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The Role of Orexin in Avian Migration

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ABSTRACT

Songbirds are typically diurnal. However, during the migratory period, they will adopt a nocturnal pattern and fly throughout the night. Songbirds kept in a laboratory environment, though unable to migrate, will still exhibit nocturnal restlessness, or Zugunruhe, during their migratory period. While the exact neurobiological cause of this switch from diurnal to nocturnal behavior is yet unknown, it may be related to activity of the neuropeptide orexin, also known as hypocretin. Orexin is known to regulate appetite and feeding, arousal and energy levels, sleep/wake cycles, and dopaminergic activity in mammals. Orexin functions by binding G-protein coupled receptors (GPCRs), of which there are two subtypes, A and B, in order to activate second-messenger signaling pathways to regulate these processes. This study utilized immunohistochemistry to selectively stain against orexin receptors A and B in the brains of white-throated sparrows that were exhibiting Zugunruhe and those that were not exhibiting Zugunruhe. Results showed a consistent distribution of orexin in several regions of the avian brain, including the ventral tegmental area (VTA), periaqueductal gray (PAG), and Purkinje cells of the cerebellum. The PAG is involved in regulating rapid eye movement (REM) sleep and the VTA is involved in regulating non-REM sleep, both of which are processes that are altered during migration. Additionally, the VTA contributes to the neural reward pathway, thought to be a component of migratory drive, and the Purkinje cells of the cerebellum are thought to help regulate respiratory and cardiac function in relationship with increased locomotor activity. Taken together, these findings implicate a role for orexin in regulating nocturnal avian migration at multiple levels.

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Chapter 1

Background

Nocturnal Avian Migration

The classic depiction of avian migration is the goose, flying in a V-formation in broad daylight. The majority of migratory birds, however, migrate at night. This includes most passerine birds, small songbirds defined primarily as “perching birds,” with three toes directed forward and one directed backward (Clench & Gill, 2024). Most passerines are typically diurnal and switch their sleep/wake patterns for migration (Bartell & Moore, 2013).

Evolutionarily, there are several potential benefits to nocturnal migration. The predominant hypothesis was proposed by ornithologist William Brewster in 1886 and remains widely accepted to this day. Brewster’s hypothesis is twofold: small migratory songbirds are less likely to face predation while migrating at night and can reserve the daytime for regular diurnal foraging activities (Brewster, 1886). A study performed on migratory sandpipers supports the theory that nocturnal migrants depart based on foraging availability. Sandpipers were noted to depart from the beaches they foraged at not only at dusk, but also shortly before high tide – both times when foraging conditions become unideal (Lank, 1989). Further, research has found nocturnal activity in typically diurnal birds does not only occur during migration. A study performed on reed warblers revealed that these birds typically chose to commence shorter-distance flights for translocation and homing at night (Mukhin et al., 2009). These findings support the lower predation hypothesis, as foraging capabilities are not as important during local

flights. Another proposed benefit to nocturnal migration is that flight conditions are improved compared to daytime – the air is cooler, less turbulent, and more humid at night, reducing energy consumption and water loss (Lank, 1989; Alerstam, 2009).

Utilization of orienting cues may also contribute to the benefits of nocturnal migration. Many nocturnal migrants appear to use the sunset as a directional cue to properly orient their flight. Orientation is also possible via polarized sunlight patterns around the time of sunset and star patterns after nightfall, with potential for geomagnetic stimuli to play a role as well (Moore, 1987). However, cage orientation experiments performed on several species of passerine birds suggest that nocturnal migrants tend to make their directional determinations around sunset and stick with that direction throughout the night, regardless of other cues that may become available. Birds exposed to geomagnetic stimuli or star patterns that contrasted with the direction of a reflected sunset oriented their movement toward the sunset and did not change direction based on those additional cues (Moore, 1987). These experiments do not prove that nocturnal migrant birds cannot use cues other than the sunset to orient themselves but rather support the idea that birds make few to one directional determination per night. During prolonged periods without the ability to use visual cues, such as multiple days in which the weather is overcast, nocturnal migrants struggle to appropriately orient themselves for flight and trend downwind, even if that direction is not seasonally appropriate. Orientation was found to improve when the sun became visible again (Moore, 1987).

A commonly suggested mechanism for the cause of changes in behavior and sleep patterns leading up to and during migration is photoperiodicity. Photoperiodicity is the ability of certain organisms to recognize changes in day length and respond accordingly. In the case of

many migratory songbirds, photoreceptors in the avian brain are able to detect day length, prompting premigratory behavioral and physiological changes when the days become appropriately long or short, depending on the season of migration (Bartell & Moore, 2013). One such behavioral change caused by photoperiodicity that is critical to nocturnal migration is nocturnal restlessness, also known as Zugunruhe. Nocturnal migrants using circannual rhythm to time migration also exhibit Zugunruhe (Bartell & Moore, 2013). These birds develop nocturnal restlessness behaviors at the appropriate times of year for migration, even when kept in conditions where day length does not change. Zugunruhe has been found to be regulated by two circadian oscillators: the standard daytime circadian clock and a Zugunruhe circadian clock. When these two oscillators are in opposite phases, Zugunruhe occurs along with normal daytime activities (Bartell & Gwinner, 2005).

Zugunruhe is a migratory behavior, typically characterized by intense wing-flapping while in darkness, that persists in birds kept in a laboratory setting. It is regarded as an analog or proxy for migration in captive migratory birds (Bartell & Moore, 2013). This behavioral phenomenon allows for nocturnal migration studies to be carried out in laboratories, rather than restricting such research solely to field studies. Some studies have sought to better understand Zugunruhe by eliminating it. White-throated sparrows that underwent pinealectomy were found to lose their circadian rhythm in constant dim light conditions within three days of the surgery. This arrhythmicity included a loss of Zugunruhe (McMillan, 1972). The creation of lesions on the ventromedial hypothalamus of white-throated sparrows already exhibiting nocturnal restlessness was also shown to stop Zugunruhe. While these birds retained normal daytime activity, they failed to exhibit Zugunruhe within the first night after the surgery (Kuenzel, 1973).

In both cases, sham-operated birds retained normal circadian rhythms and Zugunruhe (McMillan, 1972; Kuenzel, 1973).

Circadian and circannual rhythms cause a myriad of changes to hormone and neurotransmitter levels that allow birds to prepare for migration. Events such as the prealternate molt and gonadal development (for spring migration) are associated with photoperiodism (Falls & Kopachena, 2020). Another important premigratory physiological change is the accumulation of fat stores that can be mobilized for energy during long flights. Increases in appetite-controlling hormones and hypothalamic neurotransmitters, such as plasma corticosterone, pituitary prolactin, and neuropeptide Y, occur shortly before migration to induce hyperphagia and premigratory fattening (Falls & Kopachena, 2020; Bartell & Moore, 2013). Adiponectin, an adipokine that promotes the mobilization of glucose and fatty acids for energy, has been found to peak during the night when birds are migrating, as opposed to a daytime peak for non-migrating birds (Stuber et al., 2013). It has been suggested that adiponectin and other such adipokines may communicate with the circadian "clock" that controls migratory nocturnal activity, implying that the premigratory accumulation of fat stores may actually play a role in the initiation of nocturnal migration (Stuber et al., 2013).

Photoperiodicity and circannual rhythms play a significant role in initiating premigratory changes. While it is clear that these mechanisms are related to the initiation of nocturnal restlessness and migration, the precise cause of the circadian rhythm switch is yet unknown. All animal behaviors have both proximate and ultimate causes. Proximate causes are the biological mechanisms that generate behaviors, while ultimate causes are the reason these behaviors evolved. Several ultimate causes for nocturnal migration have been proposed, and many are

supported by evidence, but less is known about the proximate causes. What physiological mechanism causes the sudden switch from diurnal to nocturnal activity? We believe that mechanism may be controlled, in part, through the activity of a neuropeptide known as orexin.

