2013). It has been hypothesized that because MCH neurons promote REM sleep, they may contribute to the symptoms seen in narcolepsy by inhibiting REM-off circuitry, particularly because, in narcolepsy, they are unopposed by the wake-promoting actions of orexin neurons (Hassani et al., 2009). Like hypothalamic MCH neurons, the amygdala is naturally active during REM sleep (Maquet et al., 1996; Nofzinger et al., 1997), and unit recordings in the CeA, which is largely GABAergic, have identified cells that are active during cataplexy and REM sleep (Gulyani et al., 2002). Importantly, it has recently been shown that lesions of the CeA alleviate cataplexy in narcoleptic mice (Burgess et al., 2013). Because CeA neurons also project to the REM-off regions referred to above, they are positioned to produce muscle atonia, especially if they are strongly activated by emotions (Dauvilliers et al., 2014).
Finally, GHB, the most effective drug currently available to treat cataplexy and other aspects of narcolepsy in humans, may act through a GABA mechanism. It is thought that GHB weakly agonizes GABA_B receptors on interneurons in dopaminergic regions, causing disinhibition of dopamine cells and facilitating both muscle tone and arousal (Mamelak, 2009; Kohlmeier et al., 2013a). Furthermore, a specific GHB receptor has been identified that may be related to GABA_A receptors, and activation of the GHB receptor is thought to reduce the incidence of cataplexy by dampening hypersensitive autoinhibition in the LC (Szabadi, 2015). Administering GHB to narcoleptic mice in a regimen similar to that in humans produced a significant decrease in the amount of cataplexy they experienced, including a decrease in cataplexy density (episodes per hour of wakefulness) (Black et al., 2014). Baclofen, a specific antagonist of the GABA_B receptor, produces even greater reductions in cataplexy, although its particular influence on the circuits that produce cataplexy has yet to be elucidated (Cruz et al., 2004; Black et al., 2014).

1.2.4 Other Mechanisms of Cataplexy

Many other neurotransmitter systems have been studied in the context of cataplexy, particularly those already linked to arousal such as noradrenaline, dopamine, serotonin, and acetylcholine. The noradrenergic locus coeruleus has received a great deal of attention in the study of cataplexy because it facilitates muscle tone during wakefulness (via an α_1-receptor mechanism) but is silent during REM sleep (Fung et al., 1991; Takahashi et al., 2010). Interestingly, the locus coeruleus ceases firing with the start of a cataplexy attack and resumes firing at its end, suggesting that loss of muscle tone may be due, at least in part, to a removal of the LC-mediated noradrenergic drive onto motoneurons (Wu et al., 1999). The importance of the noradrenergic system in cataplexy is underscored by past work wherein α_1-receptor agonism decreased the number of cataplexy episodes while α_1-receptor antagonism exacerbated them in narcoleptic dogs and mice, and that application of α_1-receptor agonists in the middle of a cataplexy episode is capable of rescuing muscle tone (Mignot et al., 1988; Burgess and Peever, 2013). Because the LC is a target of dense excitatory orexinergic innervation, the loss of orexin cells in narcolepsy may underlie LC hypoactivity during cataplexy (Burgess and Peever, 2013).
These observations may also explain why some of the most effective anticatatplectic medications are also SNRIs.

Dopamine is another neurotransmitter positioned to mediate cataplexy and other elements of narcolepsy, particularly because it already has established roles in modulating emotion and reward (Kvetnansky et al., 2009). The dopaminergic system is perturbed in narcolepsy as evidenced by D₂ receptor expression and activity, which is elevated in narcoleptic canines and humans (Bowersox et al., 1987; Eisensehr et al., 2003). Drugs used to combat cataplexy and EDS (e.g., modafinil and GHB) are thought to act via dopamine mechanisms (Schmidt-Mutter et al., 1999; Wisor et al., 2001; Wisor and Eriksson, 2005). Past studies have also demonstrated that modulating activity of D₁ receptors specifically influences sleep attacks, while altering activity of D₂ receptors specifically affects cataplexy (Burgess et al., 2010). More recently, work from our lab has identified the dopaminergic A11 region of the hypothalamus as important in coupling arousal and muscle tone, as semi-chronic optogenetic stimulation of this region reduces cataplexy, while specific stimulation of this region during cataplexy rescues muscle tone within seconds (Dr. Jimmy Fraigne, unpublished observations). These studies also revealed that the A11 projects to motoneurons (e.g., the trigeminal motor pool) and the CeA, and therefore possesses requisite anatomical connections to control cataplexy.

Serotonin has been studied in cataplexy due to its relationship with promoting arousal like other monoamines. In contrast to noradrenaline and dopamine, however, work done on serotonergic signaling in cataplexy has yielded mixed results. A major source of serotonin in the brainstem, the DRN, decreases its firing activity during cataplexy, but not to the same extent as REM sleep (Wu et al., 2004). In narcoleptic canines, serotonin receptor agonists greatly reduce cataplexy and influence behavior, but serotonin receptor antagonists do not exacerbate cataplexy (Nishino et al., 1995). These results led the authors to conclude that the anticatatplectic effects observed were more likely due to the changes in behavior induced by serotonin activity, rather than mediated through the serotonin receptor itself. Despite this, SSRIs are used to manage cataplexy, even though their specific method of action in narcolepsy is still unknown (Godbout and Montplaisir, 1986; Lopez and Dauvilliers, 2013).

The cholinergic system has had longstanding role in facilitating REM sleep (McCarley, 2004; Lu et al., 2006), and work over the last few decades have provided evidence of its
involvement of cataplexy as well. For example, the food-elicited cataplexy test produces pontine spikes in extracellular acetylcholine in narcoleptic canines (Reid et al., 1994a). Acetylcholinesterase inhibitors and muscarinic receptor agonists increase the frequency of cataplexy episodes in narcoleptic mice and canines (Reid et al., 1994b; Kalogiannis et al., 2011). These effects may arise due to glutamatergic potentiation at or direct activation of the SubC by nearby cholinergic nuclei such as the pedunculopontine tegmentum (PPT) and laterodorsal tegmentum (LDT), since optogenetic activation of the PPT/LDT induces REM sleep, and the SubC is activated by cholinergic agonists such as carbachol (Torontali et al., 2014; Weng et al., 2014; Van Dort et al., 2015).

1.3 The Amygdala

The amygdala refers to a structurally and functionally diverse group of nuclei in the temporal lobe that receive inputs from and send outputs to an extensive variety of brain regions (Swanson and Petrovich, 1998). The amygdala is classically associated with emotion processing and emotional behavior, particularly in the context of fear, although over the last few decades it has become increasingly apparent that the amygdala processes many other emotions and is an important component of many other brain circuits (LeDoux, 2007; Murray, 2007; Janak and Tye, 2015). In narcoleptic individuals, strong positive emotions most commonly precipitate cataplexy, and the brain areas responsible for this are now being elucidated. The amygdala in particular has become a target of study in this context due to its role in processing emotional stimuli, and there is now evidence to suggest that one of its nuclei, the CeA, is especially important in triggering cataplexy (Burgess et al., 2013). The following sections will give a brief overview of amygdala anatomy and function, followed by its association with cataplexy, with special reference made to the CeA, the brain area of interest in this thesis project.

1.3.1 Anatomy and Connectivity

Although the specific divisions of the amygdala and their nomenclature have historically been a point of contention, the region can be subdivided into three sets of nuclei based broadly
on similarities in their histological features and developmental origins: the basolateral group, the cortical-like group, and the centromedial group (Sah et al., 2003; LeDoux, 2007). The basolateral and centromedial groups have been studied in the most detail and the former typically refers to the lateral, basolateral, and basomedial nuclei, whereas the latter is commonly associated with the central and medial nuclei (Sah et al., 2003).

Despite the vast array of intrinsic and extrinsic projections the amygdala possesses, some general statements can be made about its anatomical connectivity with other brain areas and within its nuclei, in addition to the functional importance of these connections. The basolateral group is regarded as the input region of the amygdala, with the lateral nucleus specifically functioning as a “gatekeeper” where most sensory information from the cortex and thalamus relating to vision, audition, olfaction, gustation, and somatosensation arrive (Pitkanen et al., 1997; Sah et al., 2003; LeDoux, 2007) (Fig. 3). Nuclei in the basolateral group are densely interconnected and many neurons within these nuclei ultimately send projections to the centromedial group (Pitkanen et al., 1995; Savander et al., 1995, 1996; Janak and Tye, 2015). Although the centromedial group is considered the primary output region of the amygdala, it also receives inputs from the hypothalamus and brainstem that relay autonomic and behavioral information (Sah et al., 2003).

Most amygdala nuclei send reciprocal projections to areas where their afferent inputs originate, although there are several salient connections that should be noted. The lateral nucleus has some direct connections with the centromedial group, but it more often communicates via a polysynaptic pathway that involves either the basolateral nucleus or the intercalated cell group, a population of inhibitory interneurons that lie in the fibrous zone separating the basolateral nucleus from the CeA (LeDoux, 2007). The basolateral group extensively innervates areas associated with memory, including the hippocampus and perirhinal cortex, as well as the prefrontal cortex and nucleus accumbens, through predominantly glutamatergic projections (Sah et al., 2003).

The centromedial group, and particularly the CeA, densely innervates the hypothalamus, bed nucleus of the stria terminalis, and brainstem regions that regulate the autonomic system and muscle tone, which collectively produce the physiological and behavioral responses to various emotions (Davis, 1992; Sah et al., 2003) (Fig. 3). The CeA also projects to areas that mediate
arousal and muscle tone such as the LC and DRN (Sah et al., 2003). In contrast to the basolateral group, extrinsic projections of the centromedial group are predominantly GABAAergic in nature (Nitecka and Ben-Ari, 1987; McDonald and Augustine, 1993; Pitkanen and Amaral, 1994). On the basis of cytoarchitecture, the CeA has been further subdivided into as many as four interconnected parts, although in practice, reference is made primarily to lateral and medial cell groups (McDonald, 1982; Jolkkonen and Pitkanen, 1998). Neurons in the CeA are chiefly medium spiny neurons, which form some local synapses but eventually project elsewhere in the brain (Hall, 1972; McDonald, 1982). Immunohistochemical evidence indicates that other peptides (e.g., enkephalin, neurotensin, somatostatin, and corticotropin releasing hormone) are differentially co-expressed in the GABA cells of the CeA (Moga and Gray, 1985; Cassell et al., 1986; Cassell and Gray, 1989), and the various combinations of peptides expressed in each neuron may point to distinct functions those neurons subserve (Davis, 1992; Sah et al., 2003). Moreover, the results of some studies point to a separate, albeit small, population of glutamatergic CeA neurons (Boissard et al., 2003; Xi et al., 2011).
Figure 3. Schematic depicting the major afferent and efferent pathways of the amygdala. Most sensory information from the cortex and thalamus enter the amygdala through nuclei in the basolateral group, although axons originating in the brainstem and hypothalamus more often terminate in the centromedial group. The basolateral group possesses reciprocal connections with many areas from which it receives afferents. However, most information leaves the amygdala via the central nucleus of the centromedial group, which coordinates physiological and behavioral responses to various stimuli. B, basolateral nucleus; Ce, central nucleus; itc, intercalated cell group; La, lateral nucleus; M, medial nucleus. 

*Modified from LeDoux, 2007.*
1.3.2 Functions

The amygdala is part of a network of brain structures that roughly encircle the thalamus that are collectively called the limbic system (Nakano, 1998). This network is important for the processing of and reactions to emotional stimuli, as well as related functions including emotional memory, arousal, and motivation (Janak and Tye, 2015). To help facilitate these roles, the amygdala in particular is inhibited by local circuits to keep it relatively quiescent so that it responds only to strong, novel stimuli (LeDoux, 2007). The amygdala has historically been associated with fear specifically since initial studies of mice, monkeys, and humans with lesioned amygdalae revealed reduced aggression, fear, and anger, and an inability to associate stimuli with their emotional content (Sah et al., 2003; Janak and Tye, 2015). Furthermore, fear conditioning paradigms provide a relatively uncomplicated and direct way of examining the amygdala in vivo because fear produces a variety of quantifiable changes in behavior and physiology (e.g., elevated blood pressure and heart rate, release of adrenaline and corticosterone, freezing behavior, etc.) (Davis, 1992; Sah et al., 2003). Importantly, however, the amygdala plays a significant role in processing many emotions in addition to fear, including reward and anxiety, and its influence extends beyond emotion into memory, addiction, olfaction and other senses, learning-related plasticity, autonomic motor behavior, and more (Swanson and Petrovich, 1998; Murray, 2007; Janak and Tye, 2015). Strong positive emotions represent the most common trigger for cataplexy, so the role the amygdala plays in processing emotions is emphasized here.

