CIRCADIAN RHYTHMS, NEUROANATOMY OF THE SUPRACHIASMATIC NUCLEUS AND SELECTIVE BREEDING OF THE NORTHERN RED-BACKED VOLE (*CLETHRIONOMYS RUTILUS*)

A

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By

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Abstract

The experiments performed in this thesis investigated the circadian rhythms and neuroanatomy of a subarctic rodent, the northern red-backed vole (*Clethrionomys rutilus*). Arctic and subarctic light regimes are extreme, with long periods of light and dark and large daily changes in day-length, but very little is known about circadian rhythms of mammals at high latitudes. A colony of *C. rutilus* was established and proper husbandry techniques were developed to allow voles to reproduce in captivity. Wild-caught and laboratory reared animals were tested for circadian rhythms in a 16:8 hour light:dark (LD) cycle, constant dark (DD) and constant light (LL). Voles displayed predominantly nocturnal patterns of wheel-running in 16:8 LD. In LL and DD, animals displayed large phenotypic variation in circadian rhythms with many becoming non-circadian (60% in DD, 72% in LL), indicating highly labile circadian organization. The distributions of eight common neurotransmitters in the suprachiasmatic nucleus (SCN), the brain's master circadian clock, were characterized. The SCN of *C. rutilus* is similar to that found in other rodents. Larger quantities of cholecystokinin and neuropeptide Y are found in the SCN of C. *rutilus* pointing to the possible importance of non-photic cues in resetting the phase of the internal clock. An additional study also found a distinct distribution of Substance P fibers and neurokinin-1 receptors in the SCN of *C. rutilus*. Starting with the 5^{th} generation, laboratory-bred voles were selectively bred to create two lines of voles that maintained a circadian rhythm in DD, two lines that lost their circadian rhythm in DD, and a randomly bred control line. After three additional generations no significant differences were found among the lines due to the variability in the response

to selection over the first few generations. With a large phenotypic variation in circadian wheel-running rhythms and an SCN similar to other rodents studied, *C. rutilus* is an ideal candidate to study subarctic circadian adaptations. Continued selective breeding will develop a useful tool for elucidating natural genetic variation in circadian rhythm characteristics in a subarctic mammal.

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Chapter One

General Introduction

Circadian, or daily, rhythms are a nearly universal physiological pattern for all forms of life. Plants, multi-cellular animals, and both prokaryotic and eukaryotic single celled organisms display circadian rhythms in many of their physiological and behavioral functions. Plants open their leaves as the sun rises in preparation for photosynthesis to begin, while the cell machinery of animals begins to prepare for activity such as digestion and movement in the hours before they wake. These daily rhythms help the organism take advantage of predictable daily changes in their environment. Circadian rhythms are an inherent physiological feature and will continue even in the absence of any external cues. Organisms left in constant dark, with no external environmental cues, will continue to wake and be active with a nearly 24-hour schedule (reviewed in Dunlap et al., 2004).

Most organisms are either nocturnal, active during the night, or diurnal, active during the day. When placed into constant dark (DD) or constant (LL) the activity of the animal free runs at a period different than 24 hours. This period is termed tau (τ) and varies from animal to animal and by conditions (reviewed in Dunlap et al., 2004). The main entraining agent (zeitgeber) of the circadian rhythm is light. To test the effects of light on circadian rhythms of animals, a phase response curve (PRC) is often constructed. Animals are placed in DD and given a single light pulse at time points throughout the day. The shift of activity caused by each light pulse is plotted to gauge the animal's ability to reset its circadian rhythm (Daan and Pittendrigh, 1976).

Disruption of circadian rhythms can lead to many mental and physical disorders (reviewed in Klerman, 2005). Studies have shown disturbed circadian rhythms under work conditions can negatively affect safety, performance and productivity. Circadian gene mutations have been linked to cancers, metabolic problems and sleep disorders. In addition, the circadian system is involved in many disorders that affect people living in the Arctic and subarctic areas, such as Seasonal Affective Disorder (SAD) and seasonal depression.

In 1972, the location of the mammal's internal clock was confirmed with lesion studies (reviewed in Weaver, 1998). The suprachiasmatic nucleus (SCN) is a pair of small nuclei of approximately 20,000 cells located in the bottom of the hypothalamus directly above the optic chiasm. When the SCN is lesioned an animal loses all endocrine and behavioral circadian rhythms. The SCN receives the light and dark input through the optic nerves that allows it to entrain to external conditions. Light is the main entraining agent for the body's internal clock, but the SCN can also be entrained by other cues (zeitgebers) including temperature, wheel running exposure, air pressure, and food availability (reviewed in Edery, 2000).

Molecular techniques have increased the understanding of SCN function. Many studies have classified the neuropeptides, neurotransmitters and proteins present in the SCN (reviewed in Lee et al., 2003) of mammals such as the mouse (*Mus musculus*; Bult et al., 2001; Silver et al., 1999), hamster (*Mesocricetus auratus)*, rat (*Rattus norvegicus;* Moore and Silver, 1998) and African grass rat (*Arvicanthis niloticus;* Bult and Smale, 1999). The use of mutagenic animals has led to the discovery of the core "clock genes"

which oscillate to produce the 24 hour rhythm of the SCN (reviewed in Lee et al. 2003). Current research is attempting to elucidate how these neuropeptides, neurotransmitters, peptides and genes work together to set the daily rhythm of an animal. In addition, studies are being conducted to determine the output and input signals of the SCN.

In the Arctic, light varies greatly from season-to-season. The summer solstice in Fairbanks, Alaska, has over 21 hours of daylight, while the winter has days with under four hours of light. Changes can be drastic from day-to-day, with a seven minute average daily light change occurring for nine months of the year in Fairbanks. Few studies have been completed to investigate how these drastic light regimes and changes affect the circadian rhythms, health and behavior of animals that live in the Arctic and subarctic. The studies contained within this thesis were an attempt to shed light on circadian rhythms in a subarctic mammal and the effects of the light regimes on these rhythms.

1.1 Study Objectives

Core objectives of the dissertation research were:

1. To study a wild, Arctic mammal that experiences the full range of light regimes. The northern red-backed vole (*Clethrionomys rutilus*), was chosen because it is easily captured and raised in the laboratory (Chapter 2).
2. To determine the circadian characteristics of *C. rutilus*, including whether *C.rutilus* is nocturnal or diurnal, its response to DD and LL, and its response to light pulses in DD (Chapter 3).

3. To determine the distribution of common neurotransmitters, proteins and peptides in the SCN of *C. rutilus* (Chapter 4).
4. To selectively breed *C. rutilus* for circadian and non-circadian rhythms that could later be used to investigate natural allelic variation in circadian "clock genes" in the SCN (Chapter 5).

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Chapter Two

Laboratory Care of the Northern Red-Backed Vole (*Clethrionomys rutilus)*

2.1 Abstract

Northern red-backed voles were captured during the spring and summer of 2000 and a breeding colony was established. Proper husbandry techniques were developed to ensure the maintenance of a healthy captive population. Colony records were kept of all births, deaths and pairings to determine mating success (born and weaned), average weight of adults, weight gain through the first 60 days after birth, gestation period, and common causes of mortality. Weights of wild caught animals were similar to those previously reported, while lab-raised animals tended to be heavier. Gestation length was found to be between 17 and 18 days. The overall mating success was 42%, and litter sizes averaged 5.2 ± 0.3 born and 4.8 ± 0.4 weaned. The most common causes of mortality were accidental deaths, dehydration, fighting wounds, overgrown incisors, and prolapsed rectum. With proper care and attention, *C. rutilus* proved to be a valuable new animal model for studying Arctic circadian adaptations (Chapters 3-5).

Keywords: Arctic, Northern Red-Backed Vole (*Clethrionomys rutilus),* Husbandry

2.2 Introduction

Northern red-backed voles, *Clethrionomys rutilus,* are a member of the order Rodentia, family Arvicolinae. They have a Holarctic distribution and are found

throughout most of Alaska (Stenseth, 1985). *Clethrionomys* are found in the fossil record from the mid Pleistocene, approximately 125,000 years ago, and *C. rutilus* are believed to have colonized the New World during the maximum Illinois glaciation, approximately 20,000 years ago (Cook et al., 2004; reviewed in Gromov & Polyakov, 1992). They are abundant throughout Alaska where they are found in mixed forests dominated by birch (*Betula papyrifera*) and black spruce (*Picea mariana*) as well as in the Arctic tundra (Stenseth, 1985).

Northern red-backed voles have red to brownish orange dorsal fur and a lighter crème colored underside. In the wild, the adults undergo two molts, one in the spring and one in the fall (reviewed in Gromov & Polyakov, 1992). Average length of *C. rutilus* is 130-158 mm with a tail length of 30-40 mm. Their skull has two incisors and three molars per side. The molars have a flat crown with alternating triangles (Dieterich and Preston, 1977).

All voles are burrow builders, an adaptation that helps with thermoregulation as shown by the difference in burrow depths based on soil temperature (reviewed in Gromov & Polyakov, 1992). A secondary benefit of the burrows is protection from predators. However, compared to other Microtinae, *Clethrionomys* are poorly adapted to digging burrows and prefer to inhabit shallow burrows under rocks, tree roots, dead trees and other natural shelters. *C. rutilus* are more adapted to climbing than burrowing, with reduced limb lengths, and can forage in bushes and trees. In the wild, *C. rutilus*' diet consists of woody and partly grassy vegetation, mushrooms, berries and lichens. Seeds

make up a portion of their diet throughout the year (reviewed in Gromov & Polyakov, 1992).

C. rutilus inhabits the Arctic and subarctic environments of the earth. The non-hibernating nature of *C. rutilus,* their distribution and life cycles have made them an ideal candidate for studies on Arctic physiology (Zuercher et al., 1999; White and Feist, 1998; Feist and Feist, 1978, Feist and Rosenmann, 1976; Sealander, 1972; Andrews, 1970) and *Microtine* population fluctuations in the Arctic (Patrick and Krebs, 2002; Schweiger and Boutin, 1995; Whitney, 1976; Fuller, 1969). They are also exposed to the extreme light:dark (LD) cycles of the Arctic and have been used to study circadian adaptations (Chapters 3-5; Peiponen, 1962; Pruitt, 1959; Stebbins, 1972; Swade and Pittendrigh, 1967).

Arctic and subarctic environments are exposed to extreme light:dark (L:D) regimes. Animals captured for this study in Fairbanks, Alaska, experience mean changes in day-length of approximately seven minutes a day for nine months of the year (Fig 2.3A). Day-length in Fairbanks on the summer solstice is over 21 hours and falls to just over three and a half hours during the winter solstice (Fig 2.3B).

To study the effect of these extreme light regimes on Arctic, animals were captured a population of northern red-backed voles from the interior of Alaska (N. 64°, W. 147°) during the spring and summer of 2000. A breeding colony was established and animals were kept for six years and seven generations in the laboratory.

2.3 Methods

2.3.1 *Capture of Animals*

Red-backed voles were captured from interior Alaska (N. 64°, W. 147°) during the spring and summer of 2000. Sherman live traps with a nesting chamber attached to one end containing cotton nesting material were baited with a mixture of oats, peanut butter and apple chunks. Traps were set in grids of 20 strings of five traps each off trails in areas of mixed woodland dominated by Alaska birch (*Betula neoalaskana)* and black spruce (*Picea mariana*) trees. Voles were transported to the University of Alaska Fairbanks Animal Quarters and placed in quarantine for fourteen days after capture.

Appropriate permits and approvals for capturing and housing wild animals were obtained: State of Alaska Department of Fish and Game Permit Numbers 99-121(1999), 00-001 (2000), 01-013 (2001), 02-001 (2002), 03-001 (2003), 04-031 (2004), 05-055 (2005), and 06-145 (2006), and University of Alaska Fairbanks Institutional Animal Care and Use Committee Protocol Numbers 99-27 (approved 11-12-1999), 02-47 (approved 11-12-2002), and 05-62 (approved 11-16-2005).

2.3.2 *Vole Husbandry*

Voles were housed and bred in fiberglass cages (15x92x20 cm) under 16:8 LD at room temperature (22 ± 1°C). All animals were provided with wood shavings and cotton nesting material. Food (Lab Diet Co. Mouse Diet #5015, St. Louis, MO and Purina Mills, Inc. Rabbit Chow, St. Louis, MO) and water were available *ad libitum*. Water was

provided via a water bottle with a drinking tube. Animals were also fed one slice of carrot daily at haphazard times. During feeding all animals were also checked for health. Starting with the third generation white noise generators were added to all of the animal rooms. In addition, extra care was taken not to disturb animals when daily checks and cage cleaning were performed.

Animals were first bred when they were at least 60 days old. Voles were randomly paired except siblings were never mated and first cousin pairing were avoided as much as possible (Bult and Lynch, 2000). Two weeks after pairing, males were removed to assure undisturbed delivery of the litter and reduce cannibalism. All voles were weaned at 19 ± 1 days of age and housed with same-sex litter-mates.

2.4 Results and Discussion

In addition to the normal pelage seen in the wild, a segment of our population also developed black fur on its flanks, and one grey individual was born in the 2038 voles raised over seven generations.

Wild caught males averaged 25.7 ± 1.7 (SEM; n = 21) grams and females weighed 22.0 ± 1.6 grams (n = 27). After a month in captivity males experienced a weight gain to 27.3 ± 1.6 grams (n = 16) while females showed no weight gain (n = 23). Laboratory-bred animals from the first two generations showed slightly higher average weights at 90 days of age than the wild-caught animals; 28.1 ± 1.7 grams for the males (n = 36) and 24.4 ± 1.3 grams (n = 29) for the females.

The higher weight of the laboratory raise animals may have been the result of the better nutrition as well as the reduced activity of the animals in their cages compared to activity in the wild. However, the red-backed voles tended to be very active compared to other laboratory animals. Water bottles had to be checked every day, and occasionally twice a day, due to the animals climbing on the water bottle tube and upsetting it. *C. rutilus* seemed to dehydrate quickly, and a few animals died due to this problem. Water bottles were secured with duct tape if they were found upset. The diet provided proved satisfactory. The food was placed loosely in the cages to allow animals ready access. Voles were seen to bury food and hoard uneaten portions in their nests. Cages were checked daily and more food was added as needed.

Voles were provided with wood shavings and cotton nesting material. They readily burrowed into shavings and constructed nests from the shredded cotton. Other environmental enrichment techniques were used, such as ping pong balls and PVC tubes. The PVC tubes were consistently used as parts of nests and running pathways and became a permanent part of the environment and were removed weekly for washing.

Wild-caught voles were seldom observed outside the nest and cotton had to be moved to check the animal. This often caused the vole to leap from the cage. Voles do not seem to fear heights and animals were dazed when landing. The easiest way to remove a vole for cage cleaning and weighing was to encourage the vole to enter the PVC tube and then remove the tube and vole with a hand over each end of the tube. Cages were prepared with a cover half open and nesting material under the covered end of the cage. The vole would exit the tube and enter the nest. Animals were also captured

by grasping the back of the neck fur between the thumb and forefinger, although this sometimes resulted in biting of the handler. Their small size made the use of gloves difficult. Later generations of voles became tamer and would often exit their nests when a handler entered the room. *C. rutilus* also proved adept at pushing cage tops open by jumping on and pushing the water bottle tube. In order to easily capture escaped voles, Sherman traps were placed in corners of the rooms baited with mouse food and apple slices as a water source.

