

Sleeping Beauty, Mice, & Dogs: Cell Death in Narcolepsy

D'Anne Duncan*

Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

[Role Playing Jerome M. Siegel

Department of Psychiatry,
University of California at Los Angeles
Los Angeles, California]

Summary

Sleep is important and required for the survival and normal homeostasis of vertebrates. Disturbances in the sleep-wake cycle can lead to many sleep disorders, one of which is narcolepsy. Narcolepsy is a disabling sleep disorder characterized by excessive daytime sleep, cataplexy (sudden loss of muscle tone in response to strong emotion or laughter), hallucinations, and sleep paralysis. To date, the pathological and biological basis of narcolepsy is poorly understood. My lab first discovered, in canines and humans, dysfunctional neurons that affect the sleep-wake cycle in narcolepsy, as well as neurodegeneration of brainstem and hypothalamic neurons. Other labs have identified mutations in hypocretin (orexin) receptor genes in canine narcoleptics. An orexin knockout mice model mimics the narcoleptic phenotype seen in humans and dogs. More recently, a hypocretin gene mutation has been identified in at least one human narcoleptic. Given that orexin is critical for the regulation of feeding behavior, opens the door to investigate energy homeostasis in narcolepsy, as well as in finding a treatment.

Sleep

Sleep is important and required for the survival and normal homeostasis of vertebrates¹. Sleep occurs in all mammals; if deprived of it the outcome can be fatal¹. The sequence of brain states that define sleep are governed by a series of controlled brain states, by brainstem nuclei that project throughout the brain and spinal cord¹.

Many physiological changes take place during non-REM and REM sleep. Non-REM sleep is characterized by decreases in muscle tone, heart rate, breathing, blood pressure, and metabolic rate. In contrast, during REM sleep, there is an increase in blood pressure, heart rate, and metabolism, as much as seen in the awake state¹. The most prominent area of the brain that governs sleep and wakefulness is the hypothalamus, which is the regulator of temperature, heart rate, blood pressure, food and water intake, other

brainstem nuclei, and, most importantly, the sleep-wake cycle.

Humans descend into REM after an hour or so of sleeping. In the drowsy period, also known as Stage I sleep, the frequency spectrum of the electroencephalogram (EEG) is shifted to lower numbers, and the amplitude of cortical waves slightly increases. Stage II is characterized by the further decrease in the frequency of the EEG waves, and an increase in amplitude as well as the increase of high-frequency spike clusters called sleep spindles. Sleep spindles are periodic bursts of activity at about 10-12 Hz. Stage III represents deep sleep, in which the number of sleep spindles decreases. The deepest level of sleep, known as stage IV, consists of low activity of EEG, and high-amplitude fluctuations known as delta waves. All of these stages are called non-rapid eye movements (non-REM) sleep, and the feature is slow-wave sleep; it is most difficult to awaken people from this stage of sleep. Following non-REM sleep, rapid eye movement (REM) sleep, in which EEG recordings are similar to the awake state. The brainstem nuclei responsible for wakefulness are the cholinergic nuclei of the pons-midbrain junction, locus coeruleus, and raphe nuclei; in this state these brainstem neurons are active. A malfunction in one of these brainstem nuclei can lead to one of the many sleep disorders: insomnia, sleep apnea, "restless legs syndrome" and the most well understood biologically, narcolepsy¹.

Narcolepsy

Narcolepsy is a disabling sleep disorder characterized by excessive sleepiness during normal waking hours². In other words, an individual with narcolepsy has frequent "REM sleep attacks" during the day without going through non-REM sleep¹. It affects approximately 1 in 2,000 individuals (about 125,000 individuals in the U.S.), with both sexes being equally affected, and it develops in the second or third decade of life with symptoms progressing over a period of 1 or more years and then stabilizing³.