Orexin

Orexin, also known as hypocretin, is a hypothalamic neuropeptide first identified in 1998 (Sakurai et al., 1998). There are two types of orexins, known as orexin A and B, derived from a common precursor called prepro-orexin. There are also two types of orexin receptors, orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R), which are found in different portions of the brain but can each bind both orexin A and B (Sakurai, 2014). OX1R preferably binds orexin A, while OX2R is a nonselective receptor for both orexin A and B (Marcus et al., 2001). Orexin receptor types 1 and 2 are G-protein coupled receptors (GPCRs), or integral membrane proteins capable of recognizing a wide array of signals. The binding of orexins A and B to these receptors activates signaling pathways within the brain.

Orexin-containing neurons within the brain are organized in a “bilaterally and symmetrically in a discrete set” in the hypothalamus and subthalamus (Sakurai, 1998). While orexin itself is synthesized by neurons in the lateral hypothalamus (LH), orexin receptors are distributed across the brain. In the rat, OX1R and OX2R have distinct but overlapping distributions. OX1R is found in locations including the prefrontal cortex, paraventricular thalamic nucleus, and ventromedial hypothalamic nucleus. OX2R is found in locations including the cerebral cortex, medial thalamic groups, and hypothalamic nuclei. Both receptors are found in the hippocampus (Marcus et al., 2001).

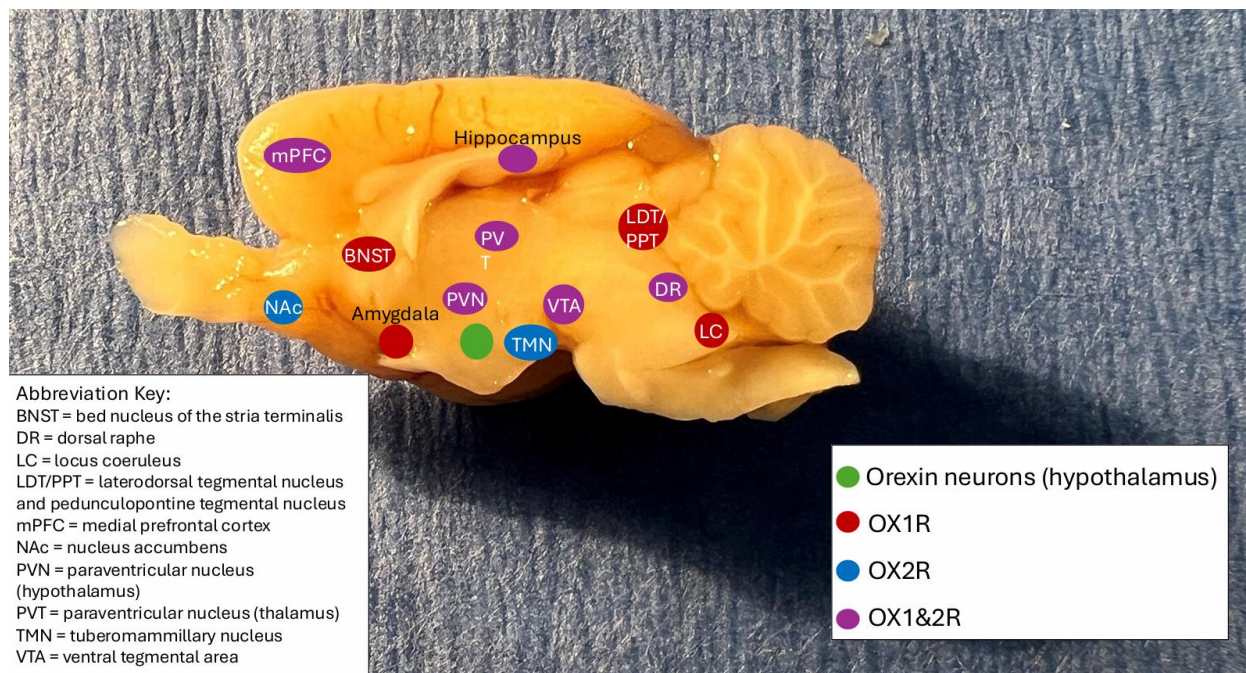


Figure 1: Distribution of orexin receptors in the rat brain (adapted from Sargin, 2019)

In humans and other mammals, orexin is known to play an important role in appetite and feeding, sleep and wakefulness, arousal and energy levels, the reward pathway, and emotional memory. The orexinergic system is also closely related to the dopaminergic system. Orexin was initially discovered as an appetite-regulating neuropeptide and was found to stimulate feeding when administered centrally to rats (Sakurai et al., 1998). The regulation of sleep and wakefulness, however, is currently the most studied and understood function of orexin. Deficiencies in orexin or OX2R have been shown to be cause of narcolepsy in humans, dogs, and rats, and orexin is thought to be critical “in the maintenance of long, consolidated awake periods” (Sakurai, 2014). While OX1R and OX2R are capable of binding both orexins A and B, it seems that the receptors have slightly different functions. Studies suggest that, in mammals, OX1R plays a larger role in appetite while OX2R plays a larger role in wakefulness (Sakurai, 2014). In birds, however, only one orexin receptor exists.

Orexin is fairly well-understood in the mammalian brain, but less research has been done on its function in the avian brain. The amino acid sequence for orexin A and B is highly conserved across vertebrates (Ramser and Dridi, 2022), but due to differences in neuroanatomy and physiology, it cannot be assumed that orexin plays the same roles or acts upon the same areas of the brain in birds as it does in mammals. For example, while mammalian orexin has been shown to play an important role in appetite and feeding, avian orexin does not appear to impact feeding behavior at all. Intracerebroventricular injection of orexin into chicks did not stimulate feeding behaviors, and orexin mRNA expression in chickens was not altered by periods of fasting (Furuse et al., 1999). The role of avian orexin in the sleep-wake cycle is yet uncertain. No differences in orexin levels were found between sleeping hens and awake hens (Miranda et al., 2013), but injections of orexin A were found to induce wakefulness in neonatal chicks (Katayama et al., 2010). Further studies on chickens suggest a potential role for avian orexin in the stress response, but the function of that mechanism remains unclear (Ramser and Dridi, 2022).

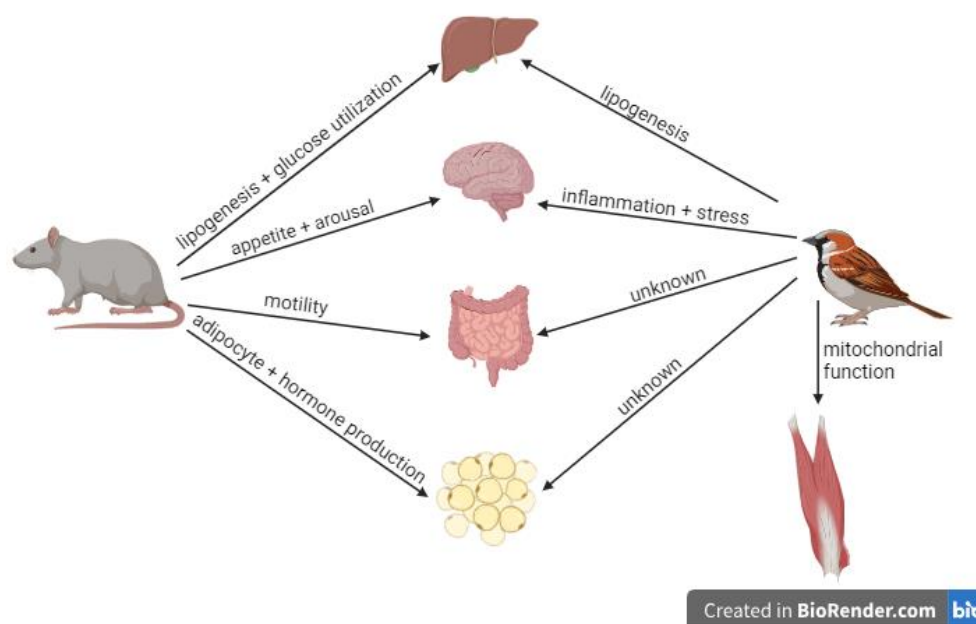


Figure 2: A comparison of the known roles of mammalian orexin and of avian orexin (adapted from Ramser and Dridi, 2022)

In both mammals and birds, orexin functions beyond just the central nervous system. Peripheral tissues also exhibit orexin. In mammals, orexin is expressed throughout much of the digestive system and has been shown to play a role in the regulation of intestinal motility (Heinonen et al., 2008). Results on other potential roles in mammalian digestion are conflicting and require further research. Despite the lack of central regulation of appetite and feeding in birds, avian orexin has also been found in the intestines and may function in avian intestinal motility as well (Arcamone et al., 2014). Orexin has also been found in the adipose tissue and livers of both mammals and birds, suggesting important functions in fat metabolism (Ramser and Dridi, 2022). Additionally, birds exhibit orexin within the muscles, where it impacts the mitochondria and energy homeostasis (Lassiter and Dridi, 2019). These peripheral functions of mammalian orexin are consistent with the central role of orexin in appetite and feeding, whereas avian orexin appears to have greater functions peripherally than centrally.