Gestation length was between 17 and 18 days with some animals having litters present on the 18th day after initial pairing. The length of gestation was similar to Morrison et al., 1976 who found the gestation length to be 17.5 days but differs from Innes and Millar, 1994 who reported a gestation period of 18.7 days Animals were weighed when weaned and averaged 14.1 ± 0.3 (SEM) grams (n = 94) with no difference between the sexes. At 40 days of age males weighed 23.6 ± 0.5 grams (n = 45) and were close to their adult weight at 60 days of age, averaging 25.7 ± 0.6 grams (n = 39). Females averaged 21.9 ± 0.6 grams (n = 42) at 40 days and 24.8 ± 0.3 grams (n = 33) at 60 days.

Mating success per generation and overall success is shown in Table 3.1. During the first few generations all animals were mated in an attempt to increase the colony size. In later generations two or three voles were randomly chosen from each litter for mating. Starting with the fifth generation a selective breeding experiment was undertaken with five lines of animals developed; two lines that maintained circadian rhythms in DD, two lines that lost circadian rhythmicity in DD, and a randomly bred control line. This

experiment resulted in a significant increase in mating attempts and the size of the colony. The overall mating success was 42%. Litter size averaged 5.2 ± 0.3 born and 4.8 ± 0.4 weaned. This litter size is probably underestimated due to mothers occasionally cannibalizing some or all of the litter.

Our overall mating success was similar to the 46% experienced by Morrison et al. (1976). Wild caught stock proved very difficult to mate with a success rate of only 13%. Improved husbandry techniques may have led to better success in later generations. The addition of the white noise generators in the colony rooms also helped to cover the noise of animal handlers and other animals in the facility. This may have reduced the stress of the animals and increased mating success. Extra care was also taken not to disturb the animals when paired. Daily animal checks were performed as quietly as possible with as little disturbance as possible. When young were present, the nest was transferred to the new cage along with fresh cotton. This seemed to reduce the cannibalism of litters. Our average litter size was slightly larger than that found by Morrison et al. (1976; 4.9 and 3.6), but lower than reported by Innes and Millar (1994; 6.6).

The most common causes of mortality in the 2038 voles raised were accidental deaths (19), dehydration (16), fighting wounds (15), escaped animals (13), overgrown incisors (5) and prolapsed rectum (4). These problems were more common in earlier generations when the animals were less tame. As mentioned above, *C. rutilus* is an active species, and individuals would often try to escape when the cage was opened. Animals were injured in falls from the cage racks or when handlers were attempting to recapture them. The use of Sherman life traps with apple chunks for moisture as well as better

attention by handlers lowered the mortality. Water bottles were often checked more than once a day and filled when below eight ounces to reduce dehydration deaths. If water bottles were knocked over they were taped to prevent this from happening again. In later generations same-sex littermates were separated earlier to prevent fighting and cannibalism. Animal were checked for overgrown incisors regularly during weekly cage cleanings, and teeth clipped if needed.

With proper care and attention *C. rutilus* proved to be amenable to life in the laboratory. Special care must be taken when handling voles due to their wild nature. With the proper care and equipment *C. rutilus* proved to be a useful model for studying circadian adaptations to the Arctic.

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Figure 2.1 The northern red-backed vole, *Clethrionomys rutilus*, with red/brown dorsal fur.

Figure 2.2 A male northern red-backed vole, *Clethrionomys rutilus.* The crème/buff undersides can be seen.

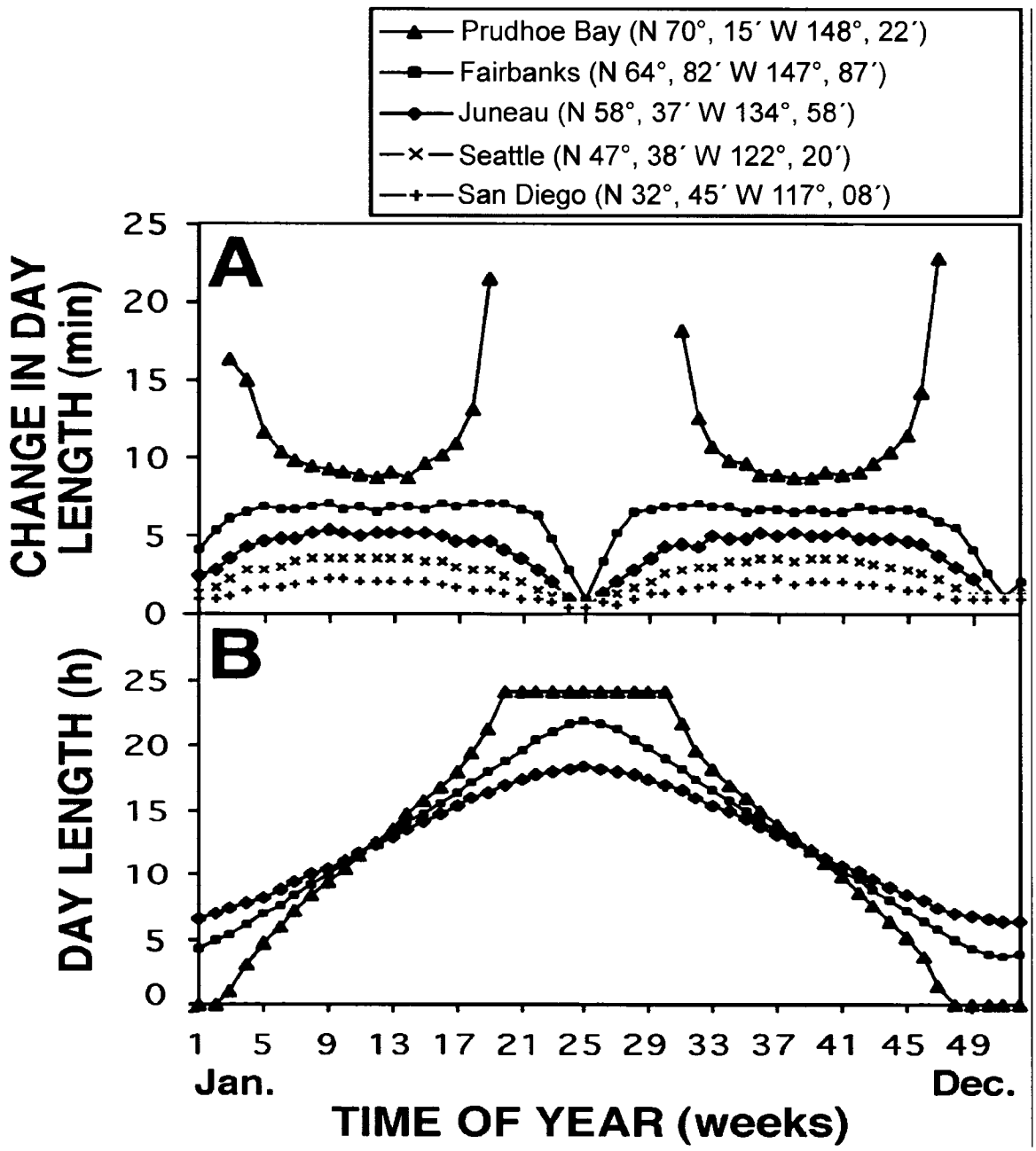

Figure 2.3 Average change in day length (A) and total day length (B) on the North Slope, AK, Fairbanks, AK, Juneau, AK, Seattle, WA, and San Diego, CA. Day length is defined as civil twilight, when the sun is below the horizon but its center is less than 6 degrees below.

Table 2.1 Red-backed vole mating success and litter size by generation (± SEM).

Red-Backed Vole Mating Success and Litter Size by Generation								
	Generation							
	1st	2nd	3rd	4th	5th	6th	7th	Overall
Total Number of Breeding Pairs	64	29	29	33	187	167	239	748
Successful Pairs	8	17	11	16	93	69	97	311
Percentage Successful	13	59	38	48	50	41	41	42
Average Litter Size Born	5.4 ± 0.7	4.9 ± 0.3	4.7 ± 0.5	5.3 ± 0.3	5.3 ± 0.2	6.3 ± 0.2	4.7 ± 0.2	5.2 ± 0.3
Range	1-7	2-7	2-7	3-7	1-9	2-12	1-10	1-12
Average Litter Size Weaned	5.4 ± 0.7	4.4 ± 0.5	4.6 ± 0.5	5.3 ± 0.3	4.6 ± 0.3	5.6 ± 0.3	4.0 ± 0.3	4.8 ± 0.4
Range	1-7	0-7	2-7	3-7	0-9	0-12	0-10	0-12

Chapter Three

Circadian Organization of a Subarctic Rodent, the Northern Red-Backed Vole (*Clethrionomys rutilus*)[1]

3.1 Abstract

Arctic environments are exposed to extreme light:dark (LD) regimes including periods of constant light (LL) and constant dark (DD) and large daily changes in day-length, but very little is known about circadian rhythms of mammals at high latitudes. We investigated the circadian rhythms of a subarctic population of northern red-backed voles (*Clethrionomys rutilus*). Both wild-caught and third-generation laboratory-bred animals showed predominantly nocturnal patterns of wheel-running when exposed to a 16:8 LD cycle. In LL and DD conditions animals displayed large phenotypic variation in circadian rhythms. Compared to wheel-running rhythms under a 16:8 LD cycle, the robustness of circadian activity rhythms decreased among all animals tested in LL and DD, i.e., decreased chi-squared periodogram waveform amplitude. A large segment of the population became non-circadian (60% in DD, 72% in LL) within eight weeks of exposure to constant lighting conditions, of which the majority became ultradian, with a few individuals becoming arrhythmic, indicating highly labile circadian organization. Wild-caught and laboratory-bred animals that remained circadian in wheel-running displayed free-running periods between 23.3 and 24.8 hours. A phase response

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curve to light pulses in DD showed significant phase delays at circadian times 12 and 15, indicating the capacity to entrain to rapidly changing day-lengths at high latitudes. Whether this phenotypic variation in circadian organization, with circadian, ultradian, and arrhythmic wheel-running activity patterns in constant lighting conditions, is a novel adaptation to life in the Arctic remains to be elucidated.

Keywords: Circadian rhythms, wheel-running activity, Arctic, light-induced phase shift, Northern Red-Backed Vole (*Clethrionomys rutilus)*

3.2 Introduction

Low temperatures and short growing seasons in the Arctic and subarctic are well described. The effects of these environmental conditions on resident mammals has been investigated (reviewed in Marchand, 1996), but few studies have focused on how Arctic mammals cope with the extreme light regimes in the Arctic. Day-length changes can be dramatic on a day-to-day basis. Animals at our trapping sites in Fairbanks, Alaska, experience mean changes in day-length of approximately seven minutes a day for nine months of the year. Day-length in Fairbanks is over 21 hours on the summer solstice and falls to just over three and a half hours at the winter solstice. These dramatic seasonal differences in photoperiod may have resulted in the evolution of unique circadian adaptations that could shed light on fundamental physiological processes.

Daan and Aschoff (1975) described increased individual variability in onset and end of locomotor activity as measures of precision of daily activity for five species of

captive birds (perch landings) and three species of captive mammals (wheel-running) from the Arctic Circle when compared to members of the same species from lower latitudes. The variability of these characteristics was highest in midsummer and midwinter when light conditions were nearly constant. Studies of a few captive *Clethrionomys rutilus* found they became arrhythmic under both the near constant light, characteristic of Arctic and subarctic summers, and the contrasting long periods of winter darkness. The same individuals showed clear nocturnal rhythms in the spring and fall when light:dark (LD) cycles were clearly defined (Peiponen, 1962 [nest box entrances]; Pruitt, 1959 [live-trapping data]; Stebbins, 1972 [nest box entrances]; Swade and Pittendrigh, 1967 [wheel-running]). Whether or not these differences in circadian characteristics between high latitudes and temperate latitudes reflect fundamental differences in circadian clock function remains to be addressed, because animals from different latitudes were subjected to different environmental conditions. In addition, most of these experiments should be viewed as anecdotal due to the lack of matched controls and small sample sizes.

Captive Svalbard ptarmigan (*Lagopus mutus hyperboreus)* from 76 – 80° N. lattitude, subjected to constant light (LL) or constant dark (DD) conditions became arrhythmic in feeding behavior and plasma melatonin levels (Reierth and Stokkan, 1998a; Reierth and Stokkan, 1998b). Captive willow ptarmigan (*Lagopus lagopus)* also became arrhythmic in activity (perch landings) during the near constant light of the Fairbanks, Alaska, summer (West, 1968). Similarly, the constant light of summer caused rhythms in plasma melatonin to disappear in female reindeer (*Rangifer tarandus)* in Finland

(Eloranta et al., 1992). However, not all animals become arrhythmic under the constant lighting conditions of the Arctic. Folk (1964) found that captive Arctic foxes (*Alopex lagopus*), wolves (*Canis lupus*) and wolverines (*Gulo gulo*) kept at Barrow, Alaska, maintained circadian rhythms in heart rate, EKG, and body temperature year-round. Arctic black-capped marmots (*Marmota camtschatica*) also kept a coherent circadian above-ground activity rhythm throughout the summer when the sun did not set (Semenov et al. 2001).

To investigate the effects of extreme light regimes on the mammalian circadian system, we tested northern red-backed voles, *C. rutilus*, under controlled laboratory conditions. *C. rutilus* is an abundant, small (20-35 gram) non-hibernating rodent distributed throughout Alaska (Stenseth, 1985). In addition, *C. rutilus* is easy to capture, has a short generation time, and breeds year round in the laboratory (this study). Voles were captured near Fairbanks, Alaska and maintained at normal room temperatures under controlled lighting conditions that included a 16:8 LD cycle, LL, and DD. A phase response curve was determined in DD with 20-minute light pulses. Both wild-caught and third-generation laboratory-bred animals were used in the experiments to determine whether circadian phenotypes in wild-caught animals were predominantly due to environmental or genetic factors. This controlled study provides baseline data for identifying a potential latitudinal cline in circadian organization within the natural distribution of this species in Alaska.

3.3 Methods

3.3.1 *Capture of Animals*

To study circadian behavioral traits, we obtained two populations of northern red-backed voles from interior Alaska (N. 64°, W. 147°). The University of Alaska Fairbanks Biological Reserve population was established during the spring and summer of 2000. The Bonanza Creek Experimental Forest population was established during the summer of 2000. Animals were captured with Sherman live traps with a nesting chamber attached to one end equipped with cotton nesting material. A mixture of oats, peanut butter and apple chunks was used for bait. Traps were set in grids of 20 strings of five traps each off trails in areas of mixed woodland dominated by Alaska birch (*Betula neoalaskana*) and black spruce (*Picea mariana*) trees. Traps were checked every eight hours and non-target species were released. Voles were transported to the University of Alaska Fairbanks Animal Quarters and placed in quarantine for fourteen days after capture. Wild-caught northern red-backed voles were kept under a 16:8 LD cycle during and after quarantine for at least four weeks to adjust to captivity. Males were used for behavioral studies performed on the wild-caught populations.

Appropriate permits and approvals for capturing and housing wild animals were obtained: State of Alaska Department of Fish and Game Permit Numbers 99-121(1999), 00-001 (2000), 01-013(2001), 02-001 (2002), and 03-001(2003) and University of Alaska Fairbanks Institutional Animal Care and Use Committee Protocol Number 99-27 (Approved November 12, 1999) and Protocol Number 02-47 (Approved November 12, 2002).

3.3.2 *Vole Husbandry*

Voles not used for behavioral testing were housed and bred in fiberglass cages (15x92x20 cm) under 16:8 LD at room temperature (22 ± 1°C). All animals were provided with wood shavings and cotton nesting material. Food (Lab Diet Co. Mouse Diet #5015, St. Louis, MO and Purina Mills, Inc. Rabbit Chow, St. Louis, MO) and water were available *ad libitum*. Animals were also fed one slice of carrot daily at haphazard times. Two weeks after pairing, males were removed to assure undisturbed delivery of the litter. All voles were weaned at 19 ± 1 days of age and housed with same sex litter-mates. Voles from each population were randomly paired except siblings were never mated and first cousin pairing were avoided as much as possible (Bult and Lynch, 2000).