Signs and Symptoms

Narcolepsy is characterized by excessive daytime sleepiness (EDS), which is the first sign and important symptom; cataplexy, which is the sudden loss of muscle tone in response to strong emotions such as laughter or anger; hyponagogic hallucinations, dream-like experiences occurring at the onset of sleep, and sleep paralysis, the inability to move while falling asleep or upon waking⁴. Narcolepsy is defined by the presence of sleepiness and cataplexy or by the polysomnographic documentation of REM sleep abnormalities. The most commonly accepted diagnostic test is the multiple sleep latency test (MSLT), in which nocturnal sleep somnography is performed followed the next day by 4 to 5 daytime naps in which sleep latency is measured⁴.

Treatments

The most common treatment of narcolepsy, EDS, is treated with amphetamine-like stimulants or modafinil, drugs that stimulate dopamine release as well as inhibit

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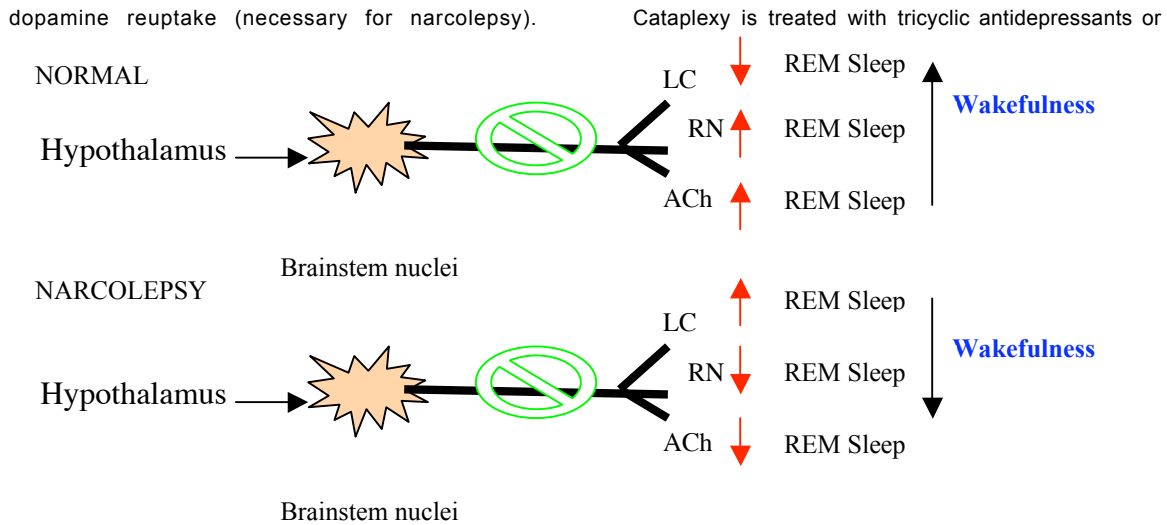


Figure 1. A Working Model for Narcolepsy

Normally, hypothalamic neurons project onto three specific brainstem nuclei involved in arousal regulation; locus coeruleus (LC), raphe nucleus (RN), and cholinergic (ACh) neurons; all of which when active induces wakefulness in organisms. In narcolepsy, the death of hypothalamic neurons may cause these brainstem nuclei to become inactive, and induce REM sleep, decreasing the onset of wakefulness.

higher doses of serotonin reuptake inhibitors⁴. Even though there are treatments to reduce the onset of the symptoms of narcolepsy, there is no specific cure.

Pathology

Hypocretin (orexin) neurons have been found to be associated with the pathology of narcolepsy. These neurons project to brainstem nuclei that are associated with motor inhibition, and more importantly to locus coeruleus (LC), raphe, and acetylcholine nuclei. Hypocretin neurons also project to forebrain regions, including the hypothalamus and amygdala. It has been illustrated that loss of the hypocretin system could in fact cause cataplexy through the inhibition of the brainstem's motor excitatory system or exciting the brainstem's motor inhibitory system (See Figure1). The loss of hypocretin facilitates sleepiness by decreasing the body's arousal system³.