Based on fear conditioning studies, the amygdala has been modeled as an essential structure for quickly detecting aversive external stimuli and producing adaptive responses to them (Davis, 1992). Indeed, electrically stimulating the amygdala produces fear responses in humans and animals (Kaada, 1951; Chapman et al., 1954; Davis, 1992). Detailed examinations of the lateral nucleus revealed many cells that were strongly activated by negative conditioned stimuli, and optogenetic activation of these neurons can take the place of either a conditioned or unconditioned stimulus and produce freezing behavior in rats that typifies the rodent fear response (Quirk et al., 1995; Johansen et al., 2010; Nabavi et al., 2014). Moreover, lesioning all or part of the amygdala attenuates fear-induced behaviors and produces deficits in fear-motivated learning (Blanchard and Blanchard, 1972; Hitchcock and Davis, 1986; Vazdarjanova et al., 2001).
However, there is growing appreciation that the amygdala is important for processing and acting on positive emotions as well. For example, neuroimaging studies in humans have shown that the amygdala is activated by positive emotions in addition to negative emotions (Breiter et al., 1996; Garavan et al., 2001). Neurons in the rat amygdala become excited during anticipation of reward (Schoenbaum et al., 1998; Tye and Janak, 2007), and neurons within the monkey amygdala fire in response to positive stimuli of various modalities (Nishijo et al., 1988; Paton et al., 2006). Lesions within the amygdala weaken reward-based behavior such as conditioned place preference using amphetamine or food-based positive reinforcement (Gallagher et al., 1990; Hiroi and White, 1991). Because the amygdala reacts to both subjectively positive and subjectively negative stimuli, some have hypothesized that this region is important in processing the intensity of certain emotions, rather than exclusively their affective valence (Breiter et al., 1996; Garavan et al., 2001; Costa et al., 2010; Bonnet et al., 2015).

1.3.3 The Amygdala and Cataplexy

As mentioned previously, cataplexy is often precipitated by strong positive emotions such as laughter and joking (Overeem et al., 2011). The amygdala, by virtue of its role in processing emotion, is therefore an important target of cataplexy research, and there is already much evidence linking this brain region to the symptom. The amygdala is anatomically well-positioned to produce muscle paralysis during cataplexy as its main output nucleus, the CeA, has extensive inhibitory projections to brainstem regions that promote muscle tone such as the LC and vIPAG (Burgess et al., 2013; Dauvilliers et al., 2014; Fraigne et al., 2015). The CeA may also possess direct excitatory projections to the SubC, which is important for generating muscle atonia during REM sleep (Hopkins and Holstege, 1978; Boissard et al., 2003; Lu et al., 2006; Xi et al., 2011).

Brain imaging studies conducted in narcoleptic individuals have found that the amygdala response to humorous or rewarding stimuli is higher than that of healthy controls (Schwartz et al., 2008; Ponz et al., 2010). Measurements of cerebral perfusion changes during cataplexy reveal hyperperfusion in the amygdala for the duration of cataplexy attacks (Hong et al., 2006; Meletti et al., 2015) (Fig. 4A). Cell recordings of narcoleptic dogs reveal that many neurons in the CeA specifically have activity that is closely associated with cataplexy attacks (Gulyani et
More recently, the first functional evidence to link the amygdala and cataplexy came with the observation that in narcoleptic mice, lesions of the CeA significantly reduced the amount of cataplexy they experienced (Burgess et al., 2013) (Fig. 4C). This same study identified strong GABAergic projections from the CeA to REM-off areas in the brainstem, suggesting that activation of the CeA may be able to inhibit these regions and so produce muscle atonia by disinhibiting REM sleep circuitry (Figs. 4D-I).

Moreover, if cataplexy and REM sleep share the same circuits, one would expect the amygdala to influence the expression of REM sleep or its features. In fact, not only does amygdala activity naturally increase during REM sleep in healthy individuals (Maquet et al., 1996; Nofzinger et al., 1997), but manipulations of the amygdala in general and the CeA specifically alter REM sleep architecture (Fraigne et al., 2015). For example, pharmacological inhibition of the CeA using TTX reduces REM sleep frequency and duration (Tang et al., 2005; Sanford et al., 2006), while electrical stimulation of the amygdala increases overall REM sleep amounts (Smith and Miskiman, 1975). Activation or inhibition of GABA\textsubscript{A} receptors in the CeA result in decreases or increases respectively in REM sleep amounts (Sanford et al., 2002). Injection of 5-HT into of the amygdala during NREM sleep leads to transitions to REM sleep (Sanford et al., 1995), while injection of carbachol into the CeA, but not other amygdala nuclei, produces more frequent REM sleep episodes (Calvo et al., 1996). Taken together, these results point to a putative role of the amygdala and specifically the CeA in regulating REM sleep in addition to cataplexy, and also underscore the notion that cataplexy and REM sleep share similar brain circuits.
Figure 4. Summary of evidence implicating the amygdala in cataplexy. Existing evidence identifies the amygdala as being functionally and anatomically well-positioned to promote emotionally-induced cataplexy. A, Functional magnetic resonance imaging demonstrated that the amygdala is highly active during cataplexy attacks in narcoleptic humans. B, Unit recordings in narcoleptic dogs identified many neurons in the CeA with activity tightly correlated to cataplexy attacks. C, Bilateral excitotoxic lesions of the CeA significantly reduced the amount of cataplexy narcoleptic mice experienced. D-F, A retrograde tracer (CTb) injected into the vlPAG/LPT labeled many neurons in the CeA, indicating the presence of CeA-vlPAG/LPT projections. Colocalization of vesicular GABA transporter (VGAT) mRNA in these cells with in situ hybridization implies these CeA projections are GABAergic. G-I, An anterograde tracer (AAV-ChR2-mCherry) injected into the CeA identified strong projections to the vlPAG and LPT, as well as the locus coeruleus and dorsal raphe nucleus. These labeling studies point towards a possible mechanism by which the CeA promotes cataplexy. Amy, amygdala; Aq, aqueduct; BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; LPT, lateral pontine tegmentum; scp, superior cerebral peduncle; vlPAG, ventrolateral periaqueductal grey. Scale bars, 250 µm. Modified from Meletti et al, 2015; Gulyani et al, 2002; Burgess et al, 2013.
1.4 Current Model of Cataplexy

The current model of cataplexy is based on the hypothesis that in narcoleptic individuals, the loss of orexin neurons destabilizes the brainstem circuitry that control muscle atonia during REM sleep, enabling losses of muscle tone to inappropriately occur during wakefulness (Dauvilliers et al., 2014; Fraigne et al., 2015) (Fig. 5). Both orexin neurons of the LH and GABA neurons of the CeA project to muscle tone-promoting regions in the brainstem such as the vlPAG, LPT, and LC. Although both the orexin and CeA GABA cells are activated by positive emotions, the excitatory projections of orexin cells to the vlPAG, LPT, and LC are thought to normally override inhibitory GABA projections from the CeA, and in this way maintain muscle tone in healthy individuals. In narcolepsy, however, orexin neurons are absent, leaving the vlPAG, LPT, and LC subject only to GABAergic inputs of the CeA. As a result, when positive emotions are experienced, GABA CeA cells become activated and inhibit the vlPAG, LPT, and LC. This leads to a disfacilitation of motoneurons, as well as direct inhibition of motoneurons via recruitment of the SubC-VMM circuit, similar to what is seen in physiological REM sleep. The combined effect of these processes is to suppress muscle tone and produce cataplexy.
Figure 5. Hypothesized circuit underlying cataplexy. Cataplexy is thought to occur when the brainstem circuitry that produces REM sleep atonia is activated during wakefulness. During REM sleep, glutamatergic cells of the SubC activate GABA/glycine cells in the VMM, which project to and inhibit spinal motoneurons to produce muscle atonia. During wakefulness, however, this SubC-VMM circuit is inhibited by brainstem regions such as the vlPAG, and muscle tone is further promoted by excitatory noradrenergic projections from the LC. In healthy individuals, the activity of the vlPAG and LC are supported by excitatory orexin inputs from the LH, which override inhibitory GABA inputs originating in the CeA, even when positive emotions are experienced. In narcolepsy, however, the orexin neurons are absent, leaving nothing to balance inputs from GABA CeA cells. As a result, when positive emotions are experienced and GABA CeA cells are strongly activated, they inhibit the vlPAG and LC, leading to the disfacilitation of motoneurons and the recruitment of the SubC-VMM circuit. The combined effect of these processes is to suppress muscle tone and produce cataplexy. CeA, central nucleus of the amygdala; LC, locus coeruleus; LH, lateral hypothalamus; MNs, motoneurons; SubC, subcoeruleus; vlPAG, ventrolateral periaqueductal grey; VMM, ventral medial medulla. From Fraigne, Torontali, Snow, and Peever, 2015.
1.5 Methodological Background

1.5.1 DREADDs

This research project involves studying the role of a discrete population of neurons in cataplexy, and so a technique with high cellular specificity must be used. Classical techniques used to study neural circuits such as electrical stimulation, pharmacological manipulation, and lesioning lack the resolution required to undertake such a study. For this reason, we have chosen to use a chemogenetic technique known as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which refers to a family of engineered G protein-coupled receptors that permit manipulation of neurons in a spatially-restricted and phenotype-specific manner (Rogan and Roth, 2011).

DREADDs, which were first developed by Bryan Roth’s group, are derived from endogenous human M3 and M4 muscarinic receptors and include the hM3Dq and hM4Di receptors (Armbruster et al., 2007; Wess et al., 2013). Directed molecular evolution of the endogenous receptors introduced two point mutations at conserved residues, resulting in a loss of affinity for their native ligand, acetylcholine, and instead rendering them responsive exclusively to the synthetic drug known as clozapine-N-oxide (CNO) (Rogan and Roth, 2011). Although these mutated receptors now interact with a different ligand, they remain coupled to the same intracellular signaling cascades as the endogenous receptors (Armbruster et al., 2007). The excitatory DREADD, hM3Dq, is coupled to the $G_q$ signaling pathway, which activates cells via intracellular calcium release and ERK1/2 activation. The inhibitory DREADD, hM4Di, is coupled to the $G_i$ signaling pathway, which inactivates cells by inhibiting adenylyl cyclase and opening potassium channels to cause hyperpolarization (Rogan and Roth, 2011).

The effects of CNO-mediated DREADD activation persist on the order of hours, making possible the examination of how persistent manipulation of a certain brain region or neuron population affects behaviors of interest (Krashes et al., 2011). Furthermore, the reversibility of DREADD-related interventions allows the brain and its circuits to remain fully intact throughout an experiment while avoiding confounding factors associated with permanent manipulations such as lesions. The genes encoding the DREADDs can be packaged into an adeno-associated virus (AAV), which can subsequently be delivered to target brain areas via stereotaxic injection.
Cellular specificity can be achieved using a Cre-Lox system in which the DREADD gene is double-floxed and inverted (DIO), and Cre recombinase is expressed in neurons of interest (Rogan and Roth, 2011) (Fig. 6). In this project, the excitatory DREADD, hM3Dq, was used to induce long-lasting activation of all neurons, or just GABA cells, in the CeA so that the role of this brain region in producing cataplexy could be examined.
Figure 6. Cre-dependent hM3Dq constructs permit manipulation of specific cell populations. The expression of genes carried by an adeno-associated virus (AAV) can be restricted to certain neuronal types (e.g., GABA cells) using a Cre-Lox system. The AAV contains a genetic construct consisting of a neuron-specific promoter (hSyn), an effector gene (i.e., hM3Dq), and a reporter gene (i.e., mCherry). However, the effector and reporter genes are in an antisense orientation relative to the promoter, such that they cannot be transcribed, and are flanked by 2 sets of Lox sites (double-floxed). Cre recombinase recognizes the Lox sequences and inverts the double-floxed genes, putting them in the same orientation as the promoter and enabling them to be transcribed. Specific expression of Cre-dependent genes becomes possible when Cre recombinase expression is localized to certain cell types (e.g., GABA cells), permitting the study of how those cells in particular influence behaviors of interest (e.g., cataplexy). To achieve expression in all cells, regardless of phenotype, a construct in which the effector and reporter genes are already in the same orientation as the promoter (i.e., they are not double-floxed) can be used instead. Such a construct would be expressed in all successfully transduced cells, regardless of phenotype and independent of Cre recombinase.
1.5.2 Mouse Model

Since human narcolepsy is associated with the loss of a functional orexin system, the animal model chosen for use in this experiment is the prepro-orexin knockout \((orexin^{-/-})\) mouse. This mouse model displays the main symptoms of human narcolepsy such as disturbed REM sleep, sleep attacks, and cataplexy (Chemelli et al., 1999; Mochizuki et al., 2004; Scammell et al., 2009). Since the focus of this project is cataplexy, it is important that the mouse model used replicates this symptom as closely as possible. Cataplexy in \(orexin^{-/-}\) mice is characterized by the abrupt loss of skeletal muscle tone during wakefulness, a theta-rich EEG, and possibly the maintenance of consciousness (Mochizuki et al., 2004; Scammell et al., 2009; Vassalli et al., 2013). As in human cataplexy, attacks usually last under 2 min, and end with a rapid restoration of muscle tone and a return to the activity being performed prior to the episode (Chemelli et al., 1999; Scammell et al., 2009). Importantly, just as cataplexy is triggered by strong positive emotions in humans, cataplexy in mice can be triggered by rewarding stimuli such as palatable food and running wheels (Espana et al., 2007; Clark et al., 2009; Burgess et al., 2013).