3.3.3 *Data Collection*

Wheel-running activity of individually housed wild-caught voles was measured using 16-cm diameter running wheels mounted in polypropylene cages equipped with a magnetic switch (24x35x21 cm) with wood chips and cotton nesting material. Number of wheel revolutions was continuously recorded in 5-min bins by computer with the VitalView data collection system (Mini-Mitter Co., Inc., Bend, OR). All cages were visually isolated and a white noise generator (LaFayette Instrument Co., LaFayette, IN) was used to minimize auditory cues.

Wheel-running activity was measured in wild-caught male voles (22-32 grams at the start of the experiment) under a 16:8 LD cycle for six weeks to allow the animals to

establish stable wheel-running patterns. This was followed by eight weeks of DD. Animals were then returned to 16:8 LD for six weeks to re-entrain to the LD cycle.

A phase response curve to twenty-minute light pulses was determined for a separate group of wild-caught voles. Males were placed in DD for 5-7 days, which provided a reliable phase reference point for their wheel-running activity rhythms (Fig. 3.1A). The animals were then given a twenty-minute 700 lux light pulse at one of the following circadian times (CTs): 3, 6, 9, 12, 15, 18, 21 and 24. Circadian time 12 is defined as time of activity onset in DD. The subsequent 5-7 days in DD were used to measure the phase shift. Although the 5-7 day period may appear rather short, it was chosen because many voles became non-circadian after 3-4 weeks in constant dark, precluding the measurement of light-induced phase shifts if longer periods were used. In addition, the Aschoff Type II procedure, which requires only 1 day in constant dark before the light pulse is given, is a valid method to assess phase shifts (Mrosovsky, 1996). Therefore, the 5-7 day period was justified. For each circadian time point, all animals were subjected to the same number of days in DD before and after a light pulse. After each light pulse animals were returned to 16:8 LD for 7 - 21 days to re-entrain before receiving a subsequent light pulse for the remaining time points. The number of days to re-entrain was variable depending on the phase of the free-running rhythm in DD compared to the phase of the new LD cycle and the lability of the wheel-running activity rhythm. For each circadian time point, all animals were subjected to the same number of days in LD before they were subjected to DD again. Re-entrainment was confirmed by visual inspection of wheel-running records. All animals had to be stably entrained to the

LD cycle for at least 3 days before the entire group was returned to DD. Returning the animals to a LD cycle following each light pulse allowed direct comparison of phase shifts at different CTs and maintenance of the animals at comparable physiological states throughout the experiment. Animals that lost circadian rhythmicity within two weeks of DD were excluded from this experiment.

Wheel-running activity was also tested in third-generation laboratory-bred animals (20-30 grams at start of experiment) under LL and DD conditions. Animals were individually housed in polycarbonate cages (37x21x14 cm) equipped with 24-cm diameter running wheels (Nalgene, Rochester, NY). These wheels were larger, spun easier, had smaller spaces between the rungs and were made of a heavier metal than the wheels used for the wild-caught animals, because the design of the smaller wheels caused several deaths in the first study. Twelve males and twelve females, 60-90 days of age, were placed in a 16:8 LD cycle for eight weeks to establish stable wheel-running rhythms. Animals were then placed in LL for eight weeks. Following LL, the animals were returned to a 16:8 LD for twelve weeks to re-entrain to the LD cycle. Due to the strong and long-lasting effect of LL on the voles' circadian rhythms, animals required up to eight weeks to resume stable entrainment. Animals were then subjected to eight weeks of DD followed by a final six weeks of 16:8 LD.

Third-generation voles were also tested for clinging behavior (similar to Drickamer and Evans, 1996) to determine if a significant number of wheel revolutions occurred while the animals were clinging to the wheel. Clinging occurs when animals stop running and ride the wheel around for one or more turns. Such clinging has been

suggested by Drickamer and Evans (1996) to affect circadian patterns of wheel-running. Voles were observed during the 3-7 hours preceding lights off in a 16:8 LD. Each vole was observed for a total of 10-70 minutes (33.9 ± 3.2 (SEM), n=21), to a total of 12 hours of observation for all voles combined. The onset time and duration of wheel-running was recorded for each animal, along with the number of revolutions where the animal was clinging. This record was compared to the total number of revolutions collected by the computer during the same time period and clinging events were quantified as a percentage of total revolutions.

3.3.4 *Classification of Wheel-Running Rhythms*

Wheel-running data were analyzed with Vitalview and Actiview (Mini-Mitter Co, Inc.), Matlab Version 6 (The Mathworks, Inc. Natick, MA), and Clock Lab (Actimetrics, Evanston, IL) software programs. Two-week intervals were used for the analyses of rhythm type and robustness of the circadian rhythm, because rhythm data analysis methods, recommended by Levine et al. (2002), require a minimum of ten days to provide reliable results. Following the general recommendations of Levine et al. (2002), animals were classified as circadian (rhythm of approximately 24 hours), ultradian (rhythm of 2-10 hours), or arrhythmic based on wheel-running activity. Records were initially screened for rhythmicity with autocorrelation using Clock Lab. If a circadian rhythm was identified by autocorrelation, it was confirmed with chi-squared (Sokolove and Bushell 1978) and Lomb-Scargle periodograms (Van Dongen et al., 1999). These analyses also provided the free running period (τ) in hours (h) and rhythm robustness,

i.e., amplitude of the periodogram waveform. If autocorrelation identified an ultradian rhythm, this was confirmed and τ was determined by Fast Fourier Transformation and Lomb-Scargle periodogram. These analyses are more sensitive to identifying shorter periods than the chi-squared periodogram analysis (Levine et al., 2002). Levels of significance were set at $p<0.05$ for all tests. When both circadian and ultradian rhythms were present, all tests were used. Animals displaying neither circadian nor ultradian patterns of wheel-running in a two-week period using the above procedures were classified as arrhythmic.

3.3.5 *Robustness Characteristics of Wheel-Running Rhythms*

The primary assessment of the robustness of wheel-running rhythm for each two-week interval was amplitude of the chi-squared periodogram waveform. Arrhythmic and ultradian animals were assigned default amplitudes of 400, the average "noise" of an arrhythmic animal's periodogram at a τ of 24 hours (Fig. 3.1D2). Differences in rhythm robustness were further investigated by quantifying two-week average fragmentation of the wheel-running pattern (mean number of activity bouts per circadian day), total wheel revolutions per day, and amplitude of the circadian wheel-running rhythm. Wheel-running activity fragmentation was determined by counting periods of activity that exceeded fifteen percent of the daily activity maximum, were at least ten minutes in duration, and were separated from previous and/or subsequent bouts by at least twenty minutes. The daily number of bouts was averaged for each two-week period. Amplitude of the wheel-running rhythm (daily maximum minus daily minimum wheel revolutions

per 5-minute bin) was determined using 30-minute smoothed plots generated by Actiview (Mini-Mitter).

Direct comparison of individual animals was done by using weeks 3-6 of the first 16:8 LD cycle as a baseline (100%) for chi-squared periodogram amplitude, fragmentation, amplitude of circadian wheel-running activity rhythm, and activity level for each animal. Circadian activity characteristics in the subsequent periods were expressed as a percentage of this baseline.

3.3.6 *Statistical Analyses*

Differences in circadian rhythm robustness (i.e. chi-squared periodogram waveform amplitudes) between the different lighting regimes (16:8 LD, DD and LL) were investigated using the general linear model (GLM) procedure analysis of variance (ANOVA, SAS Institute Inc., Cary, NC). Population (Bonanza Creek or Fairbanks) was included as a factor in the model for the wild-caught voles. Third-generation laboratory-bred voles had sex added as a factor in the model. No population or sex effects were found (data not shown) and data were pooled for further analysis. Differences in two-week mean levels of fragmentation of the wheel-running pattern, total revolutions per day, and amplitude of the wheel-running pattern were tested for significance with the GLM procedure. When significance was found, pair-wise differences were tested for significance using the Tukey Studentized Range Test (Sokal and Rohlf, 1981). Bonferroni corrections for multiple statistical tests were employed (Holm, 1979: Rice, 1989) by multiplying the p-value by the number of tests used, i.e. three. Corrections were

used for the following variables: fragmentation of the wheel-running pattern, total revolutions per day, and amplitude of the wheel-running rhythm.

The GLM procedure was also used to test for the effect of circadian time (CT 3, 6, 9, 12, 15, 18, 21, 24) on light-induced phase shifts for the phase response curve (Bult et al., 1993; Mahoney et al., 2001). When significance was found, pair-wise differences were tested for significance using the Tukey Studentized Range Test (Sokal and Rohlf, 1981). All data are presented as means ± standard error of the mean (SEM).

3.4 Results

Wild-caught voles housed under a 16:8 LD cycle displayed a clear nocturnal pattern of wheel-running with 85.2 ± 3.0% of their activity occurring during the dark period (n=15, range 54-99%; Figs. 3.1A and 3.1C). Third generation laboratory-bred voles were also predominately nocturnal with 67.8 ± 4.0% of their activity occurring during the dark period (n=24, range 21-97%; Figs. 3.2A and 3.2C).

Sixty-seven percent (4 of 6) of wild-caught voles maintained a circadian wheel-running rhythm during the eight weeks in DD (Fig. 3.1A and 3.1B2), with an average τ of 24.0 ± 0.2 h. (range 23.5-24.3 h.). Within eight weeks of DD, 33% (2 of 6) of wild-caught voles became ultradian (τ of 4.2 and 5.6 h). Third-generation laboratory-bred voles showed similar circadian wheel-running rhythms during DD, with 40% (8 of 20) maintaining a circadian rhythm throughout the eight weeks of DD with an average τ of 23.8 ± 0.1 h (range 23.4 to 24.1 h). Fifty percent (10 of 20) of the third-generation laboratory-bred voles became ultradian during the eight weeks of DD with and average τ

of 4.8 ± 0.2 h (range 3.8 to 5.6 h). Ten percent (2 of 20) of the third-generation laboratory-bred voles became arrhythmic within eight weeks of DD.

Third-generation laboratory-bred voles showed similar phenotypic variation in circadian wheel-running characteristics during eight weeks in LL when compared to DD. Twenty-nine percent (6 of 21) of the laboratory-bred voles maintained a circadian rhythm throughout eight weeks in LL with an average τ of 24.0 ± 0.2 h. (range 23.3 to 24.8 h., Fig. 3.2A and 3.2B2). Sixty-two percent (13 of 21) of the third-generation voles became ultradian within eight weeks of LL with an average τ of 4.9 ± 0.3 h. (range 3.7 to 6.7 h., Figs. 3.2C and 3.2D2). Ten percent (2 of 21) of the third-generation laboratory-bred voles became arrhythmic within eight weeks of LL. There was no relationship between voles behavior in DD and LL with different voles displaying differing circadian rhythm characteristics under each condition.

Results of the DD experiment using wild-caught voles are summarized in Table 3.1. Wild-caught voles showed significantly lower amplitudes of the chi-squared periodogram waveform during weeks seven and eight of DD, with values falling to 57% of their levels during their first four weeks in LD (LD1, Table 3.1; Figs. 3.1B and 3.1D). Although there was a tendency toward increased fragmentation of wheel-running activity, decreased daily activity level amplitude, and decreased average daily activity, these trends were not significant.

Results from the third-generation laboratory-bred voles tested in eight weeks of DD are summarized in Table 3.2. All animals showed a significant decrease in amplitude

of the chi-squared periodogram waveform, with values from weeks seven and eight of DD falling to 43% of their LD1 levels (Table 3.2). The eight weeks of DD also contributed to significant decreases in daily amplitude of wheel-running activity rhythms and average daily activity compared to levels during LD1 (Table 3.2). Table 3.2 also shows the results from the third-generation laboratory-bred animals tested through eight weeks of LL. Average amplitude of the chi-squared periodogram waveform fell to 41% of LD1 levels (Table 3.2; Figs. 3.2B and 3.2D). Constant light did not significantly alter fragmentation of activity, daily rhythm amplitude, or daily wheel-running activity. The northern red-backed vole's phase response curve is shown in Fig. 3.3A ($F_{7,41}$=6.72, p=<0.0001). Significant delays of one hour or more were found at CT 12 and CT 15 (Figs. 3.3B and 3.3D) with a phase delay of 36.4 minutes at CT 18 being intermediate between CT 12 and 15 and the rest of the time points (Fig. 3.3C). No consistent phase advances were observed in late subjective night at the end of their activity period (Fig. 3.3A).

During 12 hours of observations, 48% (10 of 21) of the voles displayed clinging behavior at various times while using the running wheels. However, clinging accounted for less than 2.1 ± 0.4% of total activity during the observation periods and, therefore, did not affect general circadian characteristics of wheel-running.

3.5 Discussion

Both wild-caught and third-generation laboratory-bred red-backed voles showed high phenotypic variation in circadian rhythms. While some voles maintained robust circadian

rhythms throughout eight weeks in constant lighting conditions, others lost circadian rhythmicity in DD or LL. All voles showed a significant decrease in robustness of their circadian rhythms under constant lighting conditions, measured by a decrease in the amplitude of the chi-squared periodogram waveform. Of voles that lost circadian rhythmicity, a large portion became ultradian. This loss of circadian rhythmicity, originally seen in the wild-caught voles, remained in the population even after three generations of random breeding in captivity, suggesting a genetic basis. The loss of circadian rhythmicity was not correlated with the age of either the wild-caught or laboratory-bred animals (data not shown). In general, the changes in circadian organization from a LD cycle to constant lighting conditions were not as robust in the wild-caught voles, i.e., no significant change in average daily amplitude and activity (see Tables 3.1 and 3.2), compared to their descendants. This may have been due to the smaller sample size of the wild-caught group. In addition, different sized wheels provided to the wild-caught and laboratory-bred voles may also have had an effect. However, we have no empirical data to compare circadian rhythm data for the two wheel types within the same group of animals.

Careful evaluation of the circadian rhythms of the northern red-backed voles under the controlled environmental conditions in the laboratory agrees with other past studies of voles in their natural lighting conditions. Four *C. rutilus* caged with running wheels under natural summer lighting at Point Barrow, Alaska (N. 70°, W. 148°), showed the same propensity for arrhythmicity that was found in this study when their actograms were visually inspected. More activity occurred during the middle of the day during the LL of

the summer months than during the spring and fall when the LD cycle was more defined and the activity pattern was mainly nocturnal (Swade and Pittendrigh, 1967). Peiponen (1962) also found *C. rutilus* to be arrhythmic in entering nest boxes during the constant light of summer and nocturnal in the fall at Kilpisjärvi Biological Station, Finland (N. 69°, E. 20°). Nest boxes were equipped with electronic switches to monitor when the animal entered and exited the nest. At Heart Lake, Northwest Territories, Alberta, (N. 60°, W. 116°) four *C. rutilus* in outdoor cages showed an activity rhythm in nest box entrances and exits that changed from arrhythmic in winter to nocturnal in spring (Stebbins, 1972). Pruitt (1959) also commented on *C. rutilus'* apparent lack of a daily rhythm in interior Alaska during the middle of winter and middle of summer using live-trapping data. Our study, consistent with earlier work, demonstrates the ability of *C. rutilus* to maintain a circadian rhythm under a defined LD cycle but also to have the tendency to become non-circadian under constant lighting conditions.