Notably, the majority of existing studies on avian orexin have been performed on the chicken (*Gallus gallus*), a non-migratory Galliform bird. Current research on the role of orexin in Passerine birds is minimal. A study of five wild-caught male house finches (*Carpodacus mexicanus*) identified a distribution of immunoreactive orexin neurons throughout the brain, revealing that orexin is primarily clustered at the paraventricular nucleus of the hypothalamus. Other clustered areas included the preoptic area, the thalamus, and the third ventricle. (Singletary et al., 2006). This distribution is fairly consistent with previously identified distributions in domesticated chickens, implying that the distribution of orexin is likely quite similar across all species of birds, and the functions may be as well. However, the house finch is a generally non-migratory bird, so further research into orexin in migratory Passerine birds remains necessary.

White-Throated Sparrows

This research utilizes the white-throated sparrow (*Zonotrichia albicollis*), a small migratory North American songbird. White-throated sparrows exhibit plumage dimorphism caused by an inversion of their second chromosome encompassing around 1,000 genes in which recombination is highly suppressed (Huynh et al., 2011). Phenotypically, sparrows appear as either white-striped or tan-striped, with these stripes occurring alongside black stripes on the head. White-striped morphs are typically heterozygous for the polymorphism, with very few being homozygous (possessing two inverted chromosomes). Tan-striped morphs are homozygous for non-inverted chromosomes (Huynh et al., 2011). Though white- and tan-striped sparrows are not considered separate subspecies, these types of birds do exhibit some differences

in mating and parenting behaviors. White-striped males, for example, tend to be more aggressive, while tan-striped morphs tend to be more nurturing to offspring (Falls & Kopachena, 2020). However, differences in levels of aggression and dominance are only evident during the breeding season and are not exhibited in fall or winter (Mazerolle & Hobson, 2007). This dimorphism is maintained through negative assortative mating, in which birds of one morph preferentially mate with birds of the opposite morph (Huynh et al., 2011).

The white-throated sparrow is found across North America, primarily in Canada and the United States. During breeding season in the spring, these sparrows are primarily found in Canada and the northernmost parts of the United States. Winter populations tend to occur in the central and southern areas of the United States, with some birds reaching as far as northern Mexico. White-throated sparrows are typically found east of the Great Plains, but a small winter population does exist on the western edge of California and Oregon (Falls & Kopachena, 2020). These birds prefer a coniferous, deciduous, or mixed forest habitat. During breeding season, sparrows are more often found towards forest edges and areas with low growths, but they tend towards more thickly covered areas during non-breeding seasons (Falls & Kopachena, 2020).

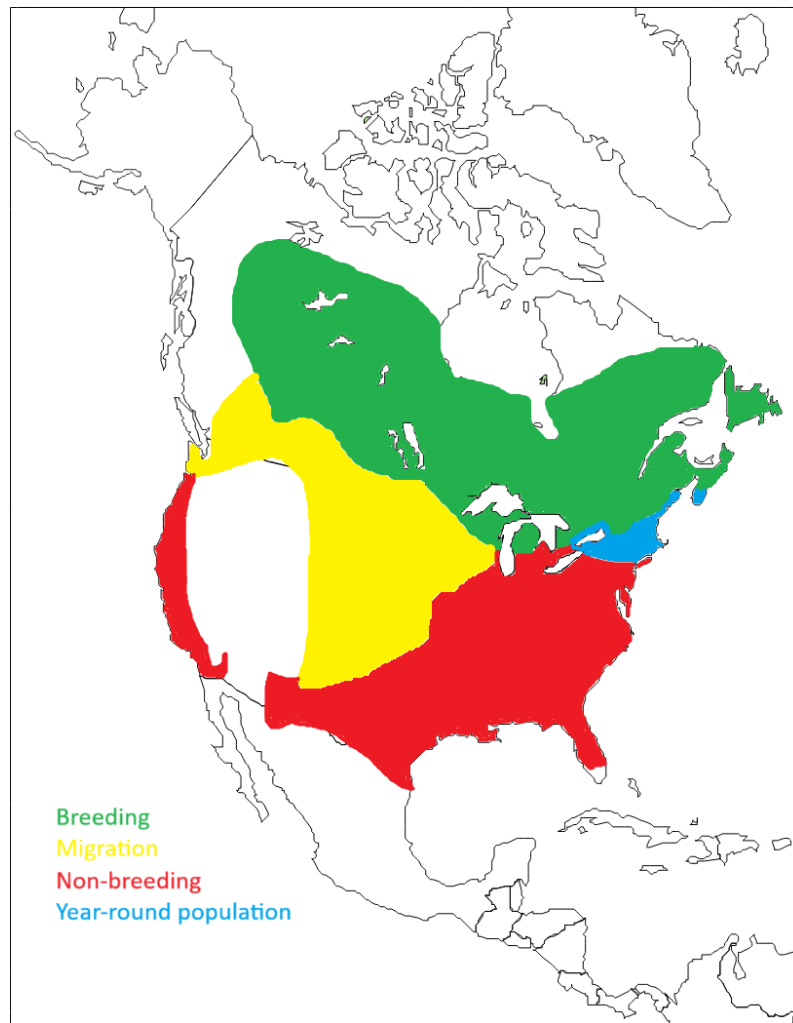


Figure 3: Map of the distribution of white-throated sparrows (*Zonotrichia albicollis*) throughout the year (adapted from Falls & Kopachena, 2020)

White-throated sparrows are considered short-distance migrants, travelling between Canada and the continental United States. Despite significant research on white-throated sparrows and their migratory behaviors, not much is known about their exact routes of travel. In the spring, birds migrate northwards from mid-March to late May, with a majority of birds beginning migration in late April and reaching their breeding ground location in mid-May (Falls & Kopachena 2020; Fink et al., 2023). Fall, southward migration occurs from late August to mid-November, taking longer and progressing more slowly than spring migration (Falls &

Kopachena, 2020). The bulk of fall migration occurs from mid-September to early November (Fink et al., 2023). White-throated sparrows have been observed to show breeding site fidelity, with individual birds tending to return to the same locations year after year (Beauchamp et al., 2020).

Additionally, white-throated sparrows are differential migrators, meaning that groups within the species migrate in different ways. Analysis of stable hydrogen isotopes from feathers collected from migrating sparrows revealed a sex-based differential migration pattern. Male white-throated sparrows arrive to their spring destinations approximately one week earlier than females and tend to winter at more northern locations (Mazerolle & Hobson, 2007). Body size also has a “moderate positive” relationship to wintering latitude, with larger birds being better able to tolerate cold weather and wintering in more northern locales (Mazerolle & Hobson, 2007). Plumage morphs, on the other hand, appear to have minimal effects on migratory behaviors and results. White-striped morphs have been observed to become active and depart for migration slightly earlier in the day than tan-striped morphs, but the overall timing of migration is similar between both morphs (Beauchamp et al., 2020). Further, no difference was noted in wintering latitudes based on morph color or aggression/dominance behaviors (Mazerolle & Hobson, 2007). This is likely due to the fact that morph differences are only prominent during breeding season.