Gaps in Knowledge

Even though there is an understanding of the pathology of narcolepsy, scientists are still unaware of the mechanism that leads to the loss of the hypocretin system. Are these neurons dying? Also, the genetics and cause of human narcolepsy are still unknown. Scientists chose to examine the cellular mechanism associated with the onset of narcolepsy.

Animals of Choice

Narcolepsy was established in canines, more specifically reported in Dachshunds and Poodles, to be transmitted through autosomal recessive inheritance³. More recently, narcolepsy has been induced in other breeds. These breeds of dogs, Doberman Pinschers and Labrador Retrievers, were used to establish a narcoleptic dog-breeding colony at Standard in 1976⁴. These narcoleptic dogs display the same symptoms as human narcoleptics; both display emotionally triggered cataplexy, fragmented sleep, and short sleep latency⁴. Cataplexy can be easily induced in these animals by presenting them with food or by play⁴. There have also been mice models that display similar narcoleptic phenotype.

First Suggestions on Narcolepsy

In narcoleptic dogs, as well as humans, cataplexy is one of the most prominent signs of narcolepsy; it was suggested that cataplectic neurons are not being triggered for a successful sleep/wake cycle. In 1991, the first investigation of cataplectic neurons took place in my lab. We investigated noncholinergic cells in the medial medulla of the narcoleptic dogs. We found that these cataplexy-off neurons had a decreased firing rate during cataplexy, while brain activity was active. These neurons were in fact active during REM sleep as well. Also discovered was that neurons did not fire at all during cataplexy, while during quiet and active waking and REM sleep specific neurons had a higher discharging rate than during cataplexy⁵.

This study suggests that there was an abnormal discharge patterns of certain neurons during the onset of cataplexy. The next approach we needed to take was to determine the specific neurons that are discharging during cataplectic attacks in canine narcoleptics.

Locus Coeruleus Neurons in Canine Narcoleptics

Following the study conducted in 1991, eight years later, the identification of locus coeruleus neurons firing during cataplexy was completed. As mentioned previously, locus coeruleus (LC) nuclei are involved with the onset of wakefulness in the sleep/wake cycle. LC neurons fire during both quiet and active waking; they discharge during arousal in response to stress as well as sensory stimulation⁶.

In this study, LC neurons were recorded during normal sleep states and during cataplexy in narcoleptic dogs to evaluate the activity of LC neurons in narcolepsy. Our results suggested that LC neurons were discharging at a lower rate during cataplexy than that seen in REM sleep. Due to the loss of LC neurons, we came to the conclusion that these neurons played a role in the loss of muscle tone (characterized by cataplexy) and REM sleep: two major symptoms of canine and human narcolepsy. The identification of

these neurons allowed us to determine if they were undergoing cell death due to the lower discharge rate⁶.

Are Cells Dying Causing Narcolepsy?

In 1999, my lab conducted another experiment in which we tested the hypothesis of neurodegeneration causing narcoleptic symptoms in canines. At that time, my lab established that narcolepsy is not a progressive disorder, meaning that once symptoms are established, they do not become worse⁷. We found elevated levels of axonal degeneration in the basal forebrain, amygdala, peduncular nucleus, and medial septal region, all of which have implications with the hypothalamus⁷. These disinhibited neurons are activated during sudden, strong emotion; which in turn inactivates the locus coeruleus, resulting in cataplexy⁷. We also discovered that the axonal degeneration coincided with the onset of narcolepsy, suggesting that the discharge rate of specific neurons in the brain near the hypothalamus may be one of the mechanisms that causes narcolepsy.

To determine whether the degeneration of neurons leads to symptom onset, we used amino-cupric silver staining that specifically stains for axonal degeneration in the brain⁷. Surprisingly, there was no axonal degeneration seen in human narcoleptics, only in canine narcoleptics at that time.

New Leads on Narcolepsy

Till date, only the identification of LC, as well as the neurons undergoing neurodegeneration was unknown until the identification of other genes involved with sleep/wake and feeding cycle by two of my colleagues: Emmanuel Mignot and Masashi Yanagisawa (1998).