Loss of circadian rhythmicity during DD is very unusual in rodents. Even the majority of subterranean mole rats, *Georychus capensis* (60-70%), *Heterocephalus glaber* (65%), and *Spalax ehrenbergi* (50-80%), display circadian rhythms in LD cycles and in DD and LL, although under natural conditions they are exposed to light infrequently (Ben-Sclomo et al., 1995; Goldman et al., 1997; Lovegrove and Muir, 1996; Riccio and Goldman, 2000a, 2000b; Tobler et al., 1998). Gerkema et al. (1990) found that 24 percent of common voles, *Microtus arvalis*, lost circadian rhythmicity within 30 days of DD. Similarly to our voles, *M. arvalis* also showed strong ultradian components.

Unlike *C. rutilus*, *M. arvalis* never became truly arrhythmic in their study. Ultradian bouts always persisted, even when the circadian component disappeared.

Ultradian rhythms appear to be a common feature of many vole species from regions other than the Arctic, and can occur simultaneously with circadian components (Daan and Slopsema, 1978; Halle, 1995; Halle and Stenseth, 1994; Kerbeshian and Bronson, 1993; Madison, 1985; Reynolds and Gorman, 1994; Zynel and Wunder, 2002). Although wheel-running patterns may be circadian, feeding bouts occur simultaneously in ultradian patterns (Daan and Slopsema, 1978; Hoogenboom et al., 1984). Therefore, ultradian rhythmicity is not likely to be a specific adaptation to the Arctic, but may be related to food intake and metabolism (Daan and Slopsema, 1978; Zynel and Wunder, 2002) and may be revealed by the loss of circadian organization. This latter interpretation is consistent with findings that the master circadian clock is not necessary for the expression of ultradian rhythms in *M. arvalis* (Gerkema et al., 1990).

Compared to *C. rutilus* in this study, similar circadian phenotypes were found in mice (*Mus musculus*). with targeted mutations of known clock genes. Mice lacking both cryptochrome genes (*Cry1 and Cry2*) became arrhythmic immediately in DD. *Cry2* mutants with one wild-type allele and a complete loss of *Cry1* lost circadian rhythmicity after two to three weeks of DD (van der Horst et al. 1999; Vitaterna et al. 1999). Oster et al. (2002) also found similar phenotypes with *Per2* mutations (circadian rhythmicity lost within a few weeks of DD) and *Per2/Cry1* mutants (circadian rhythmicity lost immediately in DD). Mice with disruptions of either *Per1* or *Per2* also showed large phenotypic variation, similar to our voles. Some maintained a weak circadian rhythm in

DD, while the majority lost their circadian rhythmicity immediately or within a few weeks (Bae et al. 2001). *BMAL1* (Bunger et al. 2000) mutant mice also lost circadian rhythmicity immediately in DD. Mice with a homozygous *Clock* mutation lost their circadian rhythm in DD after between two days (Herzog et al. 1998) and two weeks (Vitaterna et al. 1994). *Clock* mutants with one wild-type allele maintained circadian rhythmicity with a tau greater than 24 hours. Whether the *C. rutilus* circadian phenotypes in this study are due to naturally occurring mutations or differential regulation of one or more clock genes remains to be determined.

In DD, *C. rutilus* did not reveal significant light-induced phase advances. This is very similar to the PRC of some mouse lines (Bult et al., 1993). In addition, the lack of phase advances might indicate the absence of a morning oscillator which tracks the rising sun, which could be linked to the absence or differential regulation of *Per1* and/or *Cry1* clock genes (Daan et al., 2001). Whether the variability in re-entrainment to the new LD cycle after each light pulse, i.e., 7-21 day range, affected the phase shifts is unknown.

Whether the high variation in circadian rhythms of *C. rutilus* is a novel adaptation to the daylight regimes of the Arctic, due to natural allelic variation in known clock genes, or due to disruption of output mechanisms of the master circadian clock remains to be elucidated. Loss of circadian rhythmicity may be an adaptation allowing voles to exploit limited resources around the clock during the long, mostly dark winter periods. It may also allow them to take advantage of the short Arctic breeding season during the long light of the summer months when food is abundant. Alternatively, *C. rutilus* may have labile circadian organization at higher latitudes to be able to entrain to the LD cycle

in spring and fall by keeping up with rapidly changing day-lengths during these times of year. Less robustly organized circadian rhythms in laboratory mice are correlated with larger light-induced phase shifts (Amy et al., 2000; Bult et al., 1993; Mahoney et al., 2001). The intensity of light available also varies greatly from season-to-season and may effect the animal's ability to entrain.

The high variation in ultradian periods among *C. rutilus* in DD as well as in LL in the laboratory, does not support a predator-swamping hypothesis (reviewed in Ims, 1990), although synchronization of rhythms of different individuals in the wild cannot be excluded. Our voles did not show synchronization of rhythms but they were visually isolated with dividers and audibly isolated with a white noise generator. The circadian behavior of vole predators in the Arctic during the summer does not reveal consistent patterns with night active species being active during the day and others being active during the night hours. Therefore, their role in the evolution of labile circadian organization cannot easily be discerned. For example, while several owl species are active in the wild throughout the day during the Arctic summer (Armstrong, 1954), wild wolves (Mech and Merrill, 1998 [den entrances]) and captive arctic foxes (Folk, 1964 [heart rate, EKG, and body temperature]) maintain circadian rhythms with activity predominantly during the evening hours.

This study of a non-hibernating subarctic species under controlled environmental conditions develops the groundwork for future latitudinal studies of circadian characteristics. We hypothesize that the Fairbanks population may show intermediate characteristics between a North Slope Alaska Arctic population, where the majority of

the population may show highly labile circadian rhythms that disappear in constant light and dark, and a Juneau, Alaska, lower latitude population, that may show more robust circadian rhythms that are maintained in constant ligh and dark.

3.6 Acknowledgements

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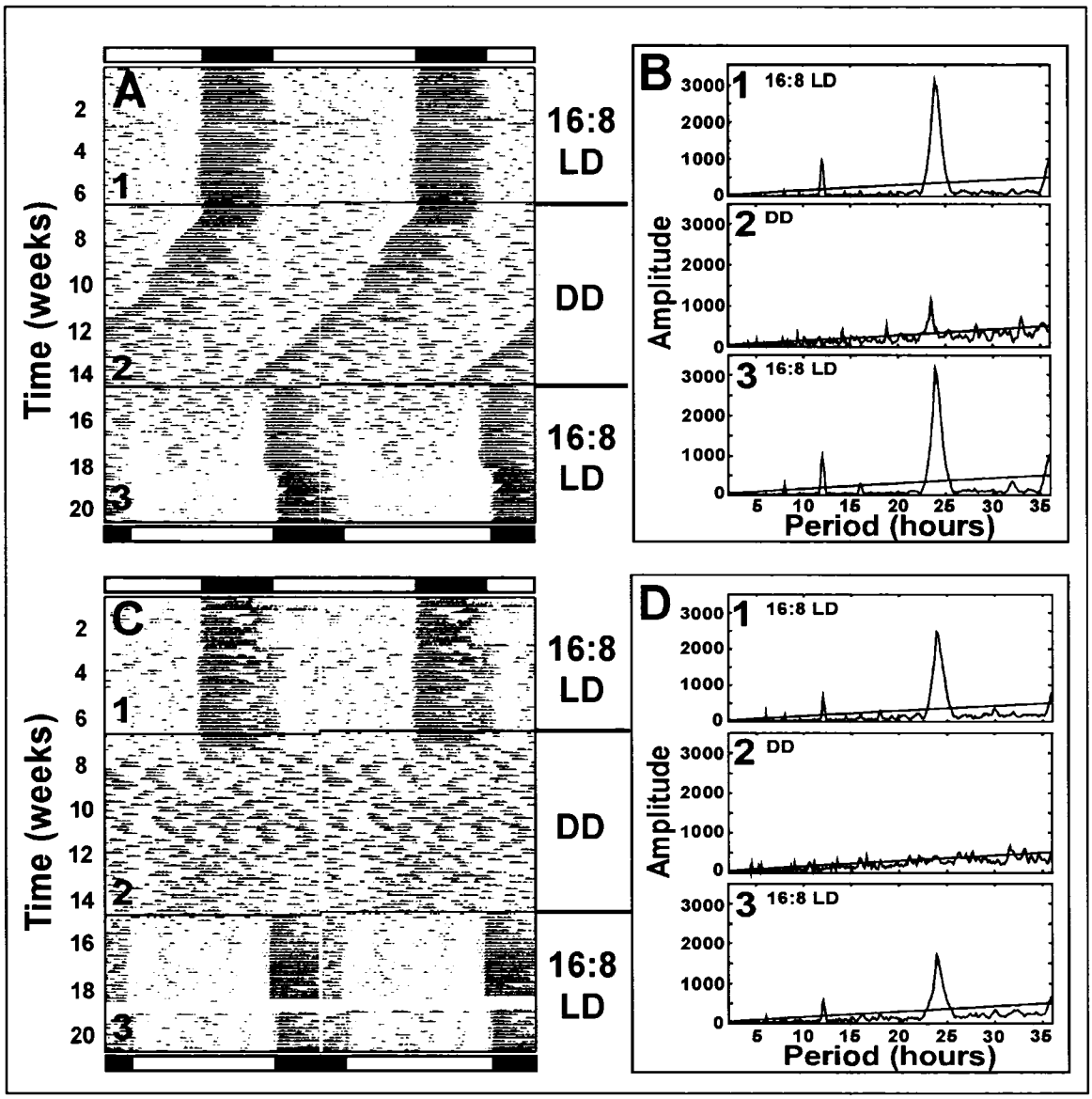

Figure 3.1 Representative constant dark (DD) double-plotted 20-week actograms (A & C), and corresponding chi-squared periodograms (B & D) from the last 14-day periods of each light regime of two wild-caught northern red-backed voles. The animals were kept under a 16:8 light-dark cycle (LD; black bar is dark period) followed by DD, then 16:8 LD. The vertical black bars indicate the number of wheel revolutions in 5-minute intervals. The amplitude is a unitless value derived from the normalized standard deviations of the activity profile as determined by the method of Sokolove and Bushell (1978). Note that one vole became weekly ultradian within one week of DD (C) and arrhythmic by the end of DD (D2), while the other vole maintained a circadian rhythm throughout DD (A & B2). The one-hour phase delay in the second LD cycle (A & C) was due to an inadvertent change of the room lighting timer related to the change to daylight savings time.

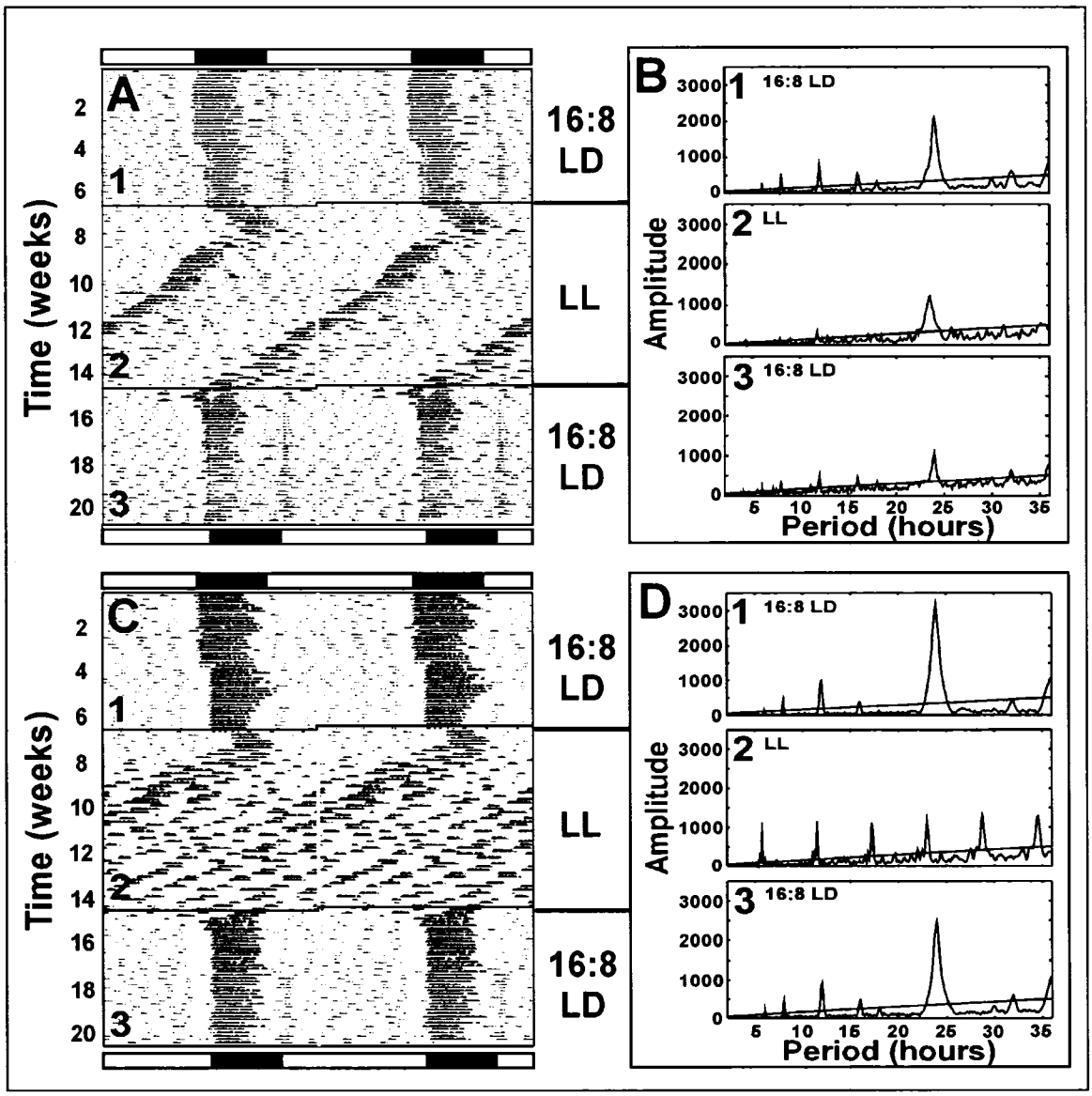

Figure 3.2 Representative constant light (LL) double-plotted 20-week actograms (A & C), and corresponding chi-squared periodograms (B & D) from the last 14-day periods of each light regime of two third-generation northern red-backed voles. The animals were kept under a 16:8 light-dark cycle (LD; black bar is dark period) followed by LL, then 16:8 LD. The vertical black bars indicate the number of wheel revolutions in 5-minute intervals. The amplitude is a unitless value derived from the normalized standard deviations of the activity profile as determined by the method of Sokolove and Bushell (1978). Note that one vole developed a strong ultradian rhythm within three weeks of LL (C & D2) and the other vole maintained circadian rhythms throughout LL (A & B2). The one-hour phase delay in the first LD cycle (A & C) was due to an inadvertent change of the room lighting timer related to the change to daylight savings time.