Data on spring arrival dates suggests that weather plays a significant role on the timing of white-throated sparrow migration. Comparison of spring arrival dates of birds migrating to the Delta Marsh observatory in Manitoba, Canada to the mean minimum temperatures in April over a nine-year period revealed a significant correlation between birds arriving at their spring locations earlier in the month and the mean minimum temperature increasing over the years

(Mazerolle & Hobson, 2007). Further, spring migratory activity increases with decreasing atmospheric pressure and the presence of south winds (Muller, 1976). As such, white-throated sparrows can be said to be “weather migrants” rather than “calendar migrants.” Calendar migrants rely on the change in day length, or photoperiodicity, as well as endogenous cues to determine when to migrate, while weather migrants can use both those and more variable cues, such as temperature or food availability (Mazerolle & Hobson, 2007). This manner of migration may help the white-throated sparrow better adapt its activities to the changing climate. Interestingly, however, fall migratory activity has not been found to significantly correlate with weather patterns (Muller, 1976).

Like most other typically diurnal species of songbird, white-throated sparrows are nocturnal migrants. Birds begin flight around dusk and fly throughout the night. Nocturnal migration in white-throated sparrows is presumed to be controlled in part by the pineal gland and the ventromedial hypothalamus, as surgical interference with these regions has been shown to eliminate nocturnal restlessness in laboratory settings (McMillan, 1972; Kuenzel, 1973). It is worth noting that Zugunruhe in white-throated sparrows is significantly more intense during the spring migratory period than during the fall, and that both spring and fall periods of Zugunruhe observed in laboratories last longer than actual migration (Kuenzel & Helms, 1974).

As with other nocturnal migrants, white-throated sparrows can utilize a variety of navigational methods to orient their flight. Cues for proper orientation include the position of sunset, polarization patterns of sunlight after sunset, and the position of stars (Able et al., 1982; Able & Cherry, 1986). Birds unable to use visual cues tend to head downwind, even if the wind is not blowing in the intended direction of migration (Able et al., 1982). Outdoor captive sparrows that cannot migrate will still orient themselves in their migratory direction by hopping

if able to use typical visual cues (Able & Cherry, 1986). Further, captive sparrows exhibit nocturnal restlessness during their migratory period regardless of whether they are kept indoors or outdoors (Falls & Kopachena, 2020).

White-throated sparrows undergo physiological changes, such as molting and fattening, prior to migration. These changes are induced by photoperiodicity and hormonal rhythms. Premigratory changes can be artificially induced by manipulating exposure of birds to light/dark cycles and hormones (Falls & Kopachena, 2020). Like Zugunruhe, physiological changes are also subject to seasonal differences. Caged white-throated sparrows were found to have greater increases of weight and body fat during the spring (Kuenzel & Helms, 1974). Research into premigratory fattening suggests that adipose tissue may play an important endocrine role in migratory behaviors. The adipokine adiponectin, which promotes energy mobilization, typically follows a circadian rhythm (increased secretion during the light period). However, adiponectin either uncouples or inverts from that rhythm during migration, causing increased secretion during the dark period (Stuber et al., 2013). Visfatin, an adipokine that can “communicate metabolic information to the molecular clock,” exhibited reduced plasma concentration during the migratory period (Stuber et al., 2013). These findings imply a connection between adipokine signaling and nocturnal migration in the white-throated sparrow, which may shed light on why both weight gain and Zugunruhe intensity are lesser during fall migration.

The white-throated sparrow was chosen as a model for this research project due to the large body of previous research on this species. White-throated sparrows have historically been a model species for avian behavioral and migration research. Only white-striped morphs were used in this study.

Research Objectives

Though there are many evolutionary benefits to nocturnal avian migration, the neurobiological cause of this phenomena and its occurrence in typically diurnal birds is yet unknown. We hypothesize that orexin plays a major role in the regulation of the circadian rhythms of migratory birds. Most research done on orexin to date has focused primarily on mammalian species, and much of the research that has been done on orexin in avians has focused on non-migratory species such as chickens. This research seeks to establish a consistent distribution of orexin receptors in the brain of the white-throated sparrow, a small migratory songbird. Analysis of this distribution will suggest possible functions of orexin in the avian brain and how these functions may contribute to migratory behaviors and circadian rhythm fluctuations.

Chapter 2

Materials and Methods

This experiment was reviewed and approved by The Pennsylvania State University Institutional Animal Care and Use Committee (proto202001473).

Brain Collection

Brain samples were collected from white-throated sparrows at different times of day. Prior to collection, birds were categorized as either exhibiting Zugunruhe or not exhibiting Zugunruhe. Categorization was determined by viewing video recordings of the birds' activities and examining actograms. Collected brain samples were perfused with heparinized saline, followed by 4% paraformaldehyde. The brains were then preserved in 4% paraformaldehyde, embedded in paraffin, and cut on a microtome. Cut samples were mounted on Superfrost Plus slides for examination via immunohistochemistry.

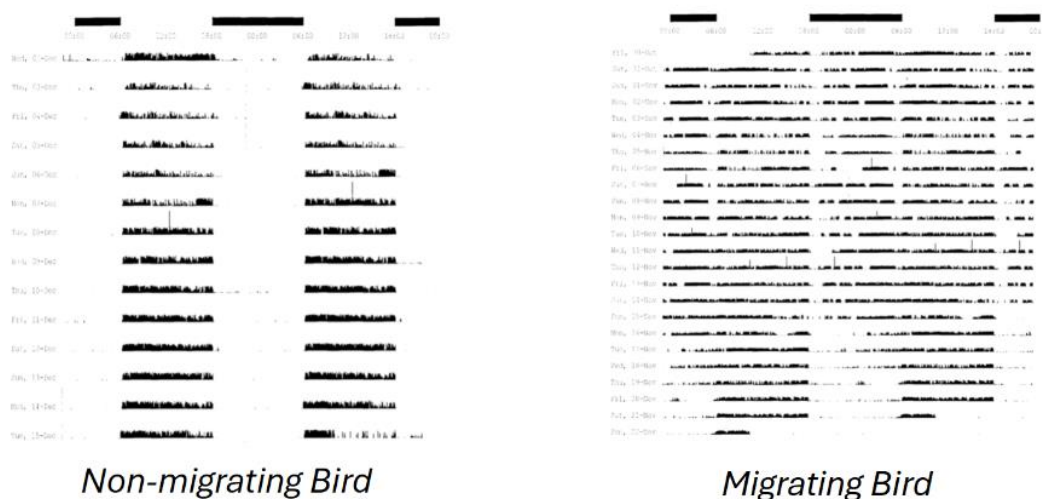


Figure 4: Examples of actograms of white-throated sparrows

Immunohistochemistry

Immunohistochemistry was used to determine the distribution and abundance of orexin receptors in the brain of the white-throated sparrow. Formalin-fixed, paraffin-embedded sample slides were deparaffinized by washing in three rounds of xylene for five minutes each, two rounds of 100% ethanol for three minutes each, and one round each of 95%, 90%, and 80% ethanol for one minute each. Sample slides were then washed in Milli-Q (deionized, demineralized, and sterilized) water for one minute. Slides were placed in a sodium citrate buffer solution in a steamer at 95° Celsius for one hour in order to retrieve antigens altered by the fixation process.