In order to manipulate specific types of neurons (i.e., GABA neurons), \(orexin^{-/-}\) mice were crossed with VGAT-Cre mice to produce a new mouse model of narcolepsy, the \(orexin^{-/-}\),VGAT-Cre mouse. VGAT-Cre mice express Cre recombinase downstream of the gene for the vesicular GABA transporter (VGAT), which is a marker of GABA cells (Wojcik et al., 2006; Vong et al., 2011). In this way, Cre recombinase is expressed only in cells where the VGAT gene is transcribed (i.e., GABA cells). Thus, using Cre-dependent AAV constructs in conjunction with \(orexin^{-/-}\),VGAT-Cre mice restricts the expression of DREADDs to GABA neurons, thereby enabling targeted manipulation of these cells in the context of cataplexy.

1.6 Specific Aim

The model outlined in Section 1.4 is consistent with many independent observations, but there are still components of the cataplexy circuit whose functions are not fully characterized. Existing evidence suggests that the CeA is an important component of the cataplexy circuit, especially in the context of emotion-triggered cataplexy attacks. However, the specific role the
CeA plays in generating cataplexy, and the cellular mechanism by which it acts, is not well understood. We tested the specific hypothesis that GABA neurons in the CeA are the mechanism through which positive emotions trigger cataplexy in narcolepsy. To do this, we used chemogenetic, electrophysiological, and behavioral techniques to examine how persistent manipulations of GABA CeA cells influence cataplexy in narcoleptic mice.
Chapter 2 – Methods

2.1 Animals

We used three different lines of mice in these experiments. Male wild-type (i.e., C57BL/6; age: 19 ± 2 weeks; mass: 25 ± 2 g) and male orexin knockout (orexin\(^{-/-}\); age: 13 ± 2 weeks; mass: 23 ± 1 g) were used to determine how CeA activation impacts behavior. We also used a new mouse line, which we developed over the last 2 years. This line was developed so that GABA cells in the CeA could be selectively targeted using either chemogenetic (or optogenetic) methods. We call these mice orexin\(^{-/-}\),VGAT-Cre (age: 13 ± 2 weeks; mass: 24 ± 2 g) because they were generated by crossing orexin\(^{-/-}\) mice (Chemelli et al., 1999) with VGAT-Cre mice (Vong et al., 2011). Throughout experiments, mice were housed individually on a 12:12 light/dark cycle (lights off at 1900h) in a temperature- and humidity-controlled environment, had food and water available ad libitum, and had access to a running wheel. All procedures and experimental protocols were approved by the University of Toronto’s Local Animal Care Committee and were in accordance with the Canadian Council on Animal Care.

2.2 Chemogenetic Methods

To activate CeA cells we drove expression of hM3Dq receptors, which when exposed to clozapine-N-oxide (CNO) cause neuronal activation (Armbruster et al., 2007; Rogan and Roth, 2011). We virally delivered hM3Dq receptors using constructs obtained from the University of North Carolina Vector Core (Chapel Hill, NC). Constructs were packaged into adeno-associated viruses with serotype 8 (AAV8), expressed downstream of a neuron-specific promoter (hSyn), and used mCherry as a reporter gene. For experiments involving orexin\(^{-/-}\) mice and wild-type mice, the construct contained an excitatory hM3Dq receptor (AAV8/hSyn-hM3Dq-mCherry, 4.5 \(\times\) 10\(^{12}\) particles/mL). For experiments involving orexin\(^{-/-}\),VGAT-Cre mice, the construct contained either hM3Dq (excitation experiments) or no hM3Dq (empty vector; control experiments). To ensure cellular specificity in these mice, viral constructs were in a double-floxed inverted orientation (DIO) such that the genes would be expressed only in cells containing
Cre recombinase (i.e., GABA cells) (excitation: AAV8/hSyn-DIO-hM3Dq-mCherry, \(5.7 \times 10^{12}\) particles/mL; control: AAV8/hSyn-DIO-mCherry, \(8.0 \times 10^{12}\) particles/mL).

### 2.3 Drug Preparation

The hM3Dq receptor is exclusively activated by the biologically inert ligand CNO. CNO injections were prepared by dissolving CNO powder (a generous donation from Dr. Bryan Roth) in a sterile solution of 0.9% saline and 0.05% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). We administered CNO i.p. in doses of 1 mg/kg for wild-type mice, or 2.5 mg/kg and 5 mg/kg for orexin/− and orexin/−, VGAT-Cre mice, in order to activate hM3Dq receptors on CeA cells; these doses have been previously shown to activate hM3Dq receptors (Farrell and Roth, 2013; Weber et al., 2015). Because we found no response difference for doses in orexin/− mice (i.e., 2.5 mg/kg and 5 mg/kg CNO all increased cataplexy by the same magnitude), we therefore combined these doses into a single CNO data set (“CeA Activation”) (see Fig. 7). In all cases, we compared effects of CNO injection to vehicle injections (“Baseline”) that contained only 0.9% saline and 0.05% DMSO, but no CNO.

### 2.4 Viral Injection Surgery

Mice were anesthetized using isoflurane (2-5%) and secured in a stereotaxic frame (model 902; David Kopf Instruments). 200 nL of virus was slowly (50 nL/min) infused into the left and right CeA (1.35 mm posterior to bregma, ±2.75 mm lateral, 4.5 mm ventral). Infusions were performed through a 28-gauge cannula connected to a digital microinjection syringe pump (Pump 11 Elite; Harvard Apparatus). Mice were administered ketoprofen (5 mg/kg, s.c.) up to 48 hours after surgery and allowed to recover for at least 14 days.
2.5 EEG and EMG Instrumentation

At the end of this 14-day recovery period, mice underwent a second surgery during which electroencephalogram (EEG) and electromyogram (EMG) electrodes were implanted in order to identify sleep states and cataplexy. Electrodes were constructed from multi-stranded stainless steel wire (AS 632; Cooner Wire). For EEG recordings, we bilaterally implanted stainless steel screws (P0090CE125; J.I. Morris) into the frontal and parietal bones (1.5 mm anterior and ± 1.5 mm lateral to bregma; 2 mm posterior and ± 2.75 mm lateral to bregma). For EMG recordings, we sutured two electrodes into the neck extensor muscles and two electrodes into the right masseter muscle. All EEG and EMG electrodes were soldered to a micro-strip connector (CLP-105-02-L-D; Electrosonic) that we affixed to the skull using dental cement (Ketac-cem and C&B Metabond Cement System; K-dental). Mice were administered ketoprofen (5 mg/kg, s.c.) up to 48 hours after surgery and allowed to recover for at least 14 days before experiments began.

2.6 Experimental Protocols

One week after the second surgery, mice were transferred into a plexi-glass recording chamber containing a running wheel (Bio-Serv). As mice require 10-12 days to maximize their wheel running (Espana et al., 2007), this provided sufficient time for them to habituate to the wheel prior to experiments, which began 14 days after being placed into the recording chamber. Orexin−/− mice experience more cataplexy when they have access to running wheels presumably because running wheels represent a rewarding stimulus (Lett et al., 2002; Espana et al., 2007; Burgess et al., 2013). Therefore, we provided mice running wheels in order to study how rewarding conditions impact cataplexy.

After being transferred to the recording chamber, we gently handled mice at the start of the dark phase (1900h) on three separate occasions to habituate them to human contact. One week after being transferred to the recording chamber, we fastened a lightweight recording cable (CW7117; Cooner Wire) to the micro-strip connector, and gave mice an additional week to habituate to this cable. During this habituation period mice were given 3 i.p. injections of 0.3 mL of lactated ringer solution (B. Braun Medical Inc.) to habituate them to i.p. injections.
At the end of this week, we recorded mice up to four consecutive nights for 12 hours each (beginning at 1900h). No intervention was performed on the first night of recording so that we could observe the natural behavior of the mice. On the subsequent nights, we randomly gave mice i.p. injections of saline or CNO at the start of the dark phase (1900h). EEG, EMG, and video were recorded each night to observe changes in cataplexy and sleep-wake behaviors.

2.7 Data Acquisition

EEG and EMG signals were passed through a Super-Z Head-Stage and a BMA-400 Bioamplifier (CWE Inc.), which amplified signals by a factor of 500. All signals were digitized at 1000 Hz (Spike 2 Software, 1401 Interface, Cambridge Electronic Design Ltd.), had a DC offset applied with a time constant of 0.4 s, and were digitally filtered (EEG, 1-100 Hz; EMG, 30-1000 Hz). In signals where electrical noise was detected, a 60 Hz Notch filter was applied in all recordings for that mouse. EMG signals were rectified. We simultaneously captured and synchronized videos to electrophysiological recordings to assist in identifying behaviors of interest.

2.8 Data Analysis

We analyzed the first 6 hours of recording for orexin⁻/⁻ mice to determine the time course of any CNO-induced effects on cataplexy and behavior (see Fig. 7E). Because we found CNO effects persisted for only 3 hours after injection, all subsequent analyses were performed only on the first 3 hours of the recording period. We scored behavioral states in 5-second epochs based on EEG, EMG, and video using a custom-made script for Spike 2 (sleepscore v1.01). We identified wakefulness, non-rapid eye movement (NREM) sleep, and REM sleep using standard criteria described previously (Burgess et al., 2010; Brooks and Peever, 2012; Burgess and Peever, 2013). Cataplexy was scored according to the consensus definition criteria: (1) an abrupt episode of nuchal atonia lasting at least 10 seconds, (2) the mouse is immobile during the episode, (3) theta activity dominates the EEG during the episode, and (4) at least 40 seconds of wakefulness precedes the episode (Scammell et al., 2009). Both the electrophysiological signals
and video recording were used to determine the activity the mouse was engaged in immediately before the attack began. Almost all cataplexy attacks were triggered by wheel running, grooming, exploring, eating, or drinking. We considered cataplexy episodes without a clear preceding behavior spontaneous attacks and had the trigger classified as “other”. We identified sleep attacks on the basis of a gradual loss of neck muscle tone and the appearance of EEG slow waves, in addition to automatic behaviors such as chewing that were evident in the masseter EMG (Burgess et al., 2010). Sleep attacks progressed into either wakefulness, NREM sleep, or REM sleep, and the subsequent state was scored as such.

We quantified the number and length of cataplexy attacks during both CNO and saline (i.e., baseline) treatments. We calculated percent time in cataplexy by summing the total time spent in cataplexy and dividing this value by the length of the recording period. The number of cataplexy episodes recorded was used to divide the total time spent in cataplexy to determine the average duration of episodes. We quantified muscle tone during cataplexy in each mouse by normalizing the average integrated EMG activity of the neck and masseter muscles during cataplexy to that muscle’s activity during NREM sleep during the saline treatment. EMG activity was quantified for each muscle using a custom-made Spike 2 script (5s epoch analysis). All calculations involving behavioral state architecture and muscle tone were done using a custom-made Excel VBA macro (see Appendix 1). We generated EEG power spectra during cataplexy by performing a fast Fourier transform of the EEG signal during cataplexy using Spike 2 software. Relative power was achieved by summing the absolute power of 1-20 Hz and dividing the absolute power of each intermediate frequency by that sum.

2.9 Histology and Fluorescence In Situ Hybridization

After experiments, mice were deeply anesthetized with Avertin (250 mg/kg, i.p.), produced from 2,2,2-tribromoethanol and 2-methyl-2-butanol (Sigma-Aldrich), and isoflurane. Upon loss of the foot withdrawal and righting reflexes, mice were immobilized and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). The brain was isolated and immersed in 4% PFA for 24 hours, then transferred to a 30% sucrose solution for 48 hours for cryoprotection. Cryoprotected brains were frozen in Tissue-Tek OCT Compound
(Electron Microscopy Sciences) and coronally sectioned at 40 µm using a cryostat (CM3050 S; Leica). Slices were subsequently mounted on glass slides with Permafluor and expression of mCherry was visually confirmed with fluorescence microscopy. Only mice in which expression encompassed the CeA bilaterally were analyzed.

For fluorescence in situ hybridization, after mice were anesthetized, brains were immediately frozen in −30°C isopentane and stored at −80°C. Brains were coronally sectioned at 16 µm and mounted on glass slides, which were stored at −80°C. Sections containing the CeA were immersed in 4% PFA for 20 min, then incubated in 0.1 M PBS containing 0.3% H$_2$O$_2$ for 10 min at room temperature. Slices were acetylated for 10 min using 0.1 M TEA buffer containing 0.25% acetic anhydride, dehydrated with ethanol, then transferred to a humid chamber saturated with formamide and incubated in a hybridization buffer (40% formamide, 10 mM Tris-HCl, pH 8.0, 200 µg/mL yeast tRNA, 10% dextran sulfate, 1× Denhardt’s solution, 600 mM NaCl, 1 mM EDTA, pH 8.0) for 2 hours at 57.5°C. Sections were next transferred to a hybridization buffer containing the antisense GAD67 riboprobe (1:1000) and incubated overnight at 57.5°C, then washed with serial SSC buffers under gentle agitation. Sections were next incubated in a blocking solution containing 4% goat serum and 0.5% blocking reagent (Roche) for 1 hour at room temperature, then incubated in a polyclonal mouse antibody to mCherry (1:1000, T513, Signalway Antibody) for 36 hours at room temperature. At the end of this period, sections were incubated in sheep anti-DIG-POD (1:500, 11207733910, Roche) overnight at 4°C, then washed five times for 5 min washes in 0.1 M PBS containing 0.1% Triton-X (PBS-T) and transferred to a solution containing the TSA Plus Cyanine 5 System (1:100, NEL745001KT, PerkinElmer) for 10 min. Slices were next incubated in Cy3-conjugated goat anti-mouse antibodies (1:200, NB7607, Cedarlane) for 3 hours at room temperature then stained with 4’,6-diamidino-2-phenylindole (DAPI, 1:1000) for 5 min. Slices were left to dry overnight, then coverslipped with Permafluor and examined with a fluorescent microscope.