The Identification of Hypocretin (Orexin)

Hypocretins (orexin) are two carboxy-terminated neuropeptides, which are produced from a common precursor known as prepro-orexin⁸. These neuropeptides have been found to be expressed only in the hypothalamus, which is the center for the sleep/wake cycle, as well as feeding and homeostasis⁸. The hypocretins have been detected in secretory vesicles at synapses of fibers that project to areas within the hypothalamus that have been identified as having implications with feeding behaviors and hormone secretion⁸. Is hypocretin involved with narcolepsy, since found in the hypothalamus?

Identification of Orexin & Orexin-Receptors

In 1998, Yanagisawa and colleagues identified orexin and orexin receptors, as well as their role in the regulation of feeding habits in mice. They identified two neuropeptides, both of which derived from prepro-orexin, which activate two G-protein coupled receptors (GPCR's) located in the hypothalamus⁹. In order to identify orexin as well as orexin receptors, they used a systematic approach for peptide ligands of multiple orphan GPCR's⁹.

Through in situ hybridization, the visualization of neurons containing prepro-orexin mRNA was characterized in hypothalamic areas of the brain⁹. Results suggested that the prepro-orexin neurons were found predominantly in these areas, suggesting that the protein would be involved in the sleep/wake cycle, as well as feeding and homeostasis⁹.

Yanagisawa and colleagues also established in rats that as the amount of food stimulus and consumption increased so did the amount of orexin-A and orexin-B in the brain⁹. In addition, they also

identified that prepro-orexin mRNA was upregulated in fasted rats as compared with fed rats. They found there was more orexin mRNA found in rats that were fasted⁹. The identification of orexin-A and orexin-B as well as their receptors was a significant finding because of its implications with feeding behaviors, which is associated with the hypothalamus. The next question to ask is, are these peptides involved with narcolepsy?

Mutation in Hypocretin Leads to Narcolepsy

In 1999, a breakthrough took place with Emmanuel Mignot and colleagues, who discovered the genetics of narcolepsy. The lab team established an autosomal recessive mutation in the hypocretin (orexin) receptor 2 gene (Hcrtr2) in canine narcoleptics¹⁰. Mignot et al., through the use of positional cloning, identified three mutations in Hcrtr2 in Doberman, Labrador, and Dachshund canines¹⁰. First, they identified a 226 bp short interspersed nucleotide element (SINE) that was inserted upstream of the 3' site of the fourth exon¹⁰. This led to the conclusion that the mutation leading to the loss of exon 4 will possibly produce truncated hypocretin protein¹¹. The SINE insertion was found in 17 narcoleptic Dobermans, however was absent in the Labrador and Dachshund narcoleptics¹¹. Second, analysis of cDNA from narcoleptic Labradors identified a 123 bp deletion of exon 6. Third, also shown in narcoleptic Labradors, a G to A point mutation was identified in the 5' splice site¹¹.

It was hypothesized that the disrupted splicing of Hcrtr2 mRNAs leads to truncated proteins that may lead to the symptoms associated with narcolepsy. Also, mutations in Hcrtr2 receptor may lead to hyperexcitability of REM sleep generator neurons, or the inhibition of REM sleep neurons, leading to narcolepsy¹¹. A gap in knowledge at that time was, is there a mutation in Hcrtr2 leading to the truncation of proteins found in human narcoleptics, not only canine narcoleptics?

Truncation of Proteins

In 2001, Mignot *et al.*, illustrated the function of mutated hypocretin in canine narcoleptics. In a human cell line, they identified a point mutation (E54K) and previously described exon-skipping mutations, leading to the truncation of Hcrtr2 protein¹². This truncated protein indicated the absence of proper membrane localization, as well as undetectable binding and signal transduction for mutants¹². The absence leads to canine narcolepsy, and addresses the gap in knowledge of the role of hypocretin truncated protein with narcolepsy.

Knock-Knock! It's Orexin...Orexin Who?