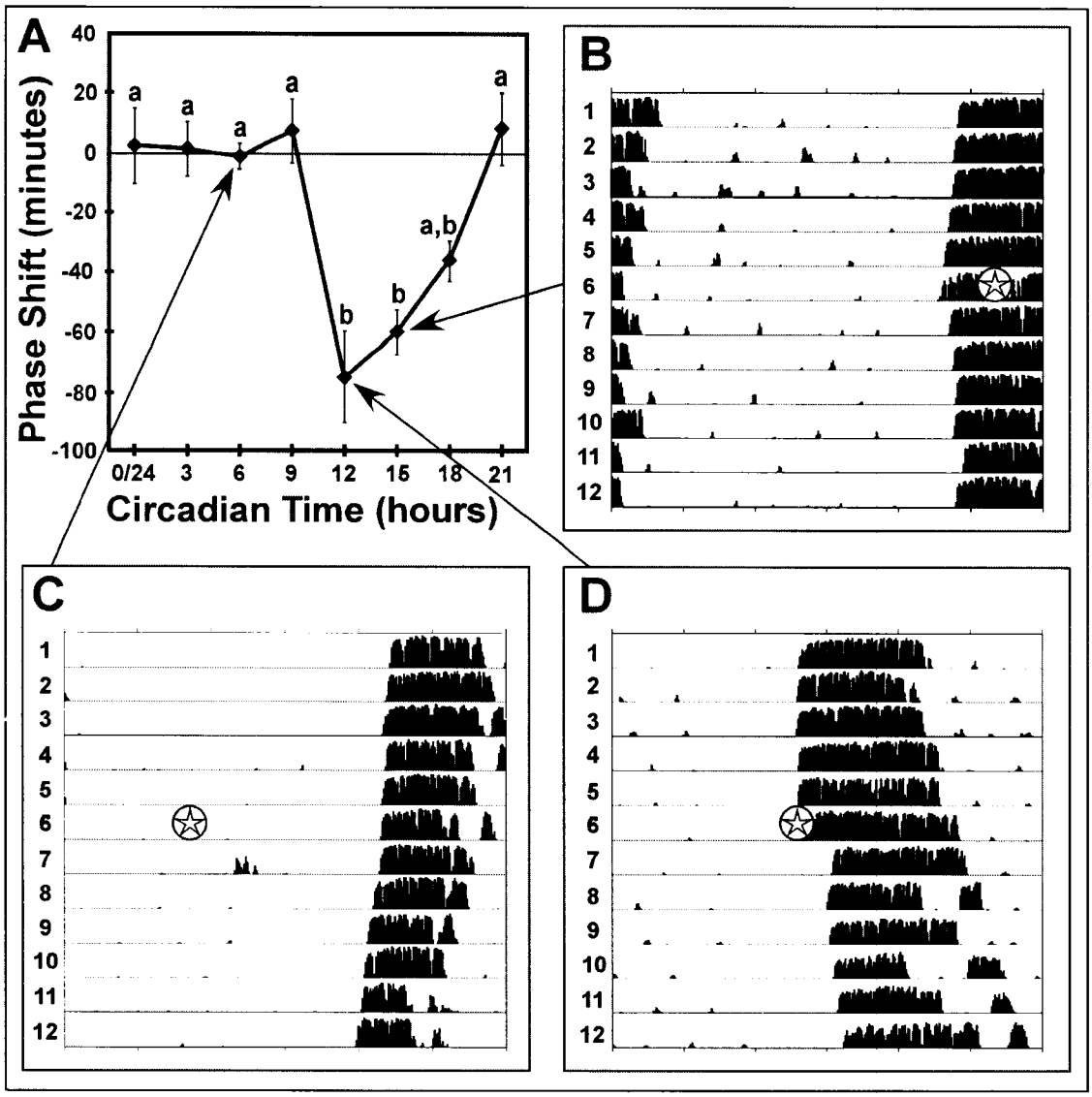

Figure 3.3 Phase response curve for wild-caught northern red-backed voles receiving a 20-minute light pulse of 700 lux in constant darkness (A). Circadian time (CT) 12 is defined as the onset of activity. Mean phase shifts are in minutes (± SEM). Representative single plotted, 12-day actograms of voles that received a light pulse at CT 15 (B), CT 6 (C) and CT 12 (D). Vertical bars indicate the number of wheel revolutions during a 5-minute interval. The star within a circle represents the time of the light pulse. Different letters indicate significant differences ($F_{7,41} = 6.72$, $p<0.0001$). Note: Although phase shifts were similar, some voles showed higher levels of activity during subjective day compared to the examples shown here in B-D.

Table 3.1 Circadian rhythm characteristics of wild-caught northern red-backed voles expressed as a percentage of normal wheel-running activity (± SEM) during the initial 16:8 LD cycle.

Wild Caught Northern Red-Backed Voles Circadian Rhythm Characteristics					
Circadian Rhythm Measure	**16:8 LD Weeks 7-8**	**DD Weeks 7-8**	**16:8 LD Weeks 5-6**	**F Value**	**Significance**
Chi Squared Periodogram Amplitude	110.1 ± 5.9 **(a)**	56.8 ± 14.3**(b)**	103.1 ± 6.1**(a)**	$F_{2,14} = 7.48$	$p<0.0186$
Average Daily Activity Bouts	101.1 ± 5.4	138.6 ± 8.1	107.3 ± 18.4	$F_{2,17} = 3.23$	$p<0.5805$
Average Daily Amplitude	96.9 ± 4.5	88.0 ± 4.1	107.3 ± 8.1	$F_{2,19} = 2.73$	$p<0.8181$
Average Daily Activity	97.1 ± 3.1	91.6 ± 4.1	92.9 ± 7.4	$F_{2,19} = 0.33$	$p<2.1618$

NOTE: Different letters in parentheses are significantly different at $p<0.05$. Significance levels are Bonferroni corrected for multiple tests.

Table 3.2 Circadian rhythm characteristics of third-generation laboratory-bred northern red-backed voles expressed as a percentage of normal wheel-running activity (± SEM) during the initial 16:8 LD cycle.

3rd Generation Laboratory-Reared Northern Red-Backed Voles Circadian Rhythm Characteristics							
Circadian Rhythm Measure	**16:8 LD Weeks 7-8**	**LL Weeks 7-8**	**16:8 LD Weeks 11-12**	**DD Weeks 7-8**	**16:8 LD Weeks 5-6**	**F Value**	**Significance**
Chi Squared Periodogram Amplitude	114.0 ± 7.3**(a)**	40.8 ± 5.6**(b)**	106.3 ± 14.2**(a)**	43.4 ± 5.4**(b)**	110.1 ± 11.1**(a)**	$F_{4,95} = 15.21$	p<0.0003
Average Daily Activity Bouts	110.8 ± 4.9	118.8 ± 8.7	141.0 ± 13.4	135.3 ± 9.0	116.2 ± 9.7	$F_{4,101}=1.96$	p<0.9513
Average Daily Amplitude	97.1 ± 2.8**(a)**	102.9 ± 7.7**(a)**	98.1 ± 6.8**(a)**	66.5 ± 4.4**(b)**	101.7 ± 7.2**(a)**	$F_{4,102}=6.29$	p<0.0009
Average Daily Activity	93.5 ± 3.1**(a)**	95.3 ± 8.7**(a)**	86.4 ± 5.7**(a)**	59.2 ± 4.7**(b)**	78.2 ± 8.2**(a,b)**	$F_{4,98}=4.97$	p<0.0099

NOTE: Different letters in parentheses are significantly different at p<0.05. Significance levels are Bonferroni corrected for multiple tests.

Chapter Four

Immunocytochemistry of the Suprachiasmatic Nucleus of the Northern Red-Backed Vole (*Clethrionomys rutilus*)

4.1 Abstract

The suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian clock in mammals. It is responsible for synchronizing daily rhythms in many physiological and endocrine processes, including melatonin secretion, feeding, and sleep. The SCN is entrained predominantly by exposure to light. The northern red-backed vole (*Clethrionomys rutilus*) experiences the extreme light:dark (LD) regimes of the Arctic and subarctic, including periods of constant light (LL) and constant dark (DD) above the Arctic Circle. How these light regimes affect the daily rhythms of mammals and their suprachiasmatic nuclei is largely unknown. Earlier work has shown that wild-caught and 3rd generation laboratory-bred *C. rutilus* voles have divergent circadian wheel-running characteristics from maintenance of circadian rhythmicity to ultradian and arrhythmic patterns in DD and LL (Tavernier et al., 2004; Chapter 3). Brains were collected from wild and laboratory-bred voles to investigate the distribution of common neurotransmitters, peptides, and proteins in the SCN. Immunocytochemistry was performed for vasoactive intestinal polypeptide (VIP), arginine-vasopressin (AVP), neuropeptide Y (NPY), cholecystokinin (CCK), calbindin, gastrin-related peptide (GRP), protein kinase Cα, and PKCβ1. Results show that the neuroarchitecture of the vole SCN is similar to that found in other species, notably the rat (*Rattus norvegicus*), hamster (*Mesocricetus auratus*) and mouse (*Mus musculus*). A calbindin-rich area was found in

the ventral region mid-way through the SCN, very similar to the hamster. The vole SCN contained more GRP fibers than most species studied. In addition, heavier staining was found for CCK and NPY suggesting an importance of non-photic cues, such as food, in altering the SCN and circadian rhythms of northern red-backed voles.

Keywords: Suprachiasmatic Nucleus, Subarctic, Northern Red-Backed Vole (*Clethrionomys rutilus)*, circadian, neuroanatomy

4.2 Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian clock in mammals. Lesion studies (Moore and Eichler, 1972; Stephan and Zucker, 1972) and *in vitro* (Inouye and Kawamura, 1979; Green and Gillette, 1982; Newman and Hospod, 1986) and *in vivo* (Moore and Klein, 1974; Schwartz and Gainer, 1977) studies have proven that these paired nuclei of 8,000 – 10,000 cells each are responsible for generating the daily rhythms of sleep wake cycles, feeding and many hormones, such as melatonin. The SCN prepares the animal physiologically for predictable daily changes in the environment. The main entraining signal to the SCN is light. Daily light cycles are able to entrain the SCN to the local time (Reviewed in Edery, 2000).

The Arctic and Sub-Arctic regions experience extreme light-dark cycle regimes. Day-to day and seasonal changes in day-length can be dramatic. Animals at our trapping sites in Fairbanks, Alaska, experience mean changes in day-length of approximately seven minutes a day for nine months of the year. Day-length in Fairbanks on the summer

solstice is over 21 hours and falls to about three and a half hours at the winter solstice. These dramatic seasonal differences in photoperiod may have resulted in the evolution of unique circadian adaptations and corresponding changes in SCN function. No studies have been completed on the distribution of common neurotransmitters, peptides, and proteins in the SCN of an Arctic mammal that experiences these extreme light regimes.

C. rutilus were captured during the spring and summer of 2000. Animals were translocated to UAF Animal Quarters, and a breeding colony was established. Studies of wild-caught and third generation laboratory-bred animals showed a large phenotypic variation in their response to constant dark (DD) and constant light (LL), using circadian wheel-running activity patterns as a measure to characterize circadian behavior. Some voles maintained a stable circadian rhythm throughout long periods in DD and LL, while others became arrhythmic or ultradian within a few weeks of DD and LL (Tavernier et al., 2004; chapter 3).

Brains were harvested from animals in the colony that were not being tested for circadian rhythms, and immunocytochemistry was performed for common markers of the SCN. This study shows successful staining for vasoactive intestinal polypeptide (VIP), arginine-vasopressin (AVP), neuropeptide Y (NPY), cholecystokinin (CCK), calbindin, gastrin-related peptide (GRP), protein kinase Cα (PKCα), and PKCβ1. The staining patterns will be discussed in the context of those described for other rodent species.

4.3 Methods

4.3.1 *Animals*

Individuals from two northern red-backed vole populations from interior Alaska (N. 64°, W. 147°) were captured and a colony from each location was established (Tavernier et al., 2004; chapter 2). Appropriate permits and approvals for capturing and housing wild animals were obtained: State of Alaska Department of Fish and Game Permit Numbers 99-121(1999), 00-001 (2000), 01-013 (2001), 02-001 (2002), 03-001 (2003), 04-031 (2004), 05-055 (2005), and 06-145 (2006), and University of Alaska Fairbanks Institutional Animal Care and Use Committee Protocol Numbers 99-27 (approved 11-12-1999), 02-47 (approved 11-12-2002), and 05-62 (approved 11-16-2005).

Voles were housed and bred in fiberglass cages (15x92x20 cm) under 16:8 LD at room temperature (22 ± 1°C). All animals were provided with wood shavings and cotton nesting material. Food (Lab Diet Co. Mouse Diet #5015, St. Louis, MO and Purina Mills, Inc. Rabbit Chow, St. Louis, MO) and water were available *ad libitum*. Animals were also fed one slice of carrot daily at random times. Voles from each population were randomly paired except siblings were never mated and first cousin pairings were avoided as much as possible (Bult and Lynch, 2000; Tavernier et al., 2004; chapter 2). Two weeks after pairing, males were removed to assure undisturbed delivery of the litter. All voles were weaned at 19 ± 1 days of age and then housed with same sex litter-mates.

4.3.2 *Immunocytochemistry*

After four or more weeks in a 16:8 LD cycle, animals were euthanized, brains removed and immunocytochemically stained using standard free-floating techniques (Amy et al., 2000; Bult et al., 2001; Castillo et al., 2005; Hochstetler et al., 2004; Samuels et al., 2006; Van der Veen et al., 2005). In short; voles were anesthetized with sodium pentobarbital (390 mg per ml, 200 mg per kg dosage, Euthasol, Delmarva Laboratories, Midlothian, VA) and perfused intracardially with 0.9% saline for 2 minutes followed by 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO) in 0.1 M phosphate buffer (PB) for 8 minutes. Brains were post-fixed overnight in 4% paraformaldehyde in 0.1 M PB and then stored in 30% sucrose in phosphate-buffered saline (PBS) overnight at 4°C. Brains were cut into 20-μm sections on a Reichert-Jung cryostat (Leica Microsystems, Bannockburn, IL) and divided into three vials of alternating slices for three sets from each animal. Sections were stored at -20°C in cryoprotectant (0.05 M PBS, pH 7.4, 1% polyvinyl pyrrolidone, 30% sucrose, 30% ethylene glycol) until processed for immunocytochemistry.

Animals were euthanized at ZT8 for the following peptides, proteins and neurotransmitter: calbindin, substance P (SubP), NPY, PCKα, PKCβI, PKCβII, PKCγ and CCK. ZT12 is defined as the beginning of night when nocturnal animals begin activity. These markers are not known to cycle in the SCN so no differences are expected among time points (Silver et al., 1999). For markers that are known to vary on a daily basis in the SCN (Inouye, 1996), animals were sacrificed at the time of highest

expression as follows : AVP and somatostatin (ZT4); VIP (ZT20), GRP (ZT12). Animals used for Per1, Per2, Cry1 and Cry2 immunocytochemistry were euthanized at ZT12 (maximum protein expression (Reppert and Weaver, 2001)). The particular primary antibodies, their concentrations, and suppliers are listed in Table 4.1.

Standard immunocytochemical procedures were performed (Amy et al., 2000; Bult et al., 2001; Castillo et al., 2004; Hochstetler et al., 2004; Samuels et al., 2006; Van der Veen et al., 2005). In short; all rinses (unless otherwise noted) were performed six times, five minutes long each with media changed between. Triton x-100 was not used for any rinses for membrane proteins such as the PKC family to ensure that the proteins remained membrane bound and were not rinsed out. Sections were rinsed in PBS and then rinsed in PBS plus 0.1% triton X-100. Sections were quenched for 20 minutes in 30% H_2O_2 in PBS and then rinsed in PBS plus 0.1% triton X-100. Sections were then blocked for two hours in 5% serum in PBS plus 0.1% triton X-100. Sections were then incubated in primary antibody and PBS plus 0.1% triton X-100 for 48 hours and then rinsed in PBS plus 0.1% triton X-100. Incubation in secondary antibody (Vector, Burlingame, CA; 1:200) and PBS plus 0.1% triton X-100 was for 30 minutes. Sections were then rinsed in PBS plus 0.1% triton X-100. Sections were then incubated in avidin-biotinylated horseradish peroxidase (ABC Elite kit; Vector Laboratories) in PBS plus 0.1% triton X-100 for 30 minutes and then rinsed in PBS. A 5-minute diaminobenzidine (DAB; 30 mg DAB in 100 ml PBS) incubation was used, and then sections were mixed with 0.01% H_2O_2 for 3-10 minutes until staining became visible. Excess DAB was rinsed

from the sections with a series of PBS rinses. Sections were then mounted on slides and coverslipped with Permount (Biomedia, Foster City, CA).