2.10 Statistical Analysis

We performed between-group comparisons between using unpaired two-tailed t-tests and within-group comparisons using paired two-tailed t-tests. For data that was not normally
distributed, we instead used Mann-Whitney $U$ or Wilcoxon signed-rank tests. When more than two interventions within a group were compared, a one-way repeated measures ANOVA was used followed by Newman-Keuls multiple comparison post hoc tests. We compared EEG power spectra using two-way repeated measures ANOVA within groups, or two-way ANOVA without repeated measures between groups. All statistical analyses were performed using GraphPad Prism (La Jolla, CA) and had a critical alpha value of $p < 0.05$ applied. All data are presented as mean ± SEM unless otherwise indicated.
Chapter 3 – Results

3.1 CeA activation exacerbates cataplexy in narcoleptic mice

The CeA may have a causal role in triggering cataplexy. For example, CeA cells increase and decrease their discharge activity at the beginning and end of cataplexy in narcoleptic dogs (Gulyani et al., 2002), and CeA lesions suppress cataplexy amounts in orexin−/− mice (Burgess et al., 2013). Therefore, our first goal was to determine if activation of CeA neurons would promote cataplexy. The CeA is composed of at least two distinct cell populations. A minority of CeA cells are glutamatergic and have relatively sparse projections beyond the amygdala (Boissard et al., 2003; Xi et al., 2011), while the majority of CeA cells contain GABA and densely innervate multiple other brain regions, including those that control muscle tone (Swanson and Petrovich, 1998; Sah et al., 2003; Burgess et al., 2013). Instead of selectively targeting either of these cell groups we decided to first use a Cre-independent strategy to activate all CeA cells so that we could establish a functional role for the CeA in mediating cataplexy. We did this by using AAVs to drive bilateral hM3Dq receptor expression in CeA neurons of orexin−/− mice and then determined how CNO-induced activation of these receptors influenced cataplexy, sleep attacks, and general sleep-wake behavior.

We successfully targeted hM3Dq receptors to cells in the left and right CeA in 5 orexin−/− mice (Figs. 7A-B). CNO-induced activation of CeA cells had no observable impact on typical waking behaviors such as eating, drinking, exploring, grooming, or wheel running, but it induced an intense increase in the number of cataplexy attacks relative to baseline conditions (Figs. 7C-D). Cataplexy amounts were elevated for 3 hours after CNO administration, and returned to baseline levels thereafter, suggesting the stimulatory effects of CNO on hM3Dq receptors last for about 3 hours (Fig. 7E). Analysis of group data showed that CeA activation triggered up to 3.5 times more cataplexy during the 3-hour period (1-way RM ANOVA, \( F=6.288, p=0.0083 \); Fig. 7F). Importantly, we determined that the changes in cataplexy we observed after CeA activation were a consequence of CNO because saline injections alone did not alter the levels of cataplexy mice experienced (compared to when no intervention was given). We also found that both 2.5 mg/kg CNO and 5 mg/kg CNO produced identical changes in cataplexy, and so combined these doses into a single CNO data set for all subsequent comparisons. CeA activation exacerbated
cataplexy by markedly increasing the number of episodes (paired \( t \)-test, \( t(4)=2.811, p=0.0483; \textbf{Fig. 7G} \)), but had no impact on the length of cataplexy events (paired \( t \)-test, \( t(4)=0.2547, p=0.8115; \textbf{Fig. 7H} \)). This observation suggests that cells in the CeA play a functional role in initiating, but not maintaining, cataplexy.

From a behavioral perspective, attacks were indistinguishable from typical baseline cataplexy in orexin\(^{-/-}\) mice. They featured the standard hallmarks of cataplexy in that they were characterized by an abrupt postural collapse and a sudden loss of EMG tone, and the appearance of theta-rich EEG activity, both of which punctuated normal waking behavior (see \textbf{Figs. 10A-B}). Like typical baseline cataplexy, attacks lasted about 50 s and ended with an abrupt and rapid return to active wakefulness (\textbf{Fig. 7H}). However, we nonetheless wanted to verify these events were indeed cataplexy and not some other form of behavioral arrest such as sleep attacks. To do this, we compared EEG power spectral profiles as well as levels of EMG tone during baseline cataplexy with that elicited during CeA activation. We quantified these two particular variables because murine cataplexy is characterized by both muscle paralysis (Burgess and Peever, 2013) and a wake-like, theta-rich cortical EEG (Vassalli et al., 2013). We found that CeA activation produced cataplexy episodes with levels of muscle tone that were comparable to those during typical baseline cataplexy (masseter tone: paired \( t \)-test, \( t(4)=0.9472, p=0.3972; \textbf{Figs. 8A-B} \)). We also found that EEG activity peaked in the theta band during baseline and CeA activation-induced cataplexy, and overall power spectral profiles were identical in both conditions (2-way RM ANOVA, \( F=3.458 \times 10^{-4}, p=0.9852; \textbf{Fig. 8C} \)). Together, these data suggest that the cataplexy produced by CeA activation is physiologically and behaviorally identical to attacks that occur during baseline conditions in orexin\(^{-/-}\) mice.

Because the amygdala has been implicated in sleep regulation (Smith and Miskiman, 1975; Calvo et al., 1996; Sanford et al., 2006), we wanted to determine if CeA activation would affect either sleep-wake behavior or sleep attacks, which are common in orexin\(^{-/-}\) mice. Although CNO-induced CeA activation triggered marked increases in cataplexy, this same intervention had no measureable effect on amounts of wakefulness (paired \( t \)-test, \( t(4)=2.315, p=0.0816 \)), NREM sleep (paired \( t \)-test, \( t(4)=1.1319, p=0.2576 \)), or REM sleep (paired \( t \)-test, \( t(4)=0.6870, p=0.5299 \)), nor did it affect sleep attacks (paired \( t \)-test, \( t(4)=2.497, p=0.0670; \textbf{Fig. 8D} \)). These findings indicate that CeA activation does not have broad effects on behavior; rather, its activation selectively targets the mechanisms mediating cataplexy.
Figure 7. CeA activation exacerbates cataplexy in narcoleptic mice. A, Stereotaxic map displaying the location of expression of the Cre-independent hM3Dq construct in the CeA of 5 orexin−/− mice. B, The extent of expression was determined on the basis of mCherry fluorescence. A representative sample of mCherry-positive neurons is shown, as is a single neuron (inset). C–D, Hypnograms displaying behavioral states of orexin−/− mice during baseline conditions (saline) or CeA activation (CNO). E, Compared to baseline, CNO produced increases in cataplexy that persisted for the first 3 hours after injection, but amounts of cataplexy during the next 3 hours were identical to that seen during baseline conditions. For this reason, only the first 3 hours after CNO injection were examined in detail. F, Following global CeA activation, a large increase in the total time spent in cataplexy was observed following CNO, but not saline, injections. There was no difference in the effects produced by 2.5 mg/kg CNO or 5 mg/kg CNO, so they were combined into a single CNO dataset for all subsequent comparisons. G, The increase in cataplexy arose from an increase in the number of cataplexy episodes mice experienced. H, The average duration of cataplexy episodes did not change. BLA, basolateral nucleus of the amygdala; ic, internal capsule; W, wakefulness; NREM, NREM sleep; REM, REM sleep; SA, sleep attacks; CAT, cataplexy. *, p < 0.05 compared to no intervention and saline or baseline as appropriate.
Figure 8. The behavioral arrests triggered by CeA activation are cataplexy. A-B, In 5 orexin−/− mice, activation of all CeA cells produced behavioral arrests characterized by similar levels of muscle tone in the masseter and neck muscles compared to typical baseline cataplexy. C, These arrests also had similar EEG spectral profiles to baseline cataplexy (data presented as mean ± SEM). D, CeA activation did not produce any changes in the overall amounts of wakefulness (W), NREM sleep (NREM), or REM sleep (REM), nor did it alter sleep attacks (inset).
3.2 CeA activation does not cause cataplexy in wild-type mice

Next, we wanted to verify that cataplexy was only triggered by CeA activation in orexin−/− mice, and that it could not be triggered by CeA stimulation in wild-type mice with an intact orexin system. Therefore, we used a Cre-independent strategy to drive hM3Dq expression in CeA cells of wild-type mice (n=4), then examined how CNO-induced activation of these cells affected behavior. Using EEG, EMG and videography, we found no evidence indicating that CeA activation causes cataplexy in wild-type mice. Unlike orexin−/− mice that experienced as many as 12 cataplexy attacks during the 3-hour recording period (see Fig. 7G), we saw no evidence of postural collapses or intermittent losses of EMG tone during waking behaviors in wild-type mice after CeA stimulation. We also found no evidence that CeA activation influenced the overall amount or architecture of sleep-wake behavior (Figs. 9A-C). These findings suggest that the orexin system protects against cataplexy, and that CeA activation only promotes cataplexy in narcoleptic mice that already lack an intact orexin signaling system (see Fig. 15). Although previous studies show that the amygdala can promote REM sleep (Calvo et al., 1996; Sanford et al., 2006), our results indicate that CeA activation does not affect sleep-wake behavior.
Figure 9. CeA activation does not alter sleep-wake architecture in wild-type mice. A, Bilateral activation of all CeA cells in wild-type mice (n=4) did not influence the amounts of time they spent in wakefulness (W), NREM sleep (NREM), or REM sleep (REM). CeA activation produced no differences in the B, frequency or C, duration of W, NREM, or REM, nor was there evidence of overt behaviors resembling cataplexy.
3.3 Orexin−/− mice that express Cre in GABA neurons exhibit typical cataplexy

Having shown that general CeA stimulation promotes cataplexy, our next step was to identify which type of CeA cell is responsible for this effect. Here, we test the role for GABA neurons because they are the primary extrinsic pathway from the CeA and they are anatomically connected to nuclei that regulate skeletal muscle tone (Swanson and Petrovich, 1998; Sah et al., 2003; Burgess et al., 2013) (see Fig. 15). However, to understand how GABA cells in the CeA regulate cataplexy, we needed to develop a new narcoleptic mouse line in which we could selectively target and manipulate these cells exclusively. To do this, we crossed orexin−/− mice with VGAT-Cre mice (Chemelli et al., 1999; Vong et al., 2011) to produce so-called orexin−/−,VGAT-Cre mice so that Cre-dependent AAVs could be used to deliver hM3Dq receptors directly to GABA cells in the CeA. However, before using these new mice we needed to determine whether they behave like typical orexin−/− mice, and we did this by comparing amounts of cataplexy, sleep attacks, NREM/REM sleep and wakefulness in orexin−/− (n=5) and orexin−/−,VGAT-Cre (n=13) mice.

First, we show that general sleep-wake behavior is unaffected in orexin−/−,VGAT-Cre mice. Specifically, we found that orexin−/− and orexin−/−,VGAT-Cre mice spent the same amount of time in wakefulness, NREM sleep, and REM sleep, and that the length and number of each behavioral state was comparable (Table 2). We also found that the number and length of sleep attacks was similar between genotypes (Table 2). Together, these results suggests that orexin−/−,VGAT-Cre mice are phenotypically indistinguishable from orexin−/− mice.

Next, we showed that cataplexy was behaviorally and electrophysiologically identical in orexin−/−,VGAT-Cre and orexin−/− mice. In both mouse lines we found that cataplexy occurred during active wakefulness when mice were engaged in purposeful activities such as wheel running, grooming, exploring, eating, and drinking. Attacks were characterized by abrupt postural collapse and rapid loss of EMG tone (Figs. 10A-B). Mice remained immobile throughout attacks, which were terminated by a swift return of skeletal muscle tone and resumption of prior waking activities (Figs. 10A-B). Levels of EMG tone and cortical EEG activity during cataplexy were similar in both orexin−/−,VGAT-Cre and orexin−/− mice (masseter
EMG: $t$-test, $t(11)=1.525$, $p=0.1554$; neck EMG: $t$-test, $t(13)=0.4438$, $p=0.6645$; EEG: 2-way ANOVA, $F=2.756 \times 10^{-3}$, $p=0.9582$; Figs. 10C-E).