In the same month as the identification of the mutation of Hcrtr2 in canine narcoleptics, Yanagisawa et al., created an orexin knock-out mouse model to further understand the genetics behind sleep regulation and narcolepsy. They reported that through the use of behavioral and EEG recordings, orexin knock-out mice exhibited a phenotype quite similar to human and canine narcoleptics¹³. Yanagisawa et al., discovered that orexin knock-out mice had a lack of orexin present in the hypothalamus through the use of immunohistochemistry¹³.

Orexin knock-out mice had very distinct motor behaviors as compared to the normal mice. Through the use of infrared videophotography, they discovered that orexin knock-out mice had multiple behavioral arrests during the dark phase (at night)¹³. All nine knock-out mice encountered several narcoleptic

attacks during the first four hours of the onset of darkness, when mice are normally most active¹³. They also reported, through EEG recordings, significantly more frequent patterns of REM sleep as compared to wild-type mice. Also, compared to wild-type mice, orexin knock-out mice had a more immediate decrease in muscle tone at the onset of a narcoleptic attack, through the observations of EEG recordings¹³.

Yanagisawa et al., also tested the hypothesis that orexin neurons innervate critical sleep-wakefulness brainstem nuclei¹³. Through the use of double immunostaining techniques, they found orexin cell bodies, as well as dendrites in histaminergic neurons; orexin immunoreactive fibers on nonadrenergic neurons of the locus coeruleus (center of wakefulness); orexin fibers also innervated serotonergic neurons in the raphe nucleus¹³. All of these brainstem nuclei have been previously hypothesized if turned off may contribute to the pathology associated with narcolepsy. To conclude this study, orexin and orexin receptors are essential pathway that lead to the regulation of the sleep/wake cycle; malfunction in this system can possibly lead to narcolepsy in humans.

Forget Dogs & Mice...Now Humans and Narcolepsy

In 2000, Mignot et al. finally established a connection in human cases of narcolepsy. They measured the amount of immunoreactive hypocretin in the cerebrospinal fluid of nine narcoleptic patients and eight-control patients¹⁴. They discovered a hypocretin deficiency in narcoleptic patients. Of the nine narcoleptics who experienced episodes of cataplexy, seven had a significantly decreased concentration of hypocretin found in the brain¹⁴. This decreased transmission of hypocretin suggested that low concentrations of hypocretin leads to the development of narcolepsy¹⁴. With this study, my lab was able to further implicate the decreased transmission of hypocretin may lead to narcolepsy in humans.

Reduced Number of Hypocretins (Orexin) in Human Narcoleptics

In 2000, my lab team established that there is a significantly decreased amount of hypocretin neurons in human narcoleptics as compared with normal patients. We found that there was an 85-95 % reduction of hypocretin neurons found in human narcoleptics, suggesting that the degeneration of these neurons is possibly responsible for the symptoms of narcolepsy¹⁵. We immunostained the hypothalamus of 16 human brains for hypocretin (Hcrt), including four narcoleptics. There was a minimal amount of Hcrt staining present in the narcoleptic brains¹⁵. In fact, there was a dramatic loss of Hcrt cells in the hypothalamus.

To assess the idea that Hcrt neurons were specifically undergoing degeneration, we stained for melanin-concentrating hormone neurons (MCH)¹⁵. We found that MCH neurons were not dying, only Hcrt neurons. We also discovered that there was an upregulation of gliosis in the hypothalamus of narcoleptic patients, as compared with controls¹⁵. The presence of gliosis suggests that neurodegeneration was taking place within the brain; more specifically, the reduced number of Hcrt neurons in narcoleptics is caused by neurodegeneration¹⁵, as seen previously in canine narcoleptics in a study conducted by my lab in 1999.