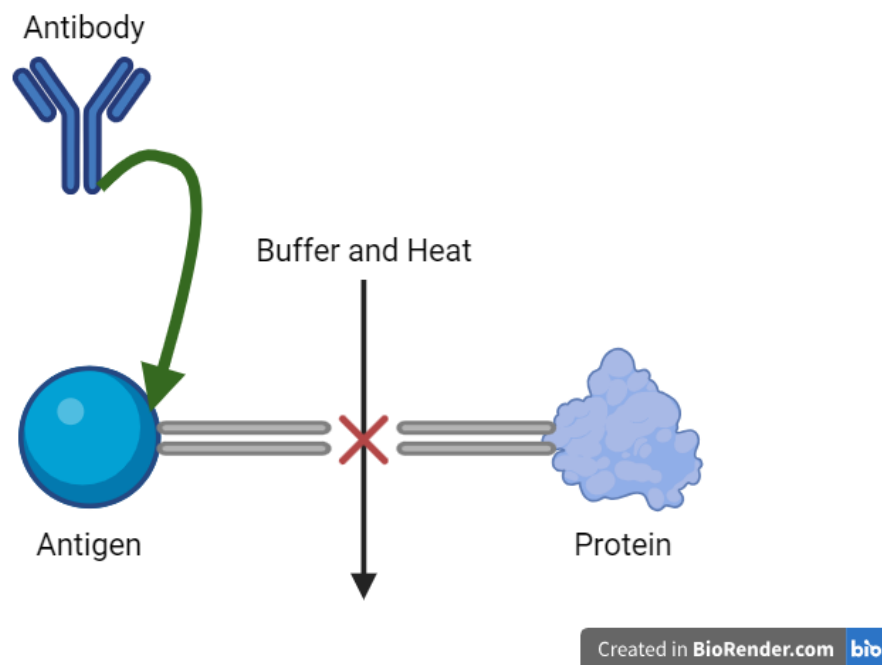


Figure 5: Antigen retrieval breaks crosslinks created by formalin fixation

After one hour in the steamer, slides were removed and cooled for twenty minutes in the buffer solution. Slides were washed twice for two minutes each in a mixture of 100 milliliters of 0.1M phosphate-buffered saline (PBS) and 50 microliters of polysorbate 20. Slides were then

washed for twenty minutes in a mixture of 87 milliliters of Tris-buffered saline (TBS) and 13 milliliters of 3% hydrogen peroxide while on a rocker. Following that, slides were washed for an additional twenty minutes in 100 milliliters of TBS while on a rocker. Slides were then placed in a slide mailer of blocking solution of 19.5 milliliters of TBS, 208 microliters of Triton X, and 1040 microliters of normal Donkey serum on a rocker for two hours in order to prevent non-specific antibody binding. After removing slides from the blocking solution, 150 milliliters of primary antibody solution containing 1380.5 microliters of TBS, 43.2 microliters of Triton X, 14.4 microliters of normal Donkey serum, and 1.92 microliters of Rabbit Anti-CD200R/Orexin receptor Polyclonal Antibody, Unconjugated (bs-1095R) was pipetted onto each slide. Slides were then covered with parafilm and placed in a 5° Celsius refrigerator for 36 hours.

After 36 hours, slides were removed from the refrigerator and placed in three consecutive washes of 100 milliliters of TBS on a rocker for five minutes each. 150 milliliters of secondary antibody solution containing 2819.5 microliters of TBS, 28.8 microliters of Triton X, 28.8 microliters of normal Donkey serum, and 2.88 microliters of Donkey Biotin labelled Anti-Rabbit Secondary Antibody was pipetted onto the slides. Slides were covered with parafilm and incubated for two hours. Slides were then washed again in three consecutive washes of 100 milliliters of TBS on a rocker for five minutes. 150 milliliters of a solution of 2.5 milliliters of TBS and 2 drops of both avidin and biotin solutions from the VECTASTAIN Elite ABC HRP Kit (Peroxidase, Standard) Kit were pipetted onto the slides, which were covered with parafilm and incubated for one hour. Slides were washed in three more washes of 100 milliliters of TBS for five minutes on a rocker.

The orexin receptors on the samples were then labeled with 3, 3'-diaminobenzidine (DAB). The DAB solution is made with 2.5 milliliters of double-distilled water (ddH₂O) and

Vector Laboratories DAB Peroxidase (HRP) Substrate Kit (With Nickel), using one drop of DAB reagent 1, two drops of DAB reagent 2, one drop of DAB reagent 3, and one drop of DAB reagent 4. 150 milliliters of DAB solution was pipetted onto each slide and slides were incubated for eight minutes. Slides were then washed in three washes of Milli-Q water for ten minutes each. Slides were dehydrated with one wash each of 50% and 70% ethanol for one minute each, two washes of 95% and 100% ethanol for one minute each, and three washes of xylene for five minutes each. Finally, slides were covered with Permount mounting medium and cover-slipped for microscopic examination.

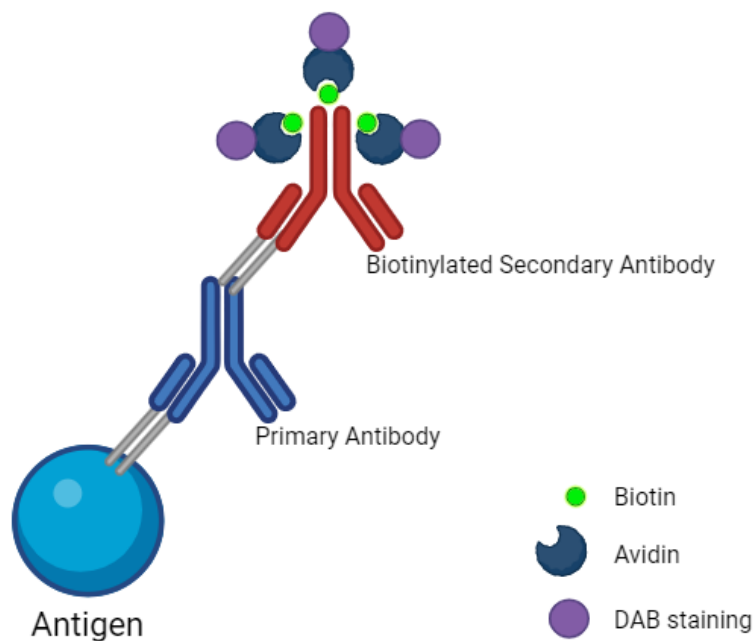


Figure 6: Immunohistochemical staining using two antibodies and an ABC complex (adapted from Im et al. 2020)

Analysis

Following immunohistochemistry, slides were examined via microscopy with an Olympus BX51 microscope. Microscopy photographs of stained regions were captured with an Olympus DP71 microscope digital camera and visualized digitally on Olympus MagnaFire. Successful stainings of orexin receptors appear as light purple dots under the microscope. Eight immunostained white-throated sparrow brains – four that were collected while the bird was exhibiting Zugunruhe and four that were collected when the bird was exhibiting non-migratory activity patterns – were visualized under the microscope and showed consistent receptor staining distribution across the same regions of the brain.

Chapter 3

Results

Visualization of stained samples revealed a consistent distribution of avian orexin receptors across multiple regions of the white-throated sparrow brain. This distribution was fairly consistent between sparrows that were exhibiting Zugunruhe at the time of brain collection and those that were not. Orexin receptors were found throughout the ventral tegmental area (VTA), periaqueductal gray (PAG), Purkinje cells of the cerebellum, optic tectum, olfactory tract, parahippocampal area, and thalamus. Some samples exhibited edge effect, a phenomenon in which the edges of the brain tissue exhibit nonselective staining. A region of the brain exhibiting staining only at the edges of visualized samples and not demonstrating any staining towards the center was not considered positive for avian orexin receptors.