Both mouse lines also spent comparable amounts of time in cataplexy. During the 3-hour recording period $orexin^{-/-}$ and $orexin^{-/-}, VGAT-Cre$ mice spent $2.3 \pm 0.5\%$ and $1.7 \pm 0.5\%$ of their time in cataplexy (Mann-Whitney U test, $U(16)=22.50$, $p=0.3488$; Fig. 10F). They also had the same number of attacks that lasted for the same amount of time. $Orexin^{-/-}$ mice experienced $5 \pm 1$ attacks that lasted for $53 \pm 8$s and $orexin^{-/-}, VGAT-Cre$ mice experienced $3 \pm 1$ attacks that lasted for $49 \pm 7$s (number: Mann-Whitney U test, $U(16)=16.50$, $p=0.1248$; duration: $t$-test, $t(16)=0.3527$, $p=0.7289$; Figs. 10G-H). Together, these data indicate that $orexin^{-/-}, VGAT-Cre$ mice exhibit cataplexy that is behaviorally and electrophysiologically indistinguishable from that of $orexin^{-/-}$ mice, making this new mouse line useful for determining how GABA cell manipulation affects cataplexy.
<table>
<thead>
<tr>
<th></th>
<th>orexin&lt;sup&gt;−&lt;/sup&gt;</th>
<th>orexin&lt;sup&gt;−&lt;/sup&gt;;VGAT-Cre</th>
<th>p-value</th>
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<tr>
<td><strong>Wake</strong></td>
<td></td>
<td></td>
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<tr>
<td>Percent of time</td>
<td>83.65 ± 5.043</td>
<td>84.60 ± 2.980</td>
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<tr>
<td>Number of episodes</td>
<td>31.20 ± 5.669</td>
<td>33.54 ± 6.721</td>
<td>0.8419</td>
</tr>
<tr>
<td>Average duration of episodes (s)</td>
<td>342.7 ± 74.49</td>
<td>483.6 ± 106.1</td>
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<tr>
<td><strong>NREM sleep</strong></td>
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<td></td>
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</tr>
<tr>
<td>Percent of time</td>
<td>10.77 ± 3.741</td>
<td>10.24 ± 2.519</td>
<td>0.9117</td>
</tr>
<tr>
<td>Number of episodes</td>
<td>25.20 ± 5.860</td>
<td>28.62 ± 6.873</td>
<td>0.7761</td>
</tr>
<tr>
<td>Average duration of episodes (s)</td>
<td>43.07 ± 5.407</td>
<td>39.04 ± 2.860</td>
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<td><strong>REM sleep</strong></td>
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</tr>
<tr>
<td>Percent of time</td>
<td>2.742 ± 1.388</td>
<td>2.365 ± 0.6213</td>
<td>0.7766</td>
</tr>
<tr>
<td>Number of episodes</td>
<td>3.400 ± 1.536</td>
<td>3.923 ± 1.195</td>
<td>0.8119</td>
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<tr>
<td>Average duration of episodes (s)</td>
<td>69.04 ± 24.39</td>
<td>65.09 ± 11.39</td>
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<tr>
<td><strong>Sleep attacks</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Percent of time</td>
<td>0.5660 ± 0.3481</td>
<td>1.059 ± 0.2183</td>
<td>0.2498</td>
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<tr>
<td>Number of episodes</td>
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<td>2.462 ± 0.4752</td>
<td>0.3405</td>
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<tr>
<td>Average duration of episodes (s)</td>
<td>23.75 ± 8.624</td>
<td>47.67 ± 9.436</td>
<td>0.1037</td>
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Figure 10. *Orexin*+ mice that express Cre in GABA neurons exhibit typical cataplexy. A, In *orexin*+ mice, cataplexy is characterized by the abrupt loss of skeletal muscle tone during wakefulness and a theta-rich electroencephalogram (EEG). B, Magnification of boxed area in A. *Orexin*+;VGAT-Cre mice express cataplexy electrophysiologically indistinguishable from that seen in conventional *orexin*+ mice as measured by C, masseter electromyogram (EMG), D, neck EMG, and E, EEG spectral power. F-H, There are no significant differences in the time spent in, number of episodes, or average duration of cataplexy attacks of *orexin*+,VGAT-Cre mice compared to *orexin*+ mice. A.U., arbitrary units.
3.4 hM3Dq receptors are expressed in GABA cells of the CeA

Our next step was to determine whether Cre-dependent AAV delivery targeted GABA cells in the CeA. In 13 orexin−/−,VGAT-Cre mice, we successfully targeted and bilaterally delivered hM3Dq receptors to GABA cells in the left and right CeA (Fig. 11A). To verify the specificity of hM3Dq expression, we used fluorescence in situ hybridization to examine the distribution of GAD67 (a specific marker of GABA cells) in the CeA. We found that not only was the CeA densely populated by GAD67-positive neurons, but that hM3Dq receptors extensively co-localized with these cells (Fig. 11B), suggesting that our subsequent CNO manipulations would primarily target GABA cells in the CeA.
Figure 11. hM3Dq receptors are expressed in GABA cells of the CeA. A, Stereotaxic map displaying the location of expression of the Cre-dependent hM3Dq construct in the CeA of 13 orexin"VGAT-Cre" mice. B, The extent of expression was determined on the basis of mCherry fluorescence (red, top left panel; single mCherry-positive neuron showed in inset), which encompassed the CeA where GAD67-positive cells predominate (green, top right panel; single GAD67-positive neuron shown in inset). Most cells (identified by DAPI, blue) expressing mCherry also expressed GAD67 (bottom left, middle, and right panels), while cells that did not express mCherry rarely expressed GAD67.
3.5 GABA cells in the CeA promote cataplexy

Our last aim was to determine whether GABA cells in the CeA are responsible for promoting cataplexy. In 13 orexin/−,VGAT-Cre mice, we successfully delivered hM3Dq receptors to GABA cells in both the left and right CeA (see Figs. 11A-B), and found that CNO-induced activation of these cells triggered a robust increase in cataplexy that was identical in effect to that produced by activating the entire CeA cell population. Cataplexy occupied 3.7 times more of the 3-hour recording period after GABA cell activation than it did before (paired t-test, t(12)=4.573; p=0.0006; Figs. 12A-C). Increases in cataplexy were due to an increase in the number of attacks, rather than the duration of attacks. Before CeA activation mice experienced 5 ± 1 episodes in 3 hours, but during GABA cell stimulation they experienced 16 ± 3 arrests during the same time period (Wilcoxon signed-rank test, W(12)=−89; p=0.0021; Fig. 12D). In contrast, CeA stimulation had no effect on the length of cataplexy episodes (baseline vs. CNO: 43 ± 6s and 42 ± 4s; paired t-test, t(12)=0.1341, p=0.8956; Fig. 12E), suggesting that GABA CeA cells gate the entrance into, but not the exit from, cataplexy.

Once again, we wanted to confirm that the behavioral arrests produced by specifically activating GABA CeA cells were in fact cataplexy. While these events were behaviorally identical to typical baseline cataplexy, we again performed a rigorous comparison of the electrophysiological features that characterized these attacks. We found that activation of GABA CeA cells produced cataplexy attacks with levels of muscle tone (masseter EMG: paired t-test, t(9)=1.056 × 10−7, p=1.0000; neck EMG: paired t-test t(11)=0.2364, p=0.8175; Figs. 13A & B) and EEG activity (2-way RM ANOVA, F=0.001451, p=0.9697; Fig. 13C) indistinguishable from baseline cataplexy. These observations suggest that activating GABA CeA cells is capable of evoking cataplexy attacks.

Next, we wanted to verify that activation of GABA CeA cells did not affect sleep-wake behavior. We found that CNO-induced CeA stimulation had no effect on overall amounts of NREM sleep (paired t-test, t(12)=1.526; p=0.1529), REM sleep (Wilcoxon signed-rank test, W(12)=24; p=0.3804) or sleep attacks (paired t-test, t(12)=0.03691; p=0.9712), but GABA cell activation did decrease amounts of wakefulness by ~7% (paired t-test, t(12)=3.437; p=0.0049; Fig. 13D). However, this change was actually due to the substantial increase in the amount of time spent in cataplexy, and this apparent decrease in wakefulness disappeared when time awake
and time in cataplexy were combined (paired \( t \)-test, \( t(12)=0.8996; p=0.3861 \); Fig. 13E). Together, these results indicate that activation of GABA CeA neurons promotes cataplexy, but does not influence sleep-wake activity.

Lastly, we needed to demonstrate that increases in cataplexy were in fact mediated by CNO-induced activation of hM3Dq receptors on CeA cells. To do this we drove bilateral expression of the physiologically inert mCherry protein in GABA CeA cells in orexin\(^-\),VGAT-Cre mice (\( n=6 \)). As expected, we found that CNO application had no effect on cataplexy amounts in these “hM3Dq-negative” mice (paired \( t \)-test, \( t(5)=0.8153, p=0.4520 \); Fig. 12C). CNO injection in hM3Dq-negative mice had no impact on either the number (paired \( t \)-test, \( t(5)=1.360, p=0.2321 \)) or duration (paired \( t \)-test, \( t(5)=1.530, p=0.1867 \)) of cataplexy attacks (Fig. 12D-E). These findings demonstrate that CNO administration triggers cataplexy by activating GABA CeA cells through an hM3Dq-dependent mechanism. They also suggest that neither CNO application nor mCherry expression within the CeA impacts cataplexy.
Figure 12. GABA cells in the CeA promote cataplexy. A-B, Hypnograms displaying behavioral states of one orexin⁻/⁻,VGAT-Cre mouse during baseline conditions and activation of GABA CeA cells. C, During activation of GABA CeA cells only in 13 orexin⁻/⁻,VGAT-Cre mice (denoted as “hM3Dq”), a large increase in the total time spent in cataplexy was observed. This same increase was not apparent in a group of 6 hM3Dq-negative orexin⁻/⁻,VGAT-Cre mice (denoted as “mCherry”). D, This cataplexy change arose from an increase in the number of cataplexy episodes hM3Dq-expressing mice experienced. Mice lacking the hM3Dq receptor experienced similar numbers of cataplexy episodes after CNO injection. E, The average duration of cataplexy episodes did not change following CNO injection in hM3Dq-positive and hM3Dq-negative mice. W, wakefulness; NREM, NREM sleep; REM, REM sleep; SA, sleep attacks; CAT, cataplexy. BLA, basolateral nucleus of the amygdala; ic, internal capsule. ***, p < 0.001; **, p < 0.01.
Figure 13. Activating GABA CeA cells does not influence sleep-wake behavior. **A-B,** In 13 orexin$^{-}$VGAT-Cre mice, activation of GABA CeA cells produced behavioral arrests characterized by similar levels of muscle tone in the masseter and neck muscles compared to typical baseline cataplexy. **C,** These arrests also had similar EEG spectral profiles to baseline cataplexy (data presented as mean ± SEM), suggesting they were indeed cataplexy. **D,** GABA CeA activation did not produce any changes in the overall amounts NREM sleep (NREM), or REM sleep (REM), nor did it alter sleep attacks (inset), but it did decrease the amount of wakefulness (W) mice experienced. **E,** However, this decrease in wakefulness was an artifact resulting from mice spending significantly more of their waking time in cataplexy, and was nullified when time awake and time in cataplexy were combined. ***, p < 0.01**
3.6 GABA cells in the CeA mediate cataplexy associated with rewarding stimuli

In narcoleptic mice cataplexy can occur spontaneously, but it is typically associated with positive or rewarding stimuli such as wheel running, grooming, and palatable food (Chemelli et al., 1999; Espana et al., 2007; Clark et al., 2009; Burgess et al., 2013). However, the neural mechanism by which such stimuli elicit cataplexy remains unidentified. Because the CeA is involved in processing positive emotions and rewarding stimuli and is anatomically connected to structures that mediate muscle tone (Murray, 2007; Burgess et al., 2013), we hypothesize that GABA CeA cells could be a neural substrate through which these stimuli trigger cataplexy. To test this hypothesis, we documented the types of behaviors that triggered cataplexy under baseline conditions and how they changed during GABA CeA activation in 13 orexin−/−, VGAT-Cre mice.