Mutations and Humans

Also in 2000, a mutation in a case of early onset of human narcolepsy was identified. Through the analysis of histopathology, seventy-four narcoleptic patients were screened for mutations of Hcrt gene, Hcrt1 gene, and Hcrt2¹⁶. Of the seventy-four patients, there was one Hcrt gene mutation (early onset of narcolepsy) that led to impaired peptide trafficking and processing¹⁶. They discovered that the wild-type Hcrt protein went through the Golgi network into secretory vesicles through the use of green fluorescent protein (GFP) or V5 epitope tags¹⁶. The mutant Hcrt protein remained in the membrane system and thus was not able to pass through the Golgi. The dominant mutation observed was a point mutation of a leucine to an arginine in one case of human narcolepsy¹⁶. A mutation in the Hcrt leads to impaired trafficking, which will then cause the onset of narcolepsy¹⁶. This mutation found in one human patient, opened the door to assess narcolepsy in humans further through future studies.

Narcolepsy, Feeding Behavior, & Orexin

In 2001, Yanagisawa and colleagues made a huge leap to associate orexin neurons and how they regulate narcolepsy, hypophagia, and obesity in mice. They generated a transgenic mouse model in which they ablated the orexin expression¹⁷. They attached an orexin promoter to a Machado-Joseph Disease (MJD) gene (ataxin-3), in which they observed that the orexin neurons were dying due, to the expression of the mutant MJD protein. These mice showed a similar phenotype to human narcoleptic patients, including behavioral arrests, premature entry in REM sleep, and late-onset obesity¹⁷.

To assess the ablation of orexin/ataxin-3 transgenic mice, immunohistochemistry was employed. They observed in the wild-type mice more orexin neurons as the mice increased in age, as compared with the transgenic mice, which had a significant number of orexin neurons at four weeks¹⁷. As time passed, however, the number of orexin significantly decreased, implying neurodegeneration¹⁷.

According to EEG/EMG recordings, they observed that even though the brain was active in Tg mice, there was a loss of muscle tone during the onset of REM sleep¹⁷. In relation to orexin and feeding behavior, this lab team found that orexin/ataxin-3 mice showed late-onset obesity¹⁷. That obesity occurred even though there was a 30% reduction in food intake, suggesting that orexin-neurons play a role in the regulation of feeding behavior and energy homeostasis¹⁷. One can speculate that the energy expenditure of these mice decreased, resulting in the differences in motor activity caused by the pathology associated with narcolepsy. Reduced feeding and obesity may reflect an underlying reduction in energy expenditure due to decreased motor activity (storing more fat), lower basal metabolic rate, or the combination of both¹⁷. This study indicates that metabolic abnormalities in food intake and/or energy expenditure may exist in human narcolepsy¹⁷. Another major conclusion that brings symptoms associated with narcolepsy is role of orexin neurons in the hypothalamus.

Another facet associated with orexins is leptin. Leptin is a hormone shown to modulate food intake and energy expenditure, as well as fat and glucose metabolism¹⁸. Basically, leptin tells the body that 'it is full' after eating¹⁸. Leptin appears to function largely within the long-term system and influences the quantity of food consumed relative to the amount of

energy expended¹⁸. Leptin has been shown to stimulate the activity of orexins in the lateral hypothalamus¹⁹, which is important for the understanding of the relationship between orexins, feeding, and narcolepsy. Orexin neurons have been associated not only with the regulation of the sleep-wake cycle, but more recently suggest a role in the regulation of energy homeostasis¹⁷, which may lead into the insight of narcolepsy and possible treatments (See Figure 2). Possibly cell death or neurodegeneration of orexin neurons may lead to obesity, which may have implications with the onset of narcolepsy.