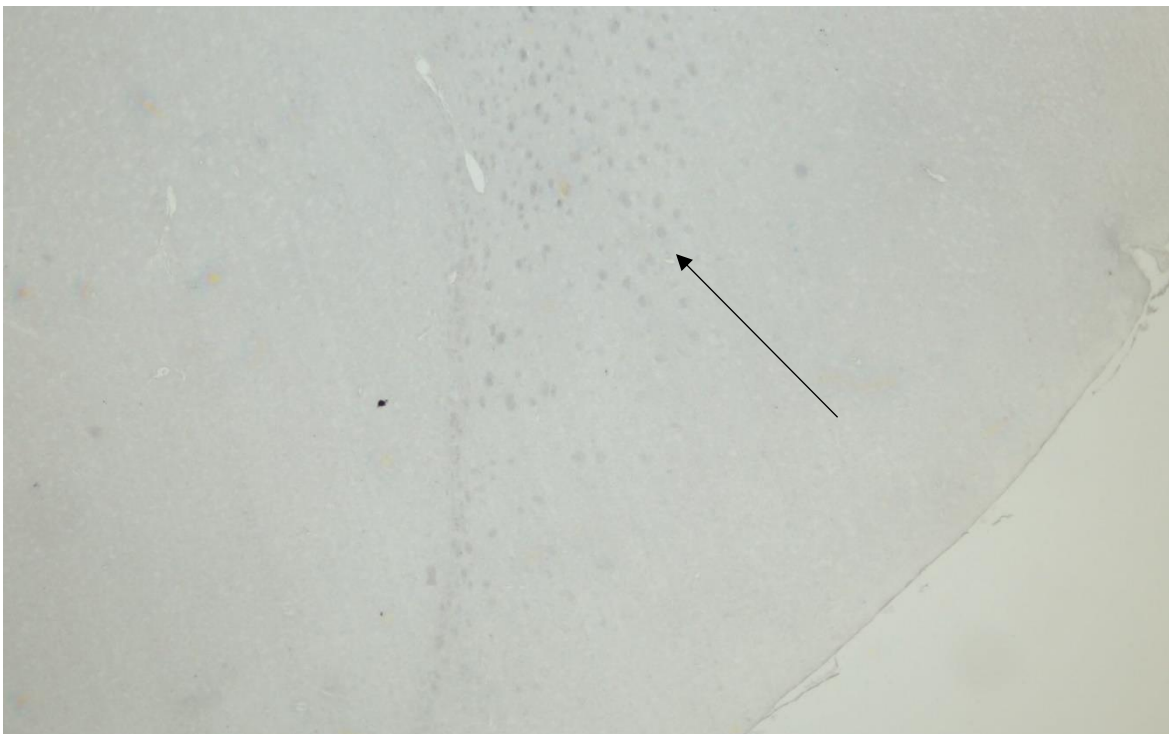


Figure 7: Ventral tegmental area (VTA) of the subpallium

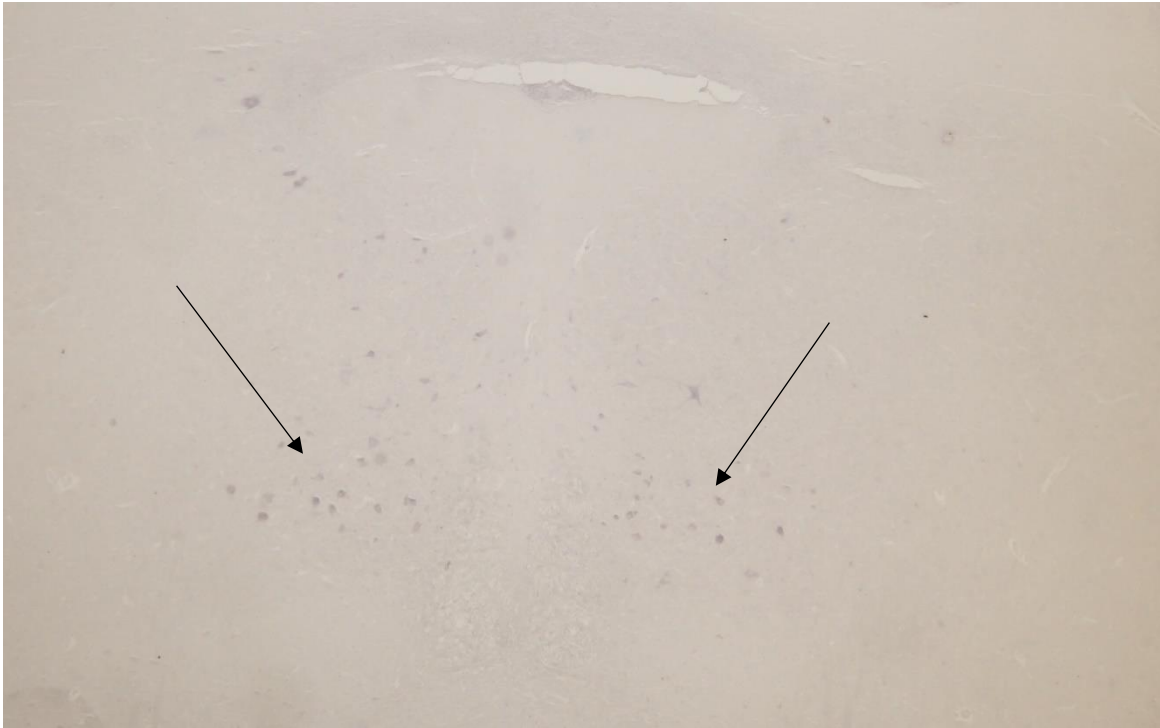


Figure 8: Periaqueductal Gray (PAG)