Controls consisted of immunocytochemistry on three separate sets of slices from the same brain collected concurrently. One set was processed as outlined above while another set had the primary antibody omitted and the other had the secondary antibody omitted.

4.3.3 *Visualization*

Slides were examined using an Axioplan 2 imaging microscope, digital AxioCam camera, and Axio Vision 3.0.6 software (Carl Zeiss, Germany). Pictures were taken of all sections containing the SCN for comparison and to determine which immunocytochemical stainings were successful.

Pictures throughout the SCN were ordered from rostral to caudal sections and printed to contact sheets containing one animal and one neurotransmitter using Adobe Photoshop Elements 2.0 (Adobe Systems, San Jose, CA). Two researchers reviewed the unlabeled contact sheets to determine which stainings were successful. Successful immunocytochemical results were then photographed at 100x and 200x for a neuroanatomical characterization of a vole's SCN. Representative sections were chosen of a rostral, two middle, and a caudal section of the SCN. A line drawing of cells and fibers containing the marker was created using Canvas 8.0 (Deneba, Miami, FL). Nerves and fibers were drawn onto a layer with the software. Additional slices were layered under the drawing to confirm that the distribution was representative of both males and

females. The following numbers of samples were used for each successful marker: VIP – 3 males and 3 females, AVP – 7 males and 5 females, NPY – 2 males and 3 females, CCK – 6 males and 5 females, calbindin – 3 males and 3 females, GRP – 6 males and 6 females, PCKα – 3 males and 3 females, PKC βI – 3 males and 3 females .

4.4 Results

Successful staining was obtained for VIP, AVP, NPY, CCK, calbindin, GRP, PCKα, and PKCβI. Their distributions are shown in Fig. 4.1 from top to bottom as they progress through the SCN from rostral to caudal aspects. The results are summarized below. No staining was visible in the control slices in which the primary or secondary antibodies were omitted from the staining procedure (Data not shown).

4.4.1 *VIP*

Large numbers of VIP positive cell bodies were located in the ventral core region of the rostral half of the SCN (Fig. 4.1A). The cells were concentrated in central and medial areas of the ventral SCN. The cell bodies became less numerous in the caudal half with the cells localized in medial areas only. Fibers containing VIP were also co-localized with the cell bodies and became more numerous in the caudal half of the SCN where they were spread uniformly throughout the whole SCN.

4.4.2 *AVP*

In the rostral SCN, the cell bodies of AVP positive neurons were spread throughout the entire SCN (Fig. 4.1B). In the middle area of the SCN, they formed a dorsal shell with more cell bodies located medially and little staining of fibers or cell bodies visible within the core region (Figs. 4.1B and 4.2A). The caudal area of the SCN had fewer cell bodies, where they were spread along the medial and dorsal aspects. AVP positive fibers were also present in greater numbers in the medial and dorsal aspects in the caudal SCN.

4.4.3 *NPY*

NPY positive fibers were found in large numbers along the medial and ventral aspects of the rostral SCN with no staining apparent in the lateral areas (Fig. 4.1C). In the middle areas they formed a dense core that moved more toward the midline in more caudal aspects (Figs. 4.1C and 4.2D). In the caudal aspects of the SCN, NPY fibers were absent from the dorsal and lateral edges.

4.4.4 *CCK*

CCK cells were present in larger numbers in the rostral part of the SCN with few to no cell bodies found in the caudal aspects (Fig. 4.1D). Fibers followed an opposite pattern with the numbers increasing around the borders of the SCN toward the caudal end (Figs. 4.1D and 4.2C). At the caudal end of the SCN, CCK fibers were mostly found along the midline of the SCN and the ventral areas.

4.4.5 *Calbindin*

A few calbindin cells were visible in the rostral SCN along the medial and ventral areas (Fig. 4.1E). The middle of the SCN had a dense core of calbindin cells located medially and ventrally (Figs. 4.1E and 4.2B). The dorsal SCN had scattered cells along the ventral areas of the SCN. Calbindin-positive fibers were sparse throughout the SCN.

4.4.6 *GRP*

GRP fiber staining was dense in the rostral half of the SCN and occurred throughout the nucleus (Fig. 4.1F). Cell bodies were present medially and ventrally. In the caudal half of the SCN, sparse numbers of cell bodies were scattered throughout the SCN with fibers also spread similarly. Fibers were localized in the ventral half of the SCN in caudal aspects with very light fiber staining in the dorsal half.

4.4.7 *PCKα*

Cell bodies that were immuno-positive for PCKα were evenly spread throughout the rostral half of the SCN (Fig. 4.1G). In the caudal half of the SCN, they were more concentrated medially and dorsally although scattered cell bodies were located throughout the SCN.

4.4.8 *PCKβI*

PCKβI cells showed a similar distribution as PCKα with scattered cells being found throughout the rostral half of the SCN (Fig. 4.1H). The caudal areas also had a few cells scattered throughout the SCN with more PCKβI positive cells along the medial and dorsal borders.

4.5 Discussion

The neurotransmitters, proteins, and peptides in the SCN of the red-backed vole showed many similarities to other rodent species. Clear core and shell subdivisions could be seen with AVP localized to the shell of the SCN and VIP localized in the core. This division didn't hold true for GRP, which was found throughout the SCN of the voles. This differs from what has been found in other species studied where GRP is only found in the shell region, such as the rat, hamster (Moore and Silver, 1998), and mouse (Silver et al., 1999).

NPY staining was distinct in the SCN of the vole, with the majority of the fibers being located along the medial and ventral areas of the rostral SCN. Staining in the caudal SCN was similar to that found in the golden hamster with fibers absent in the lateral sections of the SCN (Moore and Silver, 1998). The distribution of NPY was similar to that in the mouse, with more fibers being present in the caudal half of the vole SCN (Silver et al., 1999). NPY can cause nonphotic behavioral phase shift in both hamsters and rats, and a NPY anti-serum injected into the SCN of hamsters blocks activity mediated phase shifts (Biello et al., 1994, Biello et al., 1997, Albers and Ferris,

1984).

The distribution of CCK neurons and fibers in red-backed voles was very similar to that found in the Djungarian hamster (*Phodopus sungorus*: Reuss, 1991) and the common hamster (*Mesocricetus auratus*: Moore and Silver, 1998). The mouse contains a similar distribution to that found in red-backed voles, but the cells and fibers appear to be denser in the vole (Silver et al., 1999). CCK from the SCN has been suggested to be involved in the control of food intake. Djungarian hamsters (*Phodopus sungorus*) kept under short days (8:16 L:D) have almost a fifth the number of CCK exhibiting neurons than those kept under long days (16:8 L:D). This difference is hypothesized to lower the food intake of these animals during the winter months (Reuss, 1991). Dense staining was also found in our voles housed under a 16:8 L:D cycle, similar to the Djungarian hamsters which were housed under the same light cycle.

NPY fibers and CCK fibers and cells were more numerous in the voles than in most other species studied, such as the rat, hamster (Moore and Silver, 1998), and mouse (Silver et al., 1999). NPY injections into the SCN have been shown to mimic the effects of non-photic phase-shifts such as social interactions and mating opportunites, and are believed to mediate these type of cues (reviewed in Mrosovsky, 1996). Similar CCK injections into the SCN have been shown to suppress food intake in rats (Mori et al., 1986). Taken together, these findings suggest a possible importance of food and social cues in SCN function in *C. rutilus*.

VIP staining was very similar to what is found in the rat (*Rattus norvegicus)* with the majority of the cell bodies being found ventrally and medially with few cell bodies

located on the lateral edges of the dorsal half (Moore and Silver, 1998). The distribution was also similar to that found in the golden hamster (*Mesocricetus auratus*), but cell bodies were visible throughout the rostral half of the SCN and are absent there in the hamster SCN (Moore and Silver, 1998). Numerous studies have shown VIP is necessary for the SCN to generate and synchronize circadian rhythms (reviewed in Vosko et al., 2007). VIP was present in the SCN of voles, although other problems not tested for may exist in this system, such as a lack of the $VPAC_2$ receptor needed for the SCN to function properly (reviewed in Vosko et al., 2007).

The distribution of AVP was similar to that found in the SCN of rats, with cell bodies being more pronounced in the shell area than in the remainder of the SCN (Moore and Silver, 1998). The distribution of AVP in the red-backed vole differed from that in the golden hamster and mouse due to a lack of cells along the ventral border of the SCN in the caudal aspects. The mouse and hamster both have more cell bodies in the caudal aspect than what was found in *C. rutilus* (Bult et al., 1992; Hochstetler et al., 2004; Moore and Silver, 1998; Silver et al., 1999; Van der Veen et al., 2005). Recent evidence points to the importance of AVP in regulating luteinizing hormone release in mammals (Miller et al., 2006).

The distribution of calbindin containing cells was very similar to that found in the hamster and rat, with a dense cluster of cells located in the mid-SCN core region and disperse cells in the ventral half of the caudal SCN (Moore and Silver, 1998, Arvanitogiannis et al., 2000). In the red backed vole's rostral SCN, cells were located along the ventral edge compared to the hamster, in which cells are along the lateral edge.

Lesion and transplant studies in hamsters have shown that this dense cluster of calbindin binding cells is required for them to maintain locomotor rhythms (LeSauter and Silver, 1998). Although these cells have no pacemaker properties themselves they are believed to be essential for animals to entrain to a LD cycle and respond to light pulses (Hamada et al., 2003, Arvanitogiannis et al., 2000).

Fewer GRP immuno-positive cell bodies were found throughout the *C. rutilus* SCN and fiber staining was denser than that found in the SCN of the rat and hamster, although the distribution was similar. In the rat and hamster, GRP distribution overlaps that of VIP (Moore and Silver, 1998), while in the voles larger areas of the SCN contained GRP and the distribution overlapped both VIP and AVP. The vole SCN also contained many more GRP fibers than is found in the mouse, where fibers are mainly located along the border of the SCN (Silver et al., 1999). GRP performs many of the same tasks as VIP in the SCN of mammals, causing phase shifts (Piggins et al., 1995; McArthur et al., 2000) and synchronizing the cells (Maywood et al., 2006).

PCKβI and PCKα showed a similar distribution to that found in the mouse and the African grass rat, *Arvicanthis niloticus* (Bult and Smale, 1999; Bult et al., 2001). Cells were spread throughout the SCN with fewer cells being found in the caudal half. PCKβI cells showed more of an overlap with AVP cell distribution similar to that found in the mouse and the African grass rat. PKC isoforms are the pathway through which melatonin influences the rhythm of the SCN (McArthur et al., 1997).

In the one other immunocytochemical study done on the SCN of *C. rutilus* (Samuels et al., 2006), a difference was found in the distribution of Substance P (SubP)

compared to other mammals that have been studied. A dense cluster of fibers and terminals was found in the ventral SCN in the middle sections and no cell bodies were found throughout the SCN. This is markedly different than what has been found in other species. Differences in peptide distribution such as these may eventually lead to a greater understanding of how different distributions of cells in the SCN leads to different behaviors.

The fact that several antibodies did not work (listed in table 4.1) in this study is not surprising. No antibodies specifically made for the northern red-backed vole are available. The antibodies that worked sometimes required modifications of the immunocytochemical procedures, such as antibody concentrations, to obtain optimal staining than from those recommended or found in the literature for mice, hamsters, and other rodent species. However, the similarity in staining patterns with other rodent species and the use of control procedures validates our results.

Our results indicate that C. *rutilus* is a good model species for investigating circadian adaptations in the Arctic. Northern red-backed voles display a large phenotypic variation in the strength of their circadian rhythms that may be an adaptation to Arctic light regimes (Tavernier et al., 2004; chapter 3). In addition, the distribution of neurotranmitters, proteins, and peptides shows that the SCN is very similar to other species that have been studied in the laboratory. Differences in peptide distributions and concentrations are known to exist in animals that differ in circadian rhythm parameters (Amy et al., 2000; Bult et al., 1992, 1993, 2001; Gerkema et al., 1994; Yan et al., 2003) so studies will now focus on the distribution of peptides in the SCN of voles that are able

to maintain a circadian rhythm in constant lighting conditions and those that lose their rhythmicity.

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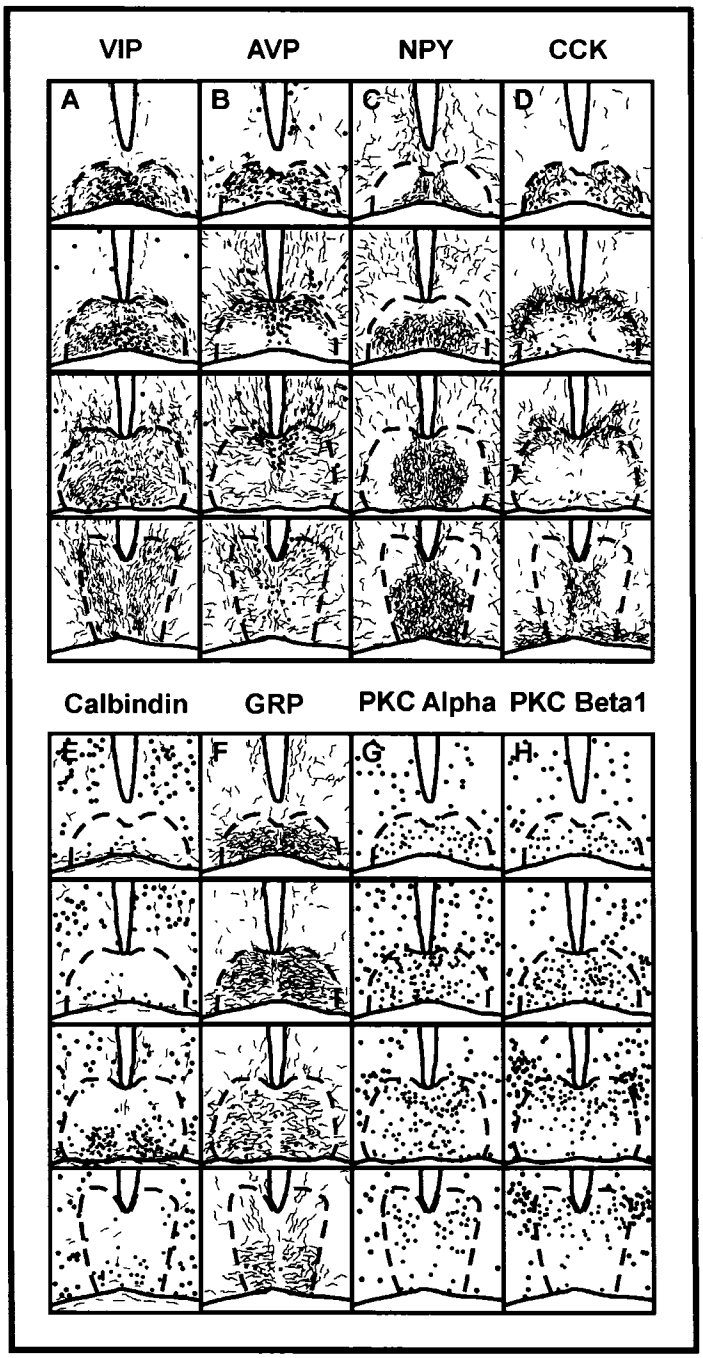

Figure 4.1 Representation of neurotransmitter, protein, and peptide distribution in the SCN of *C. rutilus* in a rostral section (row 1), two middle sections (rows 2-3), and a caudal section (row 4). Dashed lines indicate the boundaries of the SCN. A = VIP, B = AVP, C = NPY, D = CCK, E = Calbindin, F = GRP, G = PKC Alpha, H = PKC Beta1.