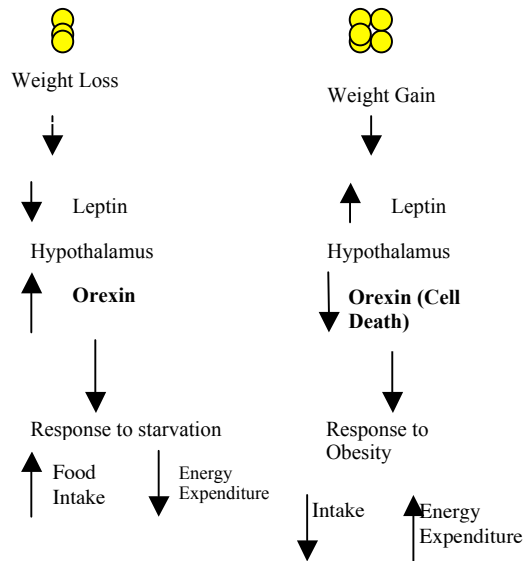


Figure 2. A New Working Model for Narcolepsy
When the body undergoes weight loss, an increase of leptin, a protein involved with the regulation of feeding, increases the amount of orexin (hypocretin) produced in the hypothalamus. The body in turn recognizes the increase in orexin as starvation, and increases the food intake and the decrease in energy expenditure. On the other hand, with weight gain, there is an increase in leptin, which decreases the amount of orexin (due to cell death) produced in the hypothalamus. The down-regulation of orexin decreases food intake and increases energy expenditure which leads to obesity, which may lead to narcolepsy.

Present Research with Orexins and Muscle Tone

My lab investigated the role of orexins and muscle tone, since loss of muscle tone is one of the symptoms of narcolepsy. We injected both orexin-A and orexin-B into the locus coeruleus in the hypothalamus²⁰. We discovered that the injections of orexin-A and orexin-B correlated with muscle-tone facilitation²⁰. Orexins are involved with muscle-tone facilitation; without these specific neurons there would be a loss of muscle tone, which may lead to symptoms of narcolepsy. The next question addressed was does is hypocretin regulated in the sleep/wake cycle in other models?

Present Day Research of Narcolepsy & Sleep/Wake Cycle

In 2002, my lab investigated the amount of Hcrt during the different sleep stages. Instead of using the canine model, looked into cats for the answer with Hcrt. We found in the hypothalamus a significant difference in

Hcrt concentrations during the active-wake, quiet-wake, slow-wave-sleep (similar to non-REM sleep), and REM sleep stages²¹. This in turn suggested that hypocretin is involved with the sleep/wake cycle, which contributes to the symptoms associated with narcolepsy²¹.

New Findings Associated With Cataplexy & Narcolepsy in 2002

Since the amygdala is associated with the emotion process in the brain, my lab hypothesized that specific neurons are changing their properties, which may be linked to cataplexy. We investigated this hypothesis in the canine model and discovered that two populations of cells showed a significant change in activity with cataplexy²². More specifically, a set of sleep-inducing neurons increased their discharge rate prior to cataplectic attack; on the other hand, a set of wake inducing neurons decreased their discharge rate prior to cataplectic attacks as seen in narcolepsy²². This study suggests that the abnormality of these neurons leads to cataplexy, which is seen in both human and canine narcoleptics.

Contradictions of Narcolepsy

There are a few contradictions in the field of narcolepsy. One major question is are these specific neurons that causes narcolepsy? One may find that neurons are dying, while another finds that these neurons have an abnormal firing rate.

Challenges Ahead in Narcolepsy

Others have illustrated that orexin plays a role in the regulation of feeding, and more recently how narcolepsy and feeding behavior is connected. The next approach will be to further assess the role of orexins (possibly cell death) in narcoleptic humans to see if that hypothesis holds true. Understanding obesity and orexins in humans will not only allow for a possible cure of an epidemic in the U.S., as well as possible treatments for narcolepsy. Also through the understanding of energy expenditure, we can learn more about obesity, more importantly narcolepsy.

Conclusion

Narcolepsy is a non-progressive neurodegenerative disorder that affects 1 in 2,000 Americans each year. Research conducted in my lab has enabled the identification of the death of specific neurons found in the hypothalamus leads to the pathology of narcolepsy seen in both canine and human narcoleptics. Other labs have established mutations in hypocretin (orexin) genes associated with narcolepsy. With the collaboration of my lab, as well as other labs, a treatment for narcolepsy can be found in the near future.

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