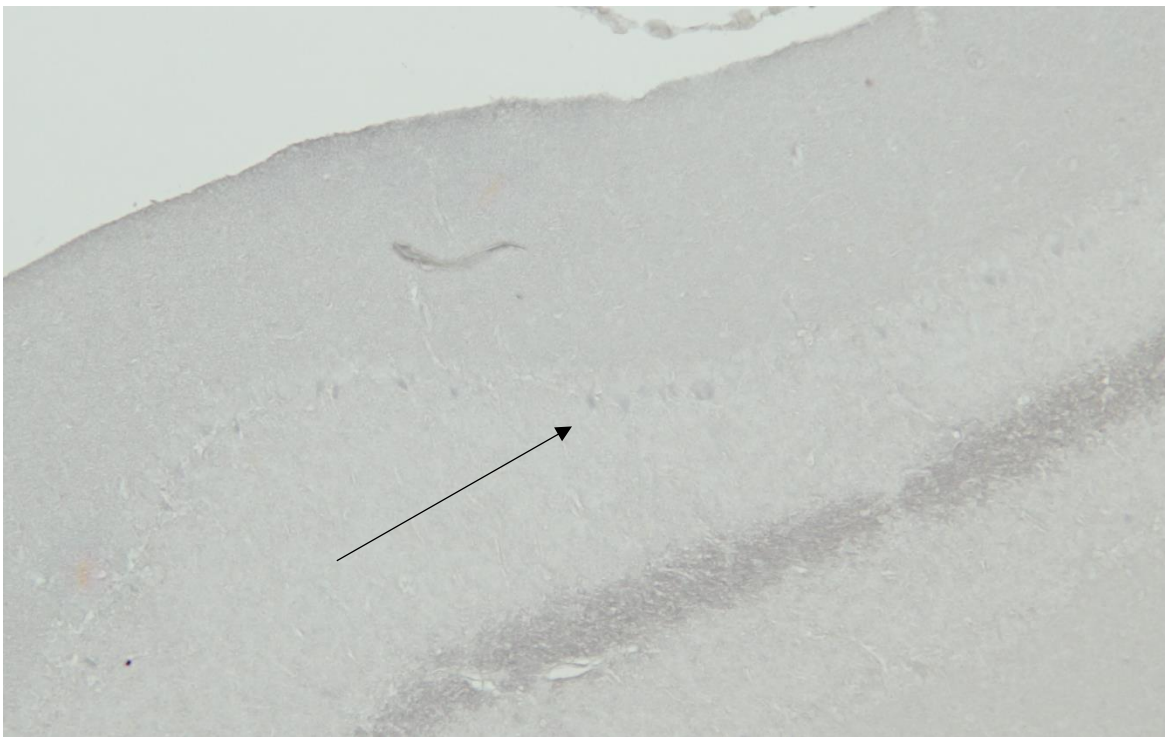


Figure 9: Purkinje cells of the cerebellum

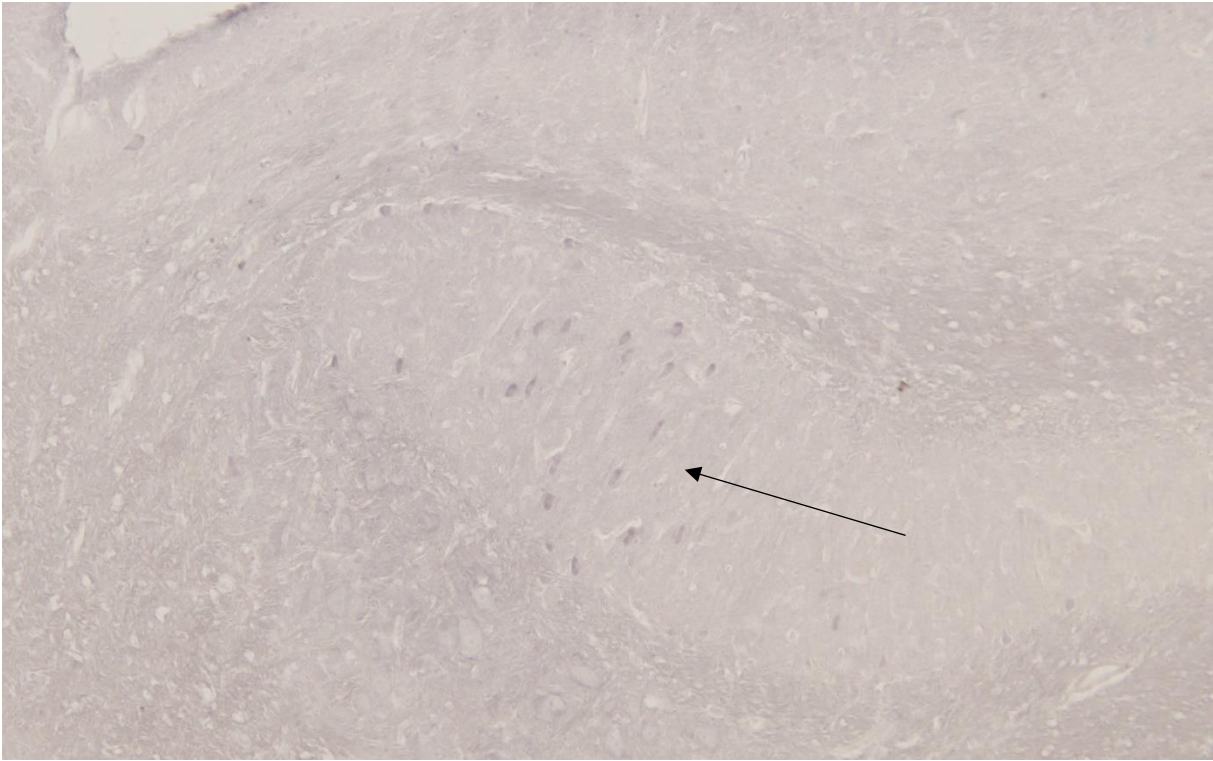


Figure 10: Optic tectum

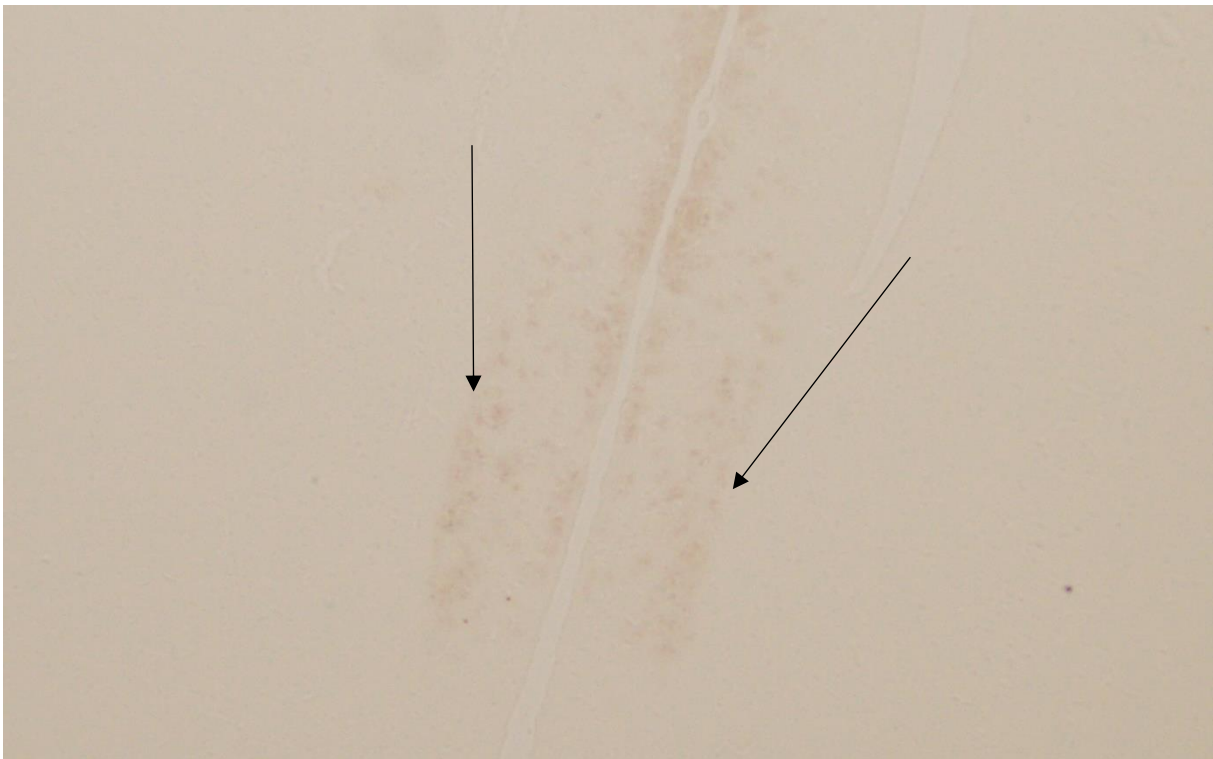


Figure 11: Olfactory tract

The brown appearance of stained orexin receptors in the olfactory tract, as opposed to the purple appearance of receptors in other areas of the brain, is due to nickel not being used as a substrate in that particular immunostaining. The reaction of nickel with DAB gives the rest of the immunostainings their purple appearance.