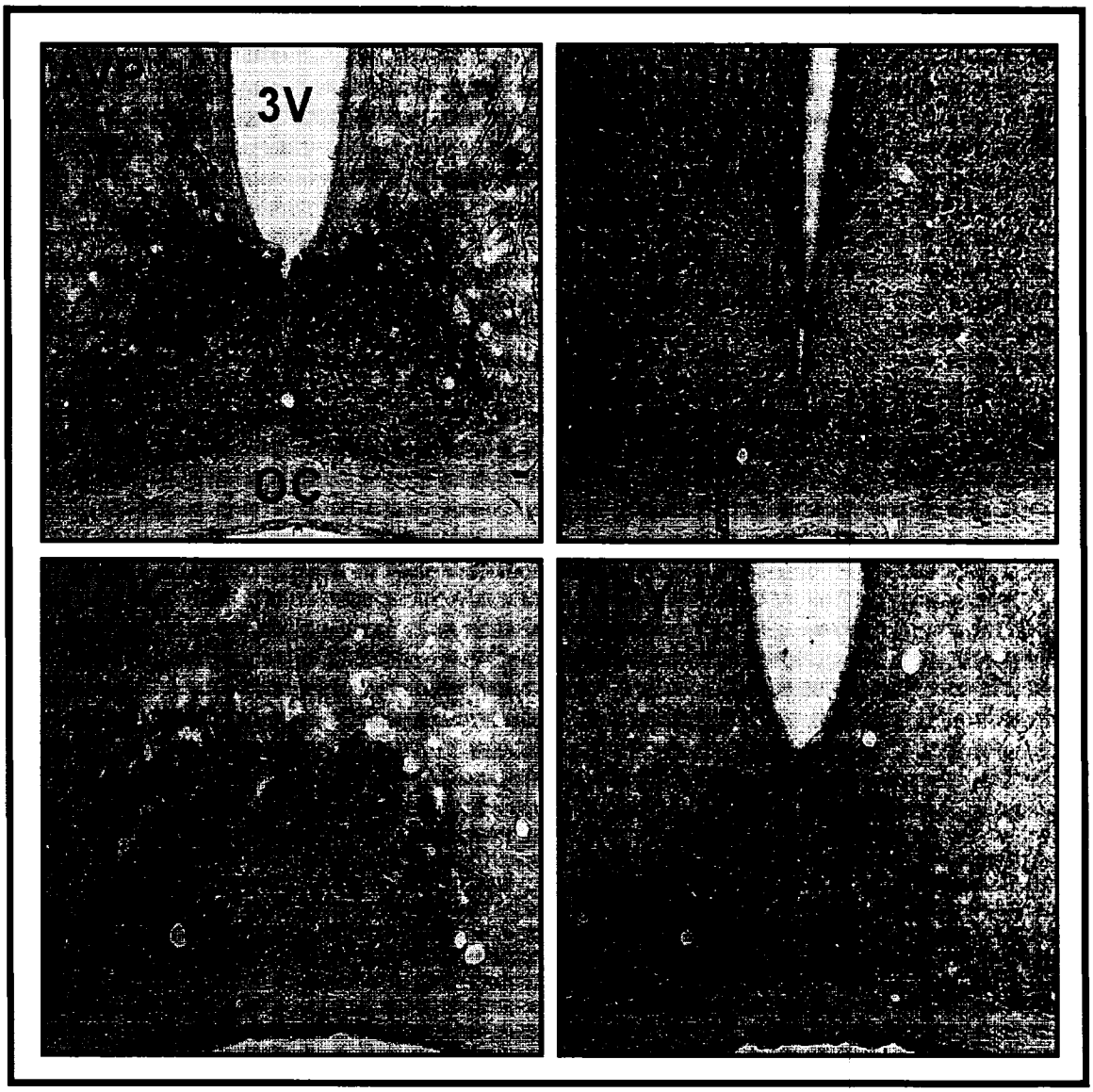

Figure 4.2 Representative pictures of immunocytochemistry in the SCN of the northern red-backed vole. A = AVP, B = Calbindin, C = CCK, D = NPY, 3V = 3rd ventricle, OC = optic chiasm.

Table 4.1 Antibodies, concentrations and suppliers of neurotransmitters, peptides and proteins tested. * = antibodies which did not function in the red-backed vole.

Marker	Antibody	Concentration	Supplier
VIP	H-5682	1:1000	Peninsula Laboratories, LLC, San Carlos, CA
AVP	PS-41 and GHC 8103	1:1000	Dr. H. Grainer and Peninsula Laboratories, LLC, San Carlos, CA
NPY	1HC 7180	1:2500	Peninsula Laboratories, LLC, San Carlos, CA
CCK	1HC 4350	1:2500	Peninsula Laboratories, LLC, San Carlos, CA
Calbindin	PC253L	1:10000	Calbiochem, San Diego, CA
GRP	H-5930	1:1500	Peninsula Laboratories, LLC, San Carlos, CA
PKCAlpha	SC-208	1:5000	Santa Cruz Biotechnology, Santa Cruz, CA
PKCBeta1	661-671	1:3000	Calbiochem, San Diego, CA
*PKCBeta2	661-673	1:1000	Calbiochem, San Diego, CA
*PKCGamma	681-697	1:3000	Calbiochem, San Diego, CA
*SubP	GHC 7451	1:1000	Peninsula Laboratories, LLC, San Carlos, CA
*Somatostatin	H-4702	1:2500	Peninsula Laboratories, LLC, San Carlos, CA
*Per1	Per11A	1:1000	Alpha Diagnostics International Inc, San Antonio, TX
*Per2	Per21A	1:1000	Alpha Diagnostics International Inc, San Antonio, TX
*Cry1	Cry11A	1:1000	Alpha Diagnostics International Inc, San Antonio, TX
*Cry2	Cry21A	1:1000	Alpha Diagnostics International Inc, San Antonio, TX

Chapter Five

Selective Breeding of Rhythmic and Non-rhythmic Northern Red-Backed Voles (*Clethrionomys rutilus*)

5.1 Abstract

Northern red-backed voles *(C. rutilus)* were captured during the spring and summer of 2000. Animals were returned to UAF Animal Quarters and a breeding colony was established. Earlier studies (Chapter 3) of wild-caught and third generation laboratory-bred animals showed large phenotypic variation in response to constant darkness (DD), using wheel-running activity patterns as a measure to characterize circadian behavior. Some voles maintained a stable circadian rhythm throughout long periods in DD, while others became arrhythmic or ultradian within a few weeks of DD. After five generations of random breeding, a selective breeding protocol began with the goal of producing two lines of voles that maintained a circadian rhythm in DD (RA and RB lines) and two lines of voles that became non-circadian (ultradian and arrhythmic) in DD (NA and NB lines). A randomly bred control line (BC line) was also maintained. After two generations of selective breeding the lines showed no significant differences in the strength of their circadian rhythm as quantified by the amplitude of the chi-squared periodogram waveform. The rhythmic lines had an average strength of 1378 ± 106 (SEM), the non-rhythmic lines had an average of 965 ± 80 and the controls were intermediate with an average of 1138 ± 95. Differences were not statistically significant due to the variability inherent at the beginning of selection experiments and the small sample sizes (n = 18 -24 individuals per line, per generation), although the trends showed

an apparent response to selection. Heritability estimates could not be obtained for circadian wheel running strength in DD due to small sample sizes. Due to space, time and funding constraints the third generation the lines were combined to create one rhythmic line, one non-rhythmic line and a control line. Additional generations of selection could allow a statistical difference to be observed between the circadian and non-circadian line if heritability is sufficiently high.

Keywords: Circadian rhythms, Arctic, Selective Breeding, Northern Red-Backed Vole (*Clethrionomys rutilus)*

5.2 Introduction

Arctic and subarctic environments are exposed to extreme light:dark (L:D) regimes with warm summers with long periods of light and the corresponding cold winters with long periods of dark. Daily changes in day-length are also large during the spring and fall. Animals captured for this study in Fairbanks, Alaska, experience mean changes in day-length of approximately seven minutes a day for most of the year. Day-length in Fairbanks on the summer solstice is over 21 hours and falls to just over three and a half hours at the winter solstice.

To study the effect of these extreme light regimes on Arctic animals, northern red-backed voles (*Clethrionomys rutilus)* were captured from two locations and their circadian rhythms were investigated under 16:8 L:D, constant light (LL) and constant dark DD (Tavernier et al., 2004; chapter 3). Under a 16:8 L:D cycle, both wild-caught

and 3rd generation laboratory-bred *C. rutilus* displayed a circadian rhythm with the majority of their activity occurring during the night. After 8 weeks of DD, wild caught (33%) and 3rd generation laboratory-bred voles (60%) became arrhythmic or ultradian. Similar results were found under LL conditions (Tavernier et al., 2004; chapter 3).

The brain's master circadian clock, the suprachiasmatic nucleus (SCN), is located at the base of the third ventricle directly over the optic chiasm. Circadian rhythms in animals are created and sustained by interlocking negative and positive feedback loops of protein production and degredation in the SCN (reviewed in Okamura, 2004). C. *rutilus* circadian rhythms are very similar to circadian phenotypes that are found in mice (*Mus musculus*) with targeted mutations of known clock genes. Mice with non-functioning cryptochrome genes (*Cry1 and Cry2*) became arrhythmic immediately in DD while *Cry2* mutants with one wild-type allele and a complete loss of *Cry1* lose circadian rhythms after two or three weeks in DD (van der Horst et al. 1999; Vitaterna et al. 1999). *Per2/Cry1* mutants also lose their circadian rhythmicity immediately in DD while *Per2* mutants take a few weeks to lose theirs (Oster et al., 2002). Disruptions of *BMAL1* (Bunger et al. 2000) *Clock* (Herzog et al., 1998; Vitaterna et al., 1994) can also cause animals to lose their rhythms in DD.

Whether mutations or differing alleles of clock genes occur in natural populations of mammals is unknown, although differences in circadian clock components have been found along a latitudinal cline in *Drosophila melanogaster* (Costa et al., 1992; Rosato et al., 1996). The clock gene period in these flies differs in the length of a threonine-glycine repeated region that controls stability to the circadian rhythm over a range of ambient

temperatures and differing lengths of the repeat region may confer adaptive advantage depending on the local environment.

To investigate this phenomenon further, a selective breeding program was started with the 5th generation of laboratory-bred red-backed voles. Our goal was to establish five lines of voles; two lines of voles that maintained a circadian rhythm in DD (RA and RB lines), two lines that were unable to maintain circadian rhythmicity in DD (ultradian and arrhythmic; NA and NB lines), and one randomly bred control line (BC). True-breeding lines of rhythmic and non-rhythmic voles would enable us to look at naturally occurring genetic variation in the circadian characteristics of an Arctic mammal for the first time.

5.3 Methods

5.3.1 *Vole Husbandry*

Two individual northern red-backed vole populations from interior Alaska (N. 64°, W. 147°) were captured and a colony from each location was established (Chapter 2). Appropriate permits and approvals for capturing and housing wild animals were obtained: State of Alaska Department of Fish and Game Permit Numbers 99-121(1999), 00-001 (2000), 01-013(2001), 02-001 (2002), 03-001(2003), 04-031(2004), 05-055(2005), and 06-145 (2006), and University of Alaska Fairbanks Institutional Animal Care and Use Committee Protocol Numbers 99-27 (approved 11-12-1999), 02-47 (approved 11-12-2002), and 05-62 (approved 11-16-2005).

Voles were housed and bred in fiberglass cages (15x92x20 cm) under 16:8 LD at room temperature (22 ± 1°C) when not being used for behavioral testing. All animals were provided with wood shavings and cotton nesting material. Food (Lab Diet Co. Mouse Diet #5015, St. Louis, MO and Purina Mills, Inc. Rabbit Chow, St. Louis, MO) and water were available *ad libitum*. Animals were also fed one slice of carrot daily at random times. Voles from each population were randomly paired except siblings were never mated and first cousin pairing were avoided as much as possible (Bult and Lynch, 2000). Two weeks after pairing, males were removed to assure undisturbed delivery of the litter. All voles were weaned at 19 ± 1 days of age and housed with same sex litter-mates. Animals were assigned toe-clip numbers for easy identification.

5.3.2 *Selection Protocol*

After five generations of random breeding in the laboratory, 24 male and 24 female animals were placed in running wheels under a 16:8 L:D cycle. Animals were given four weeks to establish stable running wheel rhythms and then placed in DD for four weeks. The last two weeks of DD were used to determine the animal's phenotype. The twelve males and twelve females with the highest amplitude of their chi-squared periodogram waveform formed the F0 generation of the RA line. The twelve males and twelve females that displayed the smallest amplitude of their chi-squared periodogram waveform, or were arrhythmic or ultradian, formed the F0 generation of the NA line. This protocol was repeated to form the F0 generation of the RB and NB lines. Each line was then randomly mated to produce the F1 generation.

Each successive generation was then selected in the following way: Twenty-four males and 24 females were randomly chosen from the line to be tested in wheels. Six control males and six control females were also tested with each line, totaling 24 males and 24 females per generation. Animals were allowed four weeks in 16:8 LD to establish stable running wheel rhythms. Animals were then placed in DD for four weeks. The last two weeks of DD were used to determine the amplitude of the chi-squared periodogram waveform. For the rhythmic lines (RA and RB) the twelve males and twelve females with the largest amplitudes of the chi-squared periodogram waveform were chosen to reproduce for the next generation. For the non-rhythmic lines (NA and NB) the twelve males and twelve females with the smallest amplitude of the chi-squared periodogram waveform, or were arrhythmic or ultradian, were chosen to reproduce for the next generation. All matings within lines were randomly determined, while avoiding the mating of siblings and minimizing cousin matings (Bult and Lynch, 2000).

5.3.3 *Data Collection*

Animals were individually housed in polycarbonate cages (37x21x14 cm) with 24-cm diameter running wheels equipped with a magnetic switch (Nalgene, Rochester, NY). Number of wheel revolutions was continuously recorded in 5-min bins by computer with the VitalView data collection system (Mini-Mitter Co., Inc., Bend, OR). All cages were visually isolated and a white noise generator (LaFayette Instrument Co., LaFayette, IN) was used to minimize auditory cues.

Wheel-running activity was measured in 24 male voles and 24 female voles (22-

32 grams at the start of the experiment) from each line under a 16:8 LD cycle for four weeks to allow the animals to establish stable wheel-running patterns. This was followed by four weeks of DD. Animals were then returned to 16:8 LD for breeding. Males and females from each line were tested simultaneously along with six randomly-bred control males and females.

5.3.4 *Classification of Wheel-Running Rhythms*

Wheel-running data were analyzed with Vitalview and Actiview (Mini-Mitter Co, Inc.), Matlab Version 6 (The Mathworks, Inc. Natick, MA), and Clock Lab (Actimetrics, Evanston, IL) software programs. Two-week intervals were used for the analyses of rhythm type and robustness of the circadian rhythm, because rhythm data analysis methods, recommended by Levine et al. (2002), require a minimum of ten days to provide reliable results. Following the general recommendations of Levine et al. (2002), animals were classified as circadian, ultradian, or arrhythmic based on wheel-running activity. Records were initially screened for rhythmicity with autocorrelation using Clock Lab. If a circadian rhythm was identified by autocorrelation, it was confirmed with chi-squared (Sokolove and Bushell 1978) and Lomb-Scargle periodograms (Van Dongen et al. 1999). These analyses also provided the free running period (τ) in hours (h) and rhythm robustness, i.e. amplitude of the periodogram waveform. If autocorrelation identified an ultradian rhythm, this was further assessed and τ was determined by Fast Fourier Transformation and Lomb-Scargle periodogram. These analyses are more sensitive to identifying shorter periods than the chi-squared periodogram analysis (Levine

et al., 2002). Levels of significance were set at $p<0.05$ for all tests. When both circadian and ultradian rhythms were identified, all tests were used. Animals displaying neither circadian nor ultradian patterns of wheel-running in a two-week period using the above procedures were classified as arrhythmic. Arrhythmic and ultradian animals were assigned default amplitudes of 400, the average "noise" of an arrhythmic animal's periodogram at a τ of 24 hours.