We found that virtually all cataplexy attacks occurred when mice were engaged in purposeful behaviors such as wheel running, grooming, exploring, eating, or drinking, and rarely occurred during non-purposeful behaviors such as sitting stationary (Fig. 14A). During baseline recordings, 77% of cataplexy events occurred when mice were either wheel running or grooming, and only 23% of events occurred spontaneously or when mice were exploring, eating, or drinking (Fig. 14A). Remarkably, CeA activation almost exclusively triggered cataplexy attacks associated with either wheel running or grooming, increasing cataplexy triggered during wheel running by 385% (Wilcoxon signed-rank test, $W(12)=-78; p=0.0025$) and during grooming by 182% (paired t-test, $t(12)=2.878; p=0.0139$) (Fig. 14B). However, there were proportionally more attacks triggered by wheel running after GABA CeA activation at the expense of attacks triggered by other activities (Fig. 14A). Cataplexy associated with exploring (paired t-test, $t(12)=0, p=1.0000$), eating (Wilcoxon signed-rank test, $W(12)=-34, p=0.0843$), drinking (Wilcoxon signed-rank test, $W(12)=-20, p=0.1725$), and other behaviors (Wilcoxon signed-rank test, $W(12)=-1, p=1.0000$), did not change during CeA activation (Fig. 14B), suggesting that GABA CeA cells selectively facilitate cataplexy associated with rewarding stimuli such as wheel running.
Because CeA stimulation proportionally increased cataplexy triggered by wheel running and produced the largest increase in running-triggered cataplexy attacks, we wanted to determine whether this intervention impacted cataplexy expression by changing levels of wheel running activity. Although CeA activation did not change overall amounts of wheel running (paired t-test, t(12)=0.5476, p=0.5940) or the number of running bouts (W(12)=-54, p=0.0639), it significantly shortened running bout length (t(12)=2.366, p=0.0356; Figs. 14C-E). Running bouts were shortened because they were punctuated by more frequent cataplexy attacks during CeA activation. During baseline conditions, cataplexy occurred every 43 ± 13 min of wheel running, but during CeA activation it occurred every 10 ± 3 min of wheel running, thereby increasing the likelihood of running-induced cataplexy by 77% (paired t-test, t(9)=3.138; p=0.0120; Fig. 14F). We therefore suggest that activation of GABA CeA neurons lowers the threshold for triggering cataplexy associated rewarding stimuli such as wheel running.
Figure 14. GABA cells in the CeA mediate cataplexy associated with rewarding stimuli. A, In orexin-/-VGAT-Cre mice (n=13), cataplexy episodes were most often triggered by rewarding behaviors such as wheel running and grooming under baseline conditions. During GABA CeA activation, proportionally more cataplexy attacks were triggered by wheel running. B, GABA CeA activation significantly increased in the number of cataplexy episodes triggered by wheel running and grooming, without influencing cataplexy triggered by other behaviors. C, The increase in running-triggered cataplexy episodes was not due to more wheel running, since mice spent the same fraction of their time awake engaged in this activity. D-E, The number of wheel running bouts did not change, but the length of running bouts significantly decreased, as cataplexy more often interrupted wheel running. F, GABA CeA activation caused mice to experience cataplexy after significantly less wheel running compared to baseline conditions, indicating a reduction in the stimulus threshold required to trigger cataplexy. *, p < 0.05; **, p < 0.01.
Chapter 4  – Discussion

Our current results indicate that positive stimuli may trigger cataplexy in orexin$^{-/}$ mice by recruiting GABA neurons in the CeA. We found that activation of GABA CeA cells potently increased the frequency of cataplexy attacks without influencing their length, demonstrating that GABA cells play a role in triggering, but not maintaining, cataplexy. We also found that GABA cell activation only promotes cataplexy associated with rewarding conditions, suggesting that positive stimuli trigger cataplexy by activating GABA cells in the CeA.

4.1 The amygdala promotes cataplexy associated with positive emotion

Although the link between positive emotions and cataplexy was identified over 130 years ago, the pathways by which emotions trigger cataplexy remains speculative. A longstanding hypothesis in sleep medicine is that the amygdala plays a causal role in mediating cataplexy (Luppi et al., 2012; Dauvilliers et al., 2014; Fraigne et al., 2015). This idea is based on the fact that the amygdala is involved in processing emotional stimuli (Murray, 2007; Bonnet et al., 2015; Janak and Tye, 2015), and that cataplexy is typically elicited by positive stimuli (Nishino and Mignot, 1997; Espana et al., 2007; Clark et al., 2009; Overeem et al., 2011). Therefore, in the present study, we set out to test the role the amygdala plays in producing cataplexy associated with positive emotional stimuli.

Evidence indicates that the amygdala is functionally capable of mediating cataplexy, and may be a key component of the pathway by which positive stimuli elicit cataplexy. For example, imaging studies indicate that amygdala activity increases during cataplexy associated with emotional stimuli in humans (Hong et al., 2006; Meletti et al., 2015), and that the intensity of the amygdala response to positive stimuli is abnormally high in narcoleptics (Schwartz et al., 2008; Ponz et al., 2010). While these observations suggest that amygdala activity in response to positive stimuli may trigger cataplexy, it is unclear whether these changes in amygdala activity are a cause or consequence of cataplexy. Our current results support the idea that the amygdala, and particularly the CeA, plays a functional role in mediating emotion-triggered cataplexy,
because, in examining the behaviors that immediately preceded cataplexy attacks, we found that CeA activation preferentially increased cataplexy attacks associated with rewarding stimuli such as wheel running. These findings are consistent with the hypothesis that the amygdala, by virtue of its role in processing emotions, is important in initiating emotionally-induced cataplexy attacks, and also complement work demonstrating that CeA lesions reduce cataplexy associated with rewarding stimuli in orexin⁻/ mice (Burgess et al., 2013).

The amygdala also influences generalized arousal and motivated behaviors (Davis and Whalen, 2001; Sah et al., 2003; Tye and Janak, 2007; Robinson et al., 2014; Janak and Tye, 2015), and may promote REM sleep (Sanford et al., 1995; Calvo et al., 1996; Maquet et al., 1996; Sanford et al., 2002; Sanford et al., 2006). However, we found that CeA stimulation had no effect on either sleep-wake activity or the time mice spent running on wheels (a potential index of motivated behaviors), suggesting that changes in cataplexy are specific and primarily associated with augmented amygdala activity rather than secondary effects arising from behavioral changes associated with CeA activation. Furthermore, the fact that CeA activation increased cataplexy without concurrently influencing REM sleep may imply that these two states, while similar behaviorally, are ultimately governed by separate executive mechanisms. However, we also performed our experiments during the dark phase when mice spend less time asleep overall, and mice were given access to a running wheel which increases and consolidates wakefulness at the expense of sleep (Espana et al., 2007). As these conditions are not permissive to REM sleep, this too may explain why we did not observe substantial changes in this sleep state. Finally, we did not detect any changes in the time mice experienced sleep attacks, the other major symptom of narcolepsy, suggesting that cataplexy and sleep attacks are distinct symptoms controlled by different neural mechanisms.

4.2 GABA cells in the CeA mediate cataplexy

Despite evidence suggesting the amygdala is the neural substrate through which emotions trigger cataplexy, the specific cellular mechanism by which the amygdala promotes cataplexy had, until now, remained unidentified. Our current data suggest that GABA cells in the CeA play a permissive role in controlling cataplexy. We show that targeted stimulation of GABA CeA
cells triggered a 3.5-fold increase in cataplexy attacks, most of which were associated with rewarding conditions like wheel running, without affecting the length of cataplexy attacks. Moreover, the magnitude of this increase was comparable to that seen when CeA cells were activated independent of their neurochemical phenotype, suggesting that the influence of this brain area on cataplexy is principally or perhaps exclusively mediated by GABA cells in the CeA. This finding is particularly interesting because it suggests that previously identified non-GABAergic, and possibly glutamatergic, neurons originating in the CeA and projecting directly to the SubC may be less important for cataplexy, despite potentially playing a role in other functions (e.g., REM sleep) (Boissard et al., 2003; Xi et al., 2011). We therefore suggest that GABA neurons represent the main cellular mechanism in the CeA through which positive stimuli trigger cataplexy.

Our results are consistent with the current mechanistic model of cataplexy, which posits that cataplexy occurs when positive emotions lead to the untimely recruitment of brainstem circuits that inhibit muscle tone (Dauvilliers et al., 2014; Fraigne et al., 2015). We hypothesize that GABA cells in the CeA function as a “relay centre” between the cortical structures that interpret emotional stimuli and the brainstem circuits that generate motor paralysis during cataplexy (Fig. 15). This hypothesis is based on data showing that rewarding conditions activate the medial prefrontal cortex (mPFC), which innervates circuits within the amygdala (Vertes, 2004; Etkin et al., 2011; Oishi et al., 2013). Connections between the mPFC and CeA are integral in promoting cataplexy because removing either of them suppresses cataplexy in orexin−/− mice (Burgess et al., 2013; Oishi et al., 2013). GABA cells, which form the primary extrinsic pathway from the CeA (Nitecka and Ben-Ari, 1987; Pitkanen and Amaral, 1994; Sah et al., 2003), innervate the LC, LPT, and vIPAG, which collectively function to facilitate waking muscle tone by silencing atonia-generating regions in the dorsal pons (e.g., the SubC). We hypothesize that positive emotions elicit cataplexy by initially activating the mPFC, which subsequently activates GABA CeA cells that inhibit the LC, LPT, and vIPAG and thereby generate muscle paralysis (Fig. 15).

However, positive emotions do not trigger cataplexy in humans or animals that do not have narcolepsy. We hypothesize that the functional orexin system healthy individuals possess is necessary to protect against CeA-mediated cataplexy. Specifically, GABA-mediated inhibition originating from the CeA is offset by excitatory signaling from orexin neurons, which are also active during rewarding conditions (e.g., grooming and social reunion) (Lee et al., 2005; Mileykovskiy et
al., 2005; Siegel and Boehmer, 2006; Blouin et al., 2013; Giardino and de Lecea, 2014), and innervate the same structures as GABA CeA cells (e.g., LC, LPT, and vlPAG) (Peyron et al., 1998; Kiyashchenko et al., 2001; Mieda and Sakurai, 2012; Hasegawa et al., 2014). The loss of orexin signaling in narcolepsy upsets this balance, enabling GABA CeA cells to inhibit the LC, LPT, and vlPAG without opposition and thereby create an environment conducive to cataplexy (Fig. 9). Furthermore, orexin neurons innervate the amygdala and may help control its activity, as orexin levels in the amygdala increase during positive emotions and are closely correlated with hypothalamic orexin activity (Blouin et al., 2013). Finally, orexin signaling may also stabilize other monoaminergic or cholinergic cell groups which themselves influence muscle tone and arousal, thereby preventing dissociated states such as cataplexy from occurring (see Section 1.2.4).
Figure 15. The amygdala acts as a “relay centre” between the cortex and the pons that enables positive emotions to elicit cataplexy. Positive emotions activate cortical areas such as the mPFC, which in turn activates the CeA. GABA cells in the CeA then inhibit midbrain regions including the LC, LPT, and vPAG, which inhibit the atonia-generating network in the brainstem or facilitate muscle tone directly. Inhibition of these midbrain regions disinhibits the SubC-GIG circuit, which produces muscle paralysis during cataplexy by inhibiting skeletal motoneurons. In healthy individuals with an intact orexin system, CeA-mediated inhibition of the LC, LPT, and vPAG is counterbalanced by excitatory orexin inputs, which prevent positive emotions from triggering muscle atonia and cataplexy. CeA, central nucleus of the amygdala; GIG, gigantocellular nucleus; LC, locus coeruleus; LPT, lateral pontine tegmentum; mPFC, medial prefrontal cortex; SubC, subcoeruleus; vPAG, ventrolateral periaqueductal grey.
4.3 GABA cells in the CeA are responsible for initiating cataplexy

Our current results indicate that the CeA plays a role in triggering, but not maintaining, cataplexy because we show that CeA activation increases the frequency, but not the duration, of cataplexy attacks. If CeA activity contributed to the length of cataplexy, its activation should have produced *status cataplecticus* (prolonged periods of cataplexy) (Overeem et al., 2001; Dauvilliers et al., 2007; Dauvilliers et al., 2014). Since we found no change in the length of cataplexy episodes, our results suggest that the CeA functions primarily as a cataplexy “on switch” by gating the entrance into cataplexy, while other brain areas are responsible for maintaining and/or terminating cataplexy attacks. The circuit mechanisms that function to switch off cataplexy remain unidentified.