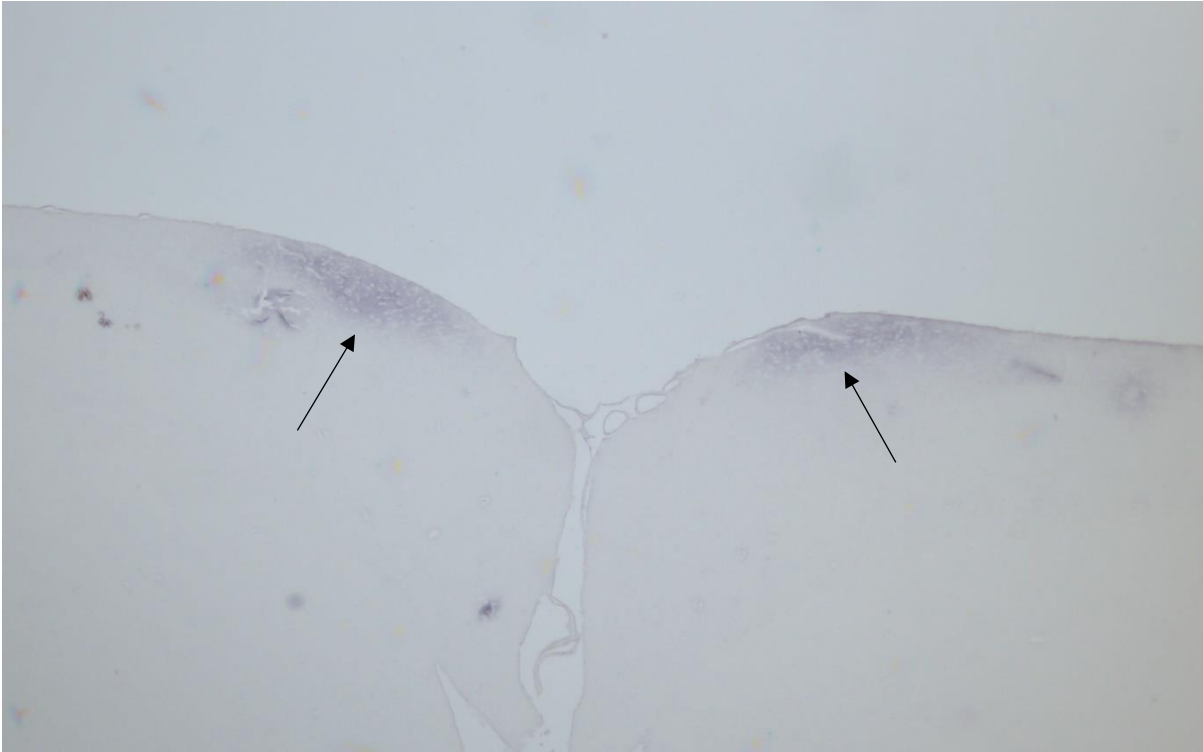


Figure 12: Parahippocampal area

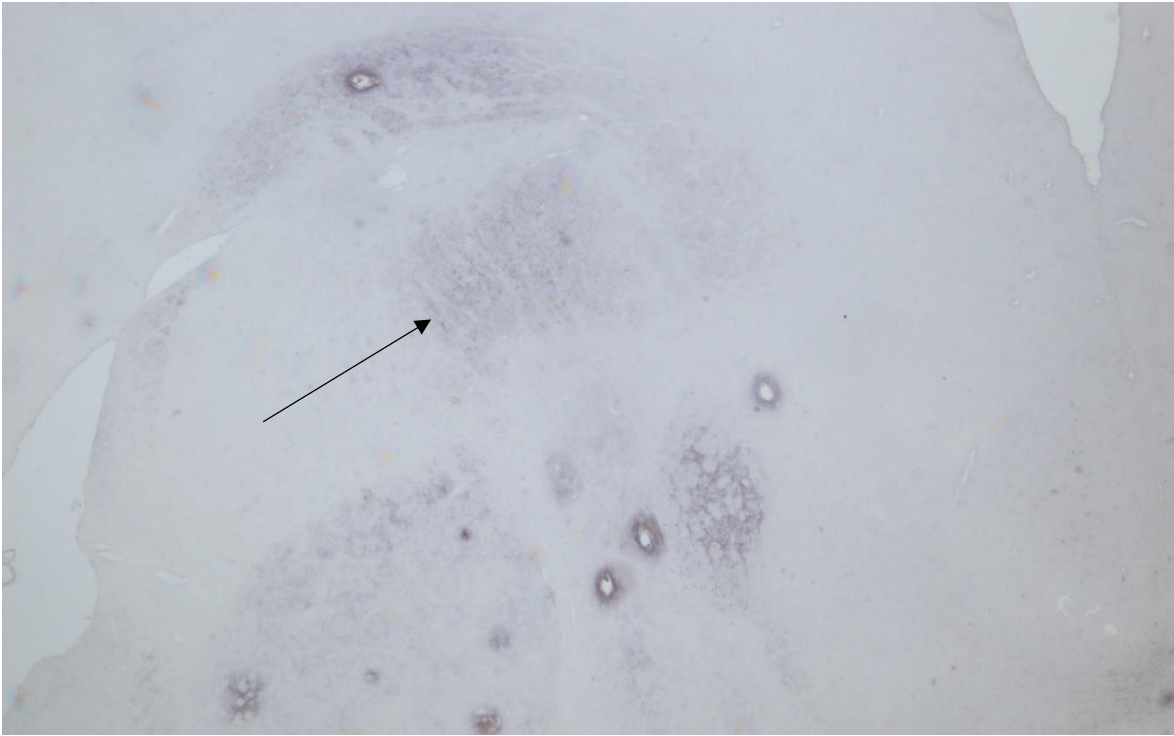


Figure 13: Thalamus



Figure 14: Pons

Chapter 4

Discussion

Implications of Findings

The distribution of orexin receptors seen in the brain of the white-throated sparrow suggests many potential functions for avian orexin, including some in nocturnal migration. Particularly of note in respect to the altered sleep-wake cycle of nocturnal migration is the presence of orexin receptors in the ventral tegmental area (VTA) of the subpallium and the periaqueductal gray (PAG).

The VTA contains dopaminergic neurons, known in mammals to be stimulated by the actions of orexinergic neurons. These neurons are important in the regulation of memory, learning, reward-seeking behaviors, and motivation (Cai & Tong, 2022). Additionally, GABAergic neurons in the VTA of mice were recently discovered to play a key role in regulating non-rapid eye movement (NREM) sleep by “directly innervat[ing] and inhibit[ing] wake-promoting orexin/hypocretin neurons by releasing GABA” (Chowdhury et al., 2019). The PAG, on the other hand, is known to regulate REM sleep in mammals, along with pain signal modulation and sympathetic nervous system responses (Mokhtar & Singh, 2023). The physiologies of the VTA and PAG in songbirds have both been found to be very similar to those of mammals (Gale & Perkel, 2006; Kingsbury et al., 2011), suggesting that avian orexin plays a similar role to mammalian orexin in these regions. This implicates avian orexin in the control of the sleep and wakefulness cycle of the white-throated sparrow – a cycle critically altered during nocturnal migration. The potential involvement of the orexin system in avian NREM sleep is also notable because some birds are able to engage in asymmetric, or unilateral, NREM sleep

during migration to compensate for the associated decrease in sleep (Rattenborg et al., 2019).

While it is not yet known if white-throated sparrows are capable of asymmetric NREM sleep, there is potential for involvement of the orexin system if they are. Outside of the regulation of sleep, the control of the VTA over the reward pathway and motivation may contribute to migratory drive, or the instinct to initiate migration.

The other regions of the brain in which orexin receptors were found may play roles in other aspects of avian migration. The Purkinje cells of the cerebellum, for example, have been found in rats to coordinate autonomic functions, such as cardiac and respiratory functions, during motion (Romano et al., 2020). Though such a coordinating role has not yet been proven in birds, the functionality of the cerebellum is thought to be highly conserved across species (Cunha et al., 2021). If this function is conserved in birds, orexin may contribute to the regulation of cardiac and respiratory functions during long-term flights. The optic tectum and olfactory tract, on the other hand, are involved in sensory processing. While this less obviously correlates to nocturnal migration, both areas have potential functions in navigation (Clark & Rose, 2023; Healy & Guilford, 1990). The parahippocampal area is involved in sensory (specifically visual) processing as well, but it is also involved in homing behaviors, which are critical in the ability of migratory birds to navigate to their final locations (Atoji & Wild, 2006).

The thalamus and pons both have a wide variety of roles and act as relay areas between other parts of the brain. The avian thalamus is known to be anatomically and functionally similar to the mammalian thalamus (Csillag & Montagnese, 2005). Functions of the thalamus include sensory processing, voluntary movement initiation, and regulation of arousal and motivation (Torrice & Munakomi, 2023). Each of these broadly important functions could also play a role in avian migration, especially in regards to voluntary movement and motivation. Less is known

about the avian pons. In mammals, the pons has functions in proprioception, auditory processing, movement and sensation in the face, and regulation of sleep and wakefulness (Rahman & Tadi, 2023; Aleksandrova et al., 2014). The pons of the barn owl is known to affect auditory processing, specifically the perception of volume (Sherry, 2021), but little else is known about the functions of the pons in birds and how those compare to mammalian functions. While proprioception and regulation of sleep and wakefulness would be important factors in avian migration, further research is needed to determine whether the avian pons is responsible for these functions.

Future Research

While identifying the distribution of avian orexin receptors is a promising step in nocturnal migration research, it does not prove that the activity of orexin directly causes Zugunruhe or nocturnal flight. A potential area for future research is the administration of orexin injections to birds not exhibiting Zugunruhe. Should such an injection be able to induce Zugunruhe or other pre- and peri-migratory behaviors, this would be strong evidence for the role of orexin in nocturnal migration. Additionally, more research still needs to be done on the functions of certain regions in the avian brain, which could provide clues to other functions of avian orexin A. There is also opportunity to expand research to other species of migratory and non-migratory birds in order to determine how similar orexin function is across avian species.

Conclusion

Though this research cannot definitively confirm the functions of avian orexin in small migratory songbirds, discovering the distribution of orexin receptors in the brain can direct future studies. The locations of orexin receptors in the brain of the white-throated sparrow imply potential functions for avian orexin in the regulation of the sleep-wake cycle, reward pathways, autonomic functions, sensory processing, and memory. While more research is still needed, there is a strong possibility for the involvement of orexin in avian nocturnal migration.

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