The CeA may increase the incidence of cataplexy by lowering the stimulus threshold for attack induction. To test this hypothesis, we examined wheel-running behavior in our *orexin*−/− mice as an index of this threshold. Although CeA activation did not change overall amounts of wheel running in our mice, it significantly shortened the length of individual running bouts because they were punctuated by more frequent cataplexy attacks. Moreover, CeA stimulation caused mice to experience cataplexy attacks after 77% less wheel running compared to control conditions, suggesting that CeA activation lowered the threshold stimulus required to trigger cataplexy. These findings are consistent with the idea that the amygdala acts as a cataplexy “on switch” during positive emotions, particularly in light of the observation that cataplexy occurs most often in response to positive emotions in humans (Overeem et al., 2011), and that the probability of an attack is significantly elevated in narcoleptic animals exposed to positive stimuli such as palatable food and running wheels (Nishino and Mignot, 1997; Espana et al., 2007; Clark et al., 2009). These findings are also consistent with studies demonstrating that the amygdala response to positive emotions is heightened in human narcolepsy (Schwartz et al., 2008; Ponz et al., 2010), which may indicate that affected individuals are “primed” to experience cataplexy.
4.4 Additional circuits involved in cataplexy

Additional brain circuits may be involved in producing cataplexy. For instance, neuroimaging studies of narcoleptic humans have identified that, like the amygdala, other emotion-processing centres such as the ventral tegmental area and nucleus accumbens are highly active during cataplexy and may be anatomically positioned to influence muscle tone independent of the CeA (Groenewegen and Russchen, 1984; Usuda et al., 1998; Meletti et al., 2015). Moreover, recent work in narcoleptic mice provides evidence for a cataplexy-promoting circuit originating in the mPFC but parallel to the CeA that involves MCH neurons of the hypothalamus (Oishi et al., 2013). Like the CeA, these neurons project to brainstem areas that regulate muscle tone and appear to be active during cataplexy. Cells in the zona incerta, laterodorsal tegmentum, and dorsal pons have been shown to suppress cataplexy (Burlet et al., 2002; Liu et al., 2011; Blanco-Centurion et al., 2013; Oishi et al., 2013). Drugs that affect cholinergic tone (e.g., cholinesterase inhibitors) can also worsen cataplexy, suggesting involvement of the cholinergic system in the cataplexy circuit (Reid et al., 1994a; Kalogiannis et al., 2011; Kohlmeier et al., 2013b), while the noradrenergic, serotonergic, and dopaminergic systems may also contribute to cataplexy since their activation can influence cataplexy (Mignot et al., 1988; Mieda et al., 2004; Liu et al., 2008; Burgess et al., 2010; Burgess and Peever, 2013; Hasegawa et al., 2014). Our current results indicate that GABA cells in the CeA play a major role initiating cataplexy attacks, but it is likely that other brain regions and neurotransmitter systems, such as those reviewed here, contribute to this in addition to other features of cataplexy attacks, such as their length. Although multiple brain circuits, including GABA cells in the CeA, are clearly involved in controlling cataplexy, it remains unclear how they communicate with and influence one another, and understanding how these systems function together represents a major challenge in identifying circuit mechanisms of cataplexy.

4.5 Technical considerations

While our study provides strong functional evidence that GABA cells in the CeA mediate cataplexy triggered by positive emotions, it cannot identify the specific extra-amygdalar circuits and brain targets by which these cells exert their effects. However, most extrinsic projections of
the CeA are descending GABAergic projections that innervate brainstem regions associated with various autonomic functions, with dense projections to those that regulate muscle tone such as the LC, LPT, and vPAG (Nitecka and Ben-Ari, 1987; Pitkanen and Amaral, 1994; Burgess et al., 2013). The CeA is therefore anatomically positioned to influence cataplexy through one or more of these nuclei, and although the contribution of CeA inputs to each of these regions in the context of cataplexy is beyond the scope of this study, it can be evaluated with other more spatially-restricted technologies currently available (e.g., optogenetics). Additionally, though we could not identify the activation profile of CeA neurons in relation to cataplexy, a great deal of evidence suggests that amygdala activity is tightly correlated to cataplexy attacks. For example, recordings of individual CeA neurons in narcoleptic dogs demonstrate a close association of firing activity to the onset and offset of cataplexy attacks, while cataplexy in humans is characterized by hyperperfusion within the amygdala (Gulyani et al., 2002; Hong et al., 2006).

To identify the functional role of GABA CeA cells in regulating cataplexy, we developed a new transgenic orexin−/− mouse line that enabled us to specifically target and manipulate GABA cells. After detailed analysis we showed that orexin−/−,VGAT-Cre mice were phenotypically indistinguishable from orexin−/− mice, because they experience the same quality and quantity of cataplexy, as well as comparable amounts of NREM sleep, REM sleep, wakefulness, and sleep attacks as conventional orexin−/− mice. We also showed that hM3Dq receptors could be virally delivered to GABA cells, and that mass activation of GABA cells in the CeA produced robust and consistent increases in cataplexy incidence. These mice could be a valuable resource for dissecting the roles that other GABA circuits (e.g., GABA vPAG cells) play in controlling cataplexy and other features of narcolepsy such as excessive sleepiness. They will also be useful for determining how GABA mechanisms influence other behaviors that are orexin-dependent (e.g., addiction).

4.6 Conclusions

Our current data provide the first evidence to show that positive or rewarding stimuli may trigger cataplexy by recruiting GABA CeA cells in orexin−/− mice. We propose that GABA CeA cells function as a “relay center” between the cortical structures that interpret emotional stimuli
and the brainstem circuits that trigger motor paralysis during cataplexy. Our findings are consistent with studies showing amygdala activation during cataplexy in narcoleptic humans and dogs, and lesion studies in narcoleptic mice demonstrating that the amygdala promotes cataplexy. The CeA is a primary output nucleus of the amygdala and is especially important in coordinating the body’s physiological response to emotion (LeDoux, 2007). Part of this response may include transient losses of muscle tone, as healthy individuals often experience feeling “weak with laughter” which is associated with a diminished monosynaptic H-reflex (Overeem et al., 1999). In narcolepsy, cataplexy may represent an extreme motor response of the body to emotion in which not just the H-reflex but all postural muscle tone is completely lost (Guilleminault et al., 1974), and the neural origin of cataplexy may involve unfettered CeA output that arises with the loss of orexin neurons. Our results also point to the GABA CeA cells as a possible target of sodium oxybate, the most effective anti-cataplectic medication to date, which is thought to inhibit neurons via a GABA$_B$ receptor-mediated pathway (Black et al., 2014), suggesting that targeted reductions in amygdala activity could be used to alleviate cataplexy in narcolepsy. In addition to identifying a novel amygdala circuit involved in generating cataplexy, our work provides a foundation with which to examine the interactions amygdala cells have with other brain areas in the context of cataplexy, and underscores the potential in developing novel cataplexy therapies aimed at mitigating amygdala activity.
References


Hitchcock J, Davis M (1986) Lesions of the amygdala, but not of the cerebellum or red nucleus, block conditioned fear as measured with the potentiated startle paradigm. Behav Neurosci 100:11-22.


Appendix 1 – Behavioral State Analysis Macro

The following is an Excel VBA macro (version date: March 17, 2015) coded by Matthew Snow that was used to automatically and accurately calculate parameters relating to behavioral state architecture and muscle tone using output generated by the “5s epoch analysis” Spike 2 script. In a dedicated Microsoft Excel file, users are prompted for the states they wish to analyze and the time intervals in which they wish to bin their data, and the macro then performs all relevant calculations based on this input.

Private Sub AnalyzeButton_Click()
    Dim StateCombo() As String
    Dim StateName() As String
    Dim TreatmentName() As String
    Dim MasseterTone() As Single
    Dim NeckTone() As Single
    Dim PercentOfTime As Single
    Dim Continue As Boolean
    Dim BinNumber As Integer
    Dim DataColumn As Integer
    Dim DataRow As Integer
    Dim i As Integer
    Dim MasseterCounter() As Integer
    Dim NeckCounter() As Integer
    Dim NumberOfBins As Integer
    Dim NumberOfEpochs As Integer
    Dim NumberOfStates As Integer
    Dim StateNamePos1 As Integer
    Dim StateNamePos2 As Integer
    Dim TableColumn As Integer
    Dim TableRow As Integer
    Dim Transitions() As Integer
    Dim TransitionsPos As Integer
    Dim TreatmentNamePos As Integer
    Dim variable As String

    Sheets(2).Cells.Clear

    'NumberOfStates = UBound(Split(StateInput, "|")) - LBound(Split(StateInput, "|")) + 1 'Defines value of NumberOfStates
    StateName() = Split(StateInput, "|") 'Populates StateName()
    NumberOfStates = UBound(StateName) + 1
ReDim StateCombo(NumberOfStates ^ 2 - 1) As String 'Defines size of StateCombo()

DataColumn = 1
TreatmentNamePos = 0
Sheets(1).Activate

Do While Cells(1, DataColumn) <> "" 'Counts the number of treatments listed on Raw Data sheet
    TreatmentNamePos = TreatmentNamePos + 1
    DataColumn = DataColumn + 6
Loop

ReDim TreatmentName(TreatmentNamePos - 1) As String
DataColumn = 1

For TreatmentNamePos = 0 To UBound(TreatmentName) 'Populates TreatmentName()
    TreatmentName(TreatmentNamePos) = Cells(1, DataColumn)
    DataColumn = DataColumn + 6
Next TreatmentNamePos

TableRow = 1
TableColumn = 1
DataColumn = 1

For TreatmentNamePos = 0 To UBound(TreatmentName) 'Repeats all calculations for each treatment

    Sheets(1).Activate
    NumberOfEpochs = WorksheetFunction.CountA(Range(Cells(5, DataColumn), Cells(130000, DataColumn))) 'Defines value of NumberOfEpochs. 130000 is an arbitrary limit and can be changed depending on need. Chosen to be suitable for up to 1 week nonstop recording (720 epochs/hour * 24 hours/day * 7 days/week = 120960)
    NumberOfBins = (NumberOfEpochs * 5 / 3600) / Val(TimeInput) 'Defines value of NumberOfBins
    If NumberOfEpochs > NumberOfBins * Val(TimeInput) * 720 Then
        NumberOfBins = NumberOfBins + 1 'Ensures the last bin is counted when it contains less than the maximum number of epochs (e.g., recording stopped in middle of bin)
    
    Sheets(2).Activate
    Columns("B").ColumnWidth = 5

With Range(Cells(TableRow, TableColumn), Cells(TableRow + 2 * NumberOfBins + 10, TableColumn)) 'creates/formats title cell for treatment on State Analysis sheet
    .MergeCells = True
    .Value = TreatmentName(TreatmentNamePos)
    .Orientation = 90
    .HorizontalAlignment = xlCenter
    .VerticalAlignment = xlCenter
    .Font.Bold = True
    .Font.Size = 30
    .Interior.Color = RGB(204, 255, 204)
    .Borders(xlEdgeBottom).LineStyle = xlContinuous
.Borders(xlEdgeTop).LineStyle = xlContinuous
.Borders(xlEdgeLeft).LineStyle = xlContinuous
.Borders(xlEdgeRight).LineStyle = xlContinuous
.Borders.Weight = xlThick
End With

For i = 1 To 6 'Creates/formats tables for non-transitions variables
to display result outputs
If i < 6 Then
    With Range(Cells(TableRow, TableColumn + 2), Cells(TableRow + 1, TableColumn + NumberOfStates + 3)) 'Creates/formats table title cells for non-transitions variables
        .MergeCells = True
        If i = 1 Then variable = "Time Spent in State (%)"
        If i = 2 Then variable = "Number of Episodes"
        If i = 3 Then variable = "Average Duration of Episodes (s)"
        If i = 4 Then variable = "Average Muscle Activity - Masseter"
        If i = 5 Then variable = "Average Muscle Activity - Neck"
        .Value = variable
        .HorizontalAlignment = xlCenter
        .VerticalAlignment = xlCenter
        .Font.Bold = True
        .Font.Size = 18
        .Interior.Color = RGB(136, 192, 254)
        .Borders(xlEdgeBottom).LineStyle = xlContinuous
        .Borders(xlEdgeTop).LineStyle = xlContinuous
        .Borders(xlEdgeLeft).LineStyle = xlContinuous
        .Borders(xlEdgeRight).LineStyle = xlContinuous
        .Borders.Weight = xlThick
    End With

    With Range(Cells(TableRow + 3, TableColumn + 4), Cells(TableRow + 3, TableColumn + NumberOfStates + 3)) 'Creates/formats state name title cell for non-transitions variables
        .MergeCells = True
        .Value = "State"
        .HorizontalAlignment = xlCenter
        .VerticalAlignment = xlCenter
        .Font.Bold = True
        .Font.Color = RGB(254, 195, 10)
        .Borders(xlEdgeBottom).LineStyle = xlContinuous
        .Borders(xlEdgeTop).LineStyle = xlContinuous
        .Borders(xlEdgeLeft).LineStyle = xlContinuous
        .Borders(xlEdgeRight).LineStyle = xlContinuous
        .Borders.Weight = xlThick
    End With

With Range(Cells(TableRow + 4, TableColumn + 4), Cells(TableRow + 4, TableColumn + NumberOfStates + 3)) 'Creates/formats/populates state name column titles for non-transitions variables
    .Value = StateName()
    .HorizontalAlignment = xlCenter
    .VerticalAlignment = xlCenter
    .Font.Bold = True
    .Borders(xlEdgeBottom).LineStyle = xlContinuous
For BinNumber = 0 To NumberOfBins - 1 'Numbers bins in all tables
    Cells(TableRow + 5, TableColumn + 3) = BinNumber + 1
    If i = 1 Then Cells(TableRow + NumberOfBins + 11, TableColumn + 3) = BinNumber + 1 'Numbers bins in transitions table during first iteration
    TableRow = TableRow + 1
Next BinNumber
TableRow = TableRow - NumberOfBins 'Resets value of TableRow
TableColumn = TableColumn + NumberOfStates + 3 'Sets
TableColumn value to the start of the next non-transitions variable table
Else
    TableColumn = 1
End If

With Range(Cells(TableRow + NumberOfBins + 7, TableColumn + 2), Cells(TableRow + NumberOfBins + 8, TableColumn + NumberOfStates ^ 2 + 3))
    'Creates/formats table title cell for transitions
    .MergeCells = True
    .Value = "Transitions"
    .HorizontalAlignment = xlCenter
    .VerticalAlignment = xlCenter
    .Font.Bold = True
    .Font.Size = 18
    .Interior.Color = RGB(136, 192, 254)
    .Borders(xlEdgeBottom).LineStyle = xlContinuous
    .Borders(xlEdgeTop).LineStyle = xlContinuous
    .Borders(xlEdgeLeft).LineStyle = xlContinuous
    .Borders(xlEdgeRight).LineStyle = xlContinuous
    .Borders.Weight = xlThick
End With

With Range(Cells(TableRow + NumberOfBins + 11, TableColumn + 2), Cells(TableRow + 2 * NumberOfBins + 10, TableColumn + 2))
    'Creates/formats bin number title cell for transitions
    .MergeCells = True
    .Value = "Bin # (" & Val(TimeInput) & ", hour bins)"
    .WrapText = True
    .HorizontalAlignment = xlCenter
    .VerticalAlignment = xlCenter
    .Font.Bold = True
    .Interior.Color = RGB(254, 195, 10)
    .Borders(xlEdgeBottom).LineStyle = xlContinuous
    .Borders(xlEdgeTop).LineStyle = xlContinuous
    .Borders(xlEdgeLeft).LineStyle = xlContinuous
    .Borders(xlEdgeRight).LineStyle = xlContinuous
    .Borders.Weight = xlThick
End With

With Range(Cells(TableRow + NumberOfBins + 10, TableColumn + 4), Cells(TableRow + NumberOfBins + 10, TableColumn + NumberOfStates ^ 2 + 3))
    'Creates/formats column title cells for transitions
End With

With Range(Cells(TableRow + NumberOfBins + 11, TableColumn + 3), Cells(TableRow + 2 * NumberOfBins + 10, TableColumn + 3))
    'Creates/formats cells for row titles in transitions tables
End With
With Range(Cells(TableRow + NumberOfBins + 11, TableColumn + 4), Cells(TableRow + 2 * NumberOfBins + 10, TableColumn + NumberOfStates ^ 2 + 3)) 'Creates/formats cells for column titles in transitions tables
.BorderAlignment = xlCenter
.VerticalAlignment = xlCenter
.Font.Bold = True
Borders(xlEdgeBottom).LineStyle = xlContinuous
.Borders(xlEdgeTop).LineStyle = xlContinuous
.Borders(xlEdgeLeft).LineStyle = xlContinuous
.Borders(xlEdgeRight).LineStyle = xlContinuous
.Borders.Weight = xlMedium
End With

With Range(Cells(TableRow + NumberOfBins + 11, TableColumn + 4), Cells(TableRow + 2 * NumberOfBins + 10, TableColumn + NumberOfStates ^ 2 + 3)) 'Creates/formats cells for column titles in transitions tables
.BorderAlignment = xlCenter
.VerticalAlignment = xlCenter
.Interior.Color = RGB(255, 255, 135)
.Borders(xlEdgeBottom).LineStyle = xlContinuous
.Borders(xlEdgeTop).LineStyle = xlContinuous
.Borders(xlEdgeLeft).LineStyle = xlContinuous
.Borders(xlEdgeRight).LineStyle = xlContinuous
.Borders.Weight = xlThin
End With

End If
Next i

For BinNumber = 0 To NumberOfBins - 1 'Calculates data for each variable for each bin
Sheets(1).Activate
ReDim Transitions(NumberOfStates ^ 2 - 1) As Integer 'Defines size of Transitions()

For StateNamePos1 = 0 To NumberOfStates - 1 'Counts the number of transitions between states. Transitions are in the form of State1 to State2. This loop changes State1 to the next state to create a new search combination.
For StateNamePos2 = 0 To NumberOfStates - 1 'Transitions are in the form of State1 to State2. This loop changes State2 to the next state to create a new search combination.
Continue = True
For DataRow = Val(TimeInput) * 720 * BinNumber + 4 To Val(TimeInput) * 720 * (BinNumber + 1) + 3 'Searches for target transition in the current bin
If BinNumber = 0 And Continue = True Then 'Populates StateCombo() and transitions table column titles during the first iteration of this loop only (i.e., the first bin of the first treatment) to be used for all remaining calculations.
StateCombo(TransitionsPos) = StateName(StateNamePos1) & " to " & StateName(StateNamePos2)
Sheets(2).Activate
Cells(TableRow + NumberOfBins + 10, TableColumn + 4) = StateCombo(TransitionsPos)
Sheets(1).Activate
Continue = False
End If

If Cells(DataRow, DataColumn) = StateName(StateNamePos1) And Cells(DataRow + 1, DataColumn) = StateName(StateNamePos2) Then 'Counts the number of target transitions by
adding 1 each time a target transition is found. Sum is stored in Transitions() at the location corresponding to the transition name in StateCombo()

\[
\text{Transitions(TransitionsPos) = Transitions(TransitionsPos) + 1}
\]

End If

Next DataRow

Sheets(2).Activate

Cells(TableRow + NumberOfBins + 11, TableColumn + 4) = Transitions(TransitionsPos) 'Fills the appropriate cell in the transitions table with the transitions number

Sheets(1).Activate

DataRow = 5 + Val(TimeInput) * 720 * BinNumber 'Resets value of DataRow to the start of the current bin

TableColumn = TableColumn + 1

TransitionsPos = TransitionsPos + 1

Next StateNamePos2

DataRow = 5 + Val(TimeInput) * 720 * BinNumber 'Resets value of DataRow to the start of the current bin

Next StateNamePos1

DataRow = 5
TableColumn = 1

TransitionsPos = 0
StateNamePos2 = 0

Sheets(2).Activate

For StateNamePos1 = 0 To UBound(StateName) 'Calculates number of episodes for each state for the current bin

For TransitionsPos = TransitionsPos To StateNamePos1 + (NumberOfStates - 1) * (StateNamePos1 + 1) 'Transitions are in the form of State1 to State2. Repeats for as many times as there are combinations beginning with the current State1.

Cells(TableRow + 5, TableColumn + NumberOfStates + 7) = Cells(TableRow + 5, TableColumn + NumberOfStates + 7) + Transitions(TransitionsPos) 'Transitions are in the form of State1 to State2. Sums the all the values in Transitions() corresponding to the same State1.

If TransitionsPos = StateNamePos1 * (NumberOfStates + 1) Then 'Subtracts the current value of Transitions() if State1=State2 (i.e., state has not changed)

Cells(TableRow + 5, TableColumn + NumberOfStates + 7) = Cells(TableRow + 5, TableColumn + NumberOfStates + 7) - Transitions(TransitionsPos)

End If

Sheets(1).Activate

If Cells(4 + Val(TimeInput) * 720 * BinNumber, DataColumn) = StateName(StateNamePos1) And Cells(5 + Val(TimeInput) * 720 * BinNumber, DataColumn) = StateName(StateNamePos2) And StateNamePos1 <> StateNamePos2 Then 'Subtracts 1 from current number of episodes count for the current State1 if it occurs in the epoch preceding the first epoch of the current bin (to account for the one more transition than epoch for any bin)

Sheets(2).Activate
Cells(TableRow + 5, TableColumn + NumberOfStates + 7) = Cells(TableRow + 5, TableColumn + NumberOfStates + 7) - 1
Else
    Sheets(2).Activate
End If

StateNamePos2 = StateNamePos2 + 1
Next TransitionsPos

StateNamePos2 = 0
Sheets(1).Activate

If WorksheetFunction.Index(Range(Cells(5, DataColumn), Cells(4 + Val(TimeInput) * 720 * (BinNumber + 1), DataColumn)), WorksheetFunction.CountA(Range(Cells(5, DataColumn), Cells(4 + Val(TimeInput) * 720 * (BinNumber + 1), DataColumn))), 1) = StateName(StateNamePos1) Then
    'Adds 1 to the current number of episodes for the current State1 if the current State1 is the final epoch of the current bin
    Sheets(2).Activate
    Cells(TableRow + 5, TableColumn + NumberOfStates + 7) =
    Cells(TableRow + 5, TableColumn + NumberOfStates + 7) + 1
Else
    Sheets(2).Activate
End If

TableColumn = TableColumn + 1
Next StateNamePos1

TableColumn = 1
StateNamePos1 = 0

For StateNamePos1 = 0 To UBound(StateName) 'Calculates the percent time spent in and average durations of each state for the current bin
    Sheets(1).Activate
    PercentOfTime =
    WorksheetFunction.CountIf(Range(Cells(Val(TimeInput) * 720 * BinNumber + 5, DataColumn), Cells(Val(TimeInput) * 720 * (BinNumber + 1) + 4, DataColumn)), StateName(StateNamePos1)) /
    WorksheetFunction.CountA(Range(Cells(Val(TimeInput) * 720 * BinNumber + 5, DataColumn), Cells(Val(TimeInput) * 720 * (BinNumber + 1) + 4, DataColumn)))
    'Calculates percent of time in state by dividing the number of epochs of that state by the total number of epochs in that bin
    Sheets(2).Activate
    With Cells(TableRow + 5, TableColumn + 4) 'Fills the corresponding cell in the percent of time table with the current value of PercentOfTime
        .Value = PercentOfTime
        .NumberFormat = "0.00%"
    End With

If Cells(TableRow + 5, TableColumn + NumberOfStates + 7) <> 0 Then
    'Calculates the average duration of episodes of the corresponding state as long as the state occurred (i.e., the number of episodes is not 0)
    With Cells(TableRow + 5, TableColumn + 2 * NumberOfStates + 10) 'Fills the corresponding cell in the average duration of episodes table
        .Value = 
        .NumberFormat = "0.00"
    End With
End If
.Value = WorksheetFunction.CountIf(Range(Cells(DataRow + Val(TimeInput) * 720 * BinNumber, DataColumn), Cells(DataRow + Val(TimeInput) * 720 * (BinNumber + 1) - 1, DataColumn)), StateName(StateNamePos1)) * 5 / Sheets(2).Cells(TableRow + 5, TableColumn + NumberOfStates + 7) 'Calculates the average duration by dividing the total time spent in the state (for the current bin) by the number of episodes of that state (for the current bin)
Sheets(2).Activate .NumberFormat = "0.00"
End With
Else
Cells(TableRow + 5, TableColumn + 2 * NumberOfStates + 10) = 0 'If there are no episodes of the corresponding state in the current bin, the average duration is set to 0
End If

TableColumn = TableColumn + 1
Next StateNamePos1
Sheets(1).Activate
ReDim MasseterTone(NumberOfStates) As Single
ReDim MasseterCounter(NumberOfStates) As Integer
ReDim NeckTone(NumberOfStates) As Single
ReDim NeckCounter(NumberOfStates) As Integer
For DataRow = Val(TimeInput) * 720 * BinNumber + 5 To Val(TimeInput) * 720 * (BinNumber + 1) + 4 'Calculates average muscle tone for masseter and neck
For StateNamePos2 = 0 To UBound(StateName)
If Cells(DataRow, DataColumn) = StateName(StateNamePos2) Then
If Cells(DataRow, DataColumn + 3) <> "" Then
MasseterTone(StateNamePos2) = MasseterTone(StateNamePos2) + Cells(DataRow, DataColumn + 3)
MasseterCounter(StateNamePos2) = MasseterCounter(StateNamePos2) + 1
End If
If Cells(DataRow, DataColumn) = StateName(StateNamePos2) And Cells(DataRow, DataColumn + 4) <> "" Then
NeckTone(StateNamePos2) = NeckTone(StateNamePos2) + Cells(DataRow, DataColumn + 4)
NeckCounter(StateNamePos2) = NeckCounter(StateNamePos2) + 1
End If
Exit For
End If
Next StateNamePos2
Next DataRow
Sheets(2).Activate
TableColumn = 1
For StateNamePos2 = 0 To UBound(StateName)
If MasseterCounter(StateNamePos2) <> 0 Then
With Cells(TableRow + 5, TableColumn + NumberOfStates * 3 + 13) 'Fills masseter activity table
.Value = MasseterTone(StateNamePos2) / 
MasseterCounter(StateNamePos2)
 .NumberFormat = "0.000000"
End With
Else
With Cells(TableRow + 5, TableColumn + NumberOfStates * 3 + 13) 'Fills masseter activity table
 .Value = "no data"
End With
End If
If NeckCounter(StateNamePos2) <> 0 Then
With Cells(TableRow + 5, TableColumn + NumberOfStates * 4 + 16) 'Fills neck activity table
 .Value = NeckTone(StateNamePos2) / 
NeckCounter(StateNamePos2)
 .NumberFormat = "0.000000"
End With
Else
With Cells(TableRow + 5, TableColumn + NumberOfStates * 4 + 16) 'Fills neck activity table
 .Value = "no data"
End With
End If
TableColumn = TableColumn + 1
Next StateNamePos2
TableRow = TableRow + 1
TableColumn = 1
TransitionsPos = 0
Next BinNumber
DataColumn = DataColumn + 6 'Sets DataColumn to the column of the next treatment
 TableRow = TableRow + NumberOfBins + 15 'Sets TableRow to the next available space to output results for the next treatment
 Next TreatmentNamePos
Unload Me
End Sub