5.3.5 *Data Analysis*

All chi-squared values were square-root transformed in an attempt to correct the heteroscedasticity of the data (Snedecor and Cochran, 1989; Bult and Lynch, 2000). For all lines, heritabilities between generations were calculated separately by linear regression of the chi-squared periodogram scores of offspring on parents for females and males separately (Falconer, 1981). Parent scores and corresponding offspring scores were corrected for the number of offspring. Regressions were calculated for each sex separately. Unfortunately, even after transformation the data was too heteroscedastic to obtain reliable estimates of heritabilities.

Differences between the lines were investigated using the general linear model (GLM) procedure analysis of variance (ANOVA, SAS Institute Inc., Cary, NC). Sex, generation, line, and replicate nested within line were included as factors in the model, as well as interaction effects between sex, line (rhythmic, non-rhythmic and control), generation and replicate (NA, NB and RA, RB). No sex effects or interaction effects were found (data not shown) and these effects were removed from the model for reanalysis.

5.4 Results

The average chi-squared periodogram values per generation of selection are shown in figure 5.1. The rhythmic lines had an average strength of 1378 ± 106 (SEM), the non-rhythmic lines had an average of 965 ± 80 and the controls were intermediate with an average of 1138 ± 95. These differences were not significant (Generation – $F_{1,2}$=1.31; Line – $F_{2,2}$=1.12; Generation x Line – $F_{2,2}$=1.30) due to variability among the replicate lines during the first generations of selection and the small sample sizes.

Heritability estimates are shown in table 5.1. The estimates were plagued by small and heteroscedastic samples. Estimates ranged from 0.13 ± 0.47 (SEM) to 0.93 ± 0.35 in the control line, from -0.42 ± 0.55 to 0.91 ± 0.52 in the rhythmic lines, and from -1.18 ± 0.88 to 0.44 ± 0.38 in the non-rhythmic lines.

5.5 Discussion

Nonsignificant results after two generations of selection are not surprising. Lynch (1980) performed selection for the amount of cotton used by mice to build large or small nests. The lines did not significantly diverge until about 5 generations of selection, probably due to the breakup of genetic linkages that created additional genetic variability that selection could act upon (Lynch, 1980). Similar results were obtained for reselection for nest-building behavior after the replicate lines were crossed (Bult and Lynch, 2000). Swallow et al. (1998) used 10 generations of selection for increased wheel running before a significant difference was observed.

Althogh differences were not significant between the selected lines, the trend in chi-squared values among the lines is promising. The average value of the rhythmic lines is 400 units higher than the average of the non-rhythmic value in the second generation of selection. The phase response curve (PRC) of *C. rutilus* does not reveal significant light-induced phase advances (Tavernier et al., 2004; chapter 3). This is very similar to the PRC of some mouse lines (Bult et al., 1993) and may indicate a compromised system for tracking sunrise. This could be due to the absence of properly functioning *Per1* and/or *Cry1* clock genes, differeing alleles, or differential regulation of these clock components. Various lines of evidence have pointed to *Per1* and/or *Cry1* as tracking sunrise within the circadian system (reviewed in Daan et al., 2001).

Multiple solutions may have been used by different lines to attain their circadian wheel-running behavior. Therefore, the genetic factors that contribute to observed phenotypes may differ among lines. When mice selected for nest-building behavior experienced renewed selection with a replicate cross within the lines, increases in nest size were observed even though an apparent plateau previously had been reached after 20 generations. This points to a differing genetic basis in nest building behavior between the replicate even though they were selected under the same conditions (Bult and Lynch, 2000).

Garland (2003) outlines many of the problems with selection experiments and why they are not used more. In this experiment, I became aware of some of the problems mentioned. Generational times averaged a year for each line. Resources only allowed for one line to be tested at a time, resulting in each generation of each line being tested once

a year. This spread the mating and testing of lines out throughout the year with a line being tested while the other lines were being mated, or waiting for equipment so they could be tested. The amount of time spent on animal care and the cost of keeping a large enough sample of animals proved prohibitive to continuation of the experiment under these conditions.

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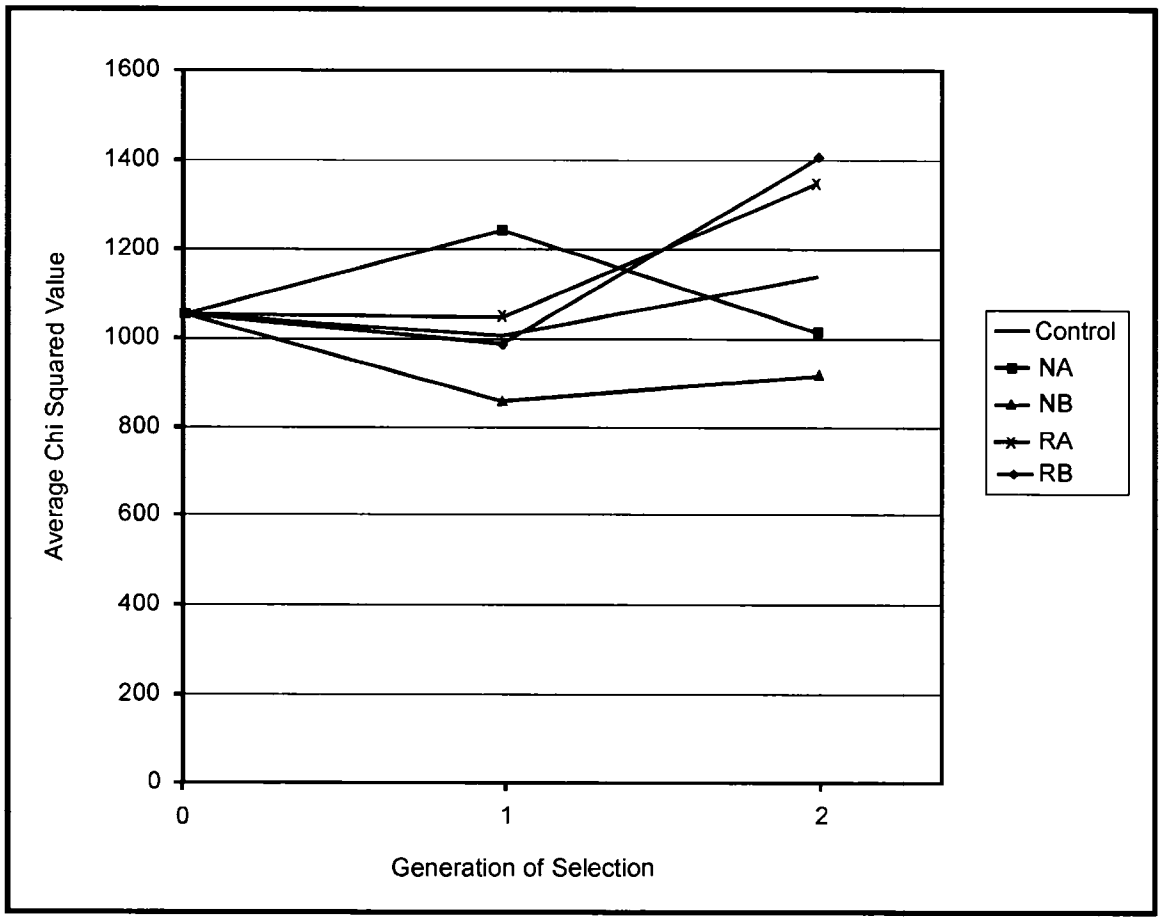

Figure 5.1 Average chi-squared periodogram values of selected lines over two generations of selection. Rhythmic lines = RA and RB. Non-rhythmic line = NA and NB. BC = randomly bred control line.

Table 5.1 Heritability estimates in selected lines over two generations. Rhythmic lines = RA and RB. Non-rhythmic line = NA and NB. BC = randomly bred control line.

	BC	RA	RB	NA	NB
Generation 0-1					
Males	0.19 ± 0.24	0.48 ± 0.24	-0.35 ± 0.39	0.13 ± 0.52	0.17 ± 0.62
Females	0.55 ± 0.29	-0.42 ± 0.55	0.91 ± 0.52	-0.29 ± 0.60	0.44 ± 0.38
Generation 1-2					
Males	0.13 ± 0.47	-0.22 ± 0.29	-0.24 ± 0.64	-0.49 ± 0.30	-1.18 ± 0.88
Females	0.93 ± 0.35	0.47 ± 0.72	-0.09 ± 0.35	-0.13 ± 0.38	-0.53 ± 0.75

Chapter Six

General Conclusions

This thesis examined the circadian rhythms of a non-hibernating arctic mammal, the red-backed vole (*Clethrionomys rutilus*), the distribution of neurotransmitters, neuropeptides and proteins in the suprachiasmatic nucleus (SCN) of this species, and our ability to selectively breed for voles that were able to maintain a circadian rhythm in constant dark (DD) and those unable to maintain a circadian rhythm in DD. Circadian rhythms are an almost universal part of life and prepare organisms for predictable daily changes in their environment. In mammals, they are controlled by the SCN, small paired nuclei located above the optic chiasma in the hypothalamus. The phase of the SCN is set by many environmental cues, of which the strongest is light (reviewed in Edery, 2000). Arctic light regimes are extreme with large daily changes in day-length and seasonal periods of (DD) and constant light (LL). The majority of circadian studies have used animals that experience more uniform light:dark (LD) cycles throughout there life. This thesis examined a non-hibernating Arctic mammal to elucidate its circadian rhythms and the neuroanatomy of its SCN. An attempt was also made to selectively breed for circadian phenotypes found in the wild.

The four main study objectives of this thesis were defined in the first chapter. Following are the experimental results I obtained to address the objectives.

My first objective was to acquire a wild Arctic mammal that experiences the full range of light regimes. For this objective, I chose the northern red-backed vole, *Clethrionomys rutilus*. *C. rutilus* is an abundant, small (20-35 gram) non-hibernating

rodent distributed throughout Alaska (Stenseth, 1985). In addition, *C. rutilus* is easy to capture, has a short generation time, and was able to breed year round in the laboratory. Being active year round, *C. rutilus* is exposed to the full range of light regimes in the Arctic.

Samples from two separate populations of *C. rutilus* were captured during the spring and summer of 2000. Animals were translocated to the University of Alaska Fairbanks, and breeding colonies were developed and maintained until the present. Experimentation was conducted to determine the proper husbandry conditions that would allow the voles to prosper in captivity. Housing, food and lighting regimes were optimized to allow for the maintenance of a healthy colony. General life history characteristics, such as animal weights, growth, breeding success, litter size and common causes of mortality were recorded. With extra care we could raise voles and maintain sufficient stock for my experiments.

Chapter three covers the results of my second objective, which was a study of the circadian rhythms in a subarctic animal. This investigation into the circadian rhythms of northern red-backed voles may have uncovered a novel way that this species deals with the extreme light and dark cycles of the subarctic. Both wild-caught and third-generation laboratory-bred animals tested in running wheels showed predominantly nocturnal patterns of wheel-running when exposed to a 16:8 LD cycle. In constant light (LL) and constant dark (DD) conditions, however, animals displayed high phenotypic variation in circadian rhythms. Compared to wheel-running rhythms under a 16:8 LD cycle, the robustness of circadian activity rhythms decreased among all animals tested in LL and

DD, i.e. decreased chi-squared periodogram waveform amplitude average. A large segment of the 3rd generation tested became non-circadian (60% in DD, 72% in LL) within eight weeks of exposure to constant lighting conditions, of which the majority became ultradian, with a few individuals becoming arrhythmic, indicating highly labile circadian organization. Wild-caught and laboratory-bred animals that remained circadian in wheel-running displayed free-running periods between 23.3 and 24.8 hours. A phase response curve to light pulses in DD showed significant phase delays at circadian times 12 and 15, indicating the capacity to entrain to rapidly changing day-lengths at high latitudes.

In order to meet the third objective, immunocytochemistry for common neurotransmitters was conducted on the SCN of *C. rutilus*. Immunocytochemistry for vasoactive intestinal peptide, vasopressin, neuropeptide Y (NPY), cholecystokinin (CCK), calbindin, gastrin-related peptide, protein kinase C Alpha, and protein kinase C Beta 2 was completed. The distribution of neurotransmitters within the SCN was similar to that found in other common laboratory rodents. Apparent larger quantities of CCK and NPY were found in the SCN of C. *rutilus*, pointing to the possible importance of food and non-photic cues in resetting the phase of the internal clock. Appendix A shows the additional experiments on voles that were sent to the laboratory of Dr. Hugh Piggins. The distribution of Substance P (SP) and the Neurokinin-1 receptor in the SCN and brain of *C. rutilus* was explored. SP in the SCN of the voles showed a novel distribution when compared to other species. These results show that although northern red-backed voles

have a distribution of neurotransmitters, neuropeptides and proteins similar to other species there are some differences to be firther investigated.

After the fifth generation of random breeding in the laboratory, a selective breeding protocol was undertaken. This experiment was conducted to address the fourth objective. Two lines of voles that maintained circadian rhythms in DD (RA and RB), two lines that showed no circadian rhythmicity in DD (NA and NB), and a randomly-bred control line were developed. After three generations of selective breeding, no significant changes were observed in the strength of circadian rhythms between the lines, probably due to small sample sizes, possible low heritability, and the large variability in the response to selection over the first few generations. We could not calculate reliable stimates of heritability due to the same problems. However, voles in the second generation of selection showed a 400 unit difference in average chi-squared values between the RA and RB lines and the NA and NB lines. These differences, although not significant, show a trend in the expected direction.

These studies of the northern red-backed vole have helped to illuminate one way in which Arctic animals may cope with the changing daylight regimes. Being active around the clock during the long days of summer may allow voles to take advantage of the abundant resources and breeding opportunities. During the winter when their energy expenditures are large (Feist and Rosenmann, 1976; Grodzinski, 1971), being active around the clock may help them acquire the energy needed to stay alive. There could be selection against maintaining a circadian rhythm under these pressures. Additional environmental factors such as predation that were not addressed in this thesis may also be

affecting the circadian rhythms of *C. rutilus*. Future studies may help illuminate the reasons for these large phenotypical variances in the circadian rhythms of *C. rutilus*.

Current studies are being conducted to investigate the melatonin rhythms of circadian and non-circadian voles and how they may affect the animal's ability to respond reproductively to long and short daylengths. Future studies will determine if differences exist in the distribution and quantity of the common neurotransmitters, proteins and pepides in the SCN between rhtymic and non-rhythmic voles. The selective breeding experiment has been contimued with the two rhythmic lines combined into one line and the non-rhythmic lines also combined into one line. It is hope that within three to five years they will be valuable tool with clear differences in circadian behavior, so possible natural allelic variation in circadian rhythm producing genes can be investigated.

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Appendix A

Substance P and Neurokinin-1 Immunoreactivities in the Neural Circadian System of the Alaskan Northern Red-Backed Vole, *Clethrionomys rutilus* [1]

A.1 Abstract

The suprachiasmatic nucleus (SCN) of the hypothalamus houses the main mammalian circadian clock. This clock is reset by light-dark cues and stimuli that evoke arousal. Photic information is relayed directly to the SCN via the retinohypothalamic tract (RHT) and indirectly via the geniculohypothalamic tract, which originates from retinally innervated cells of the thalamic intergeniculate leaflet (IGL). In addition, pathways from the dorsal and median raphe (DR and MR) convey arousal state information to the IGL and SCN respectively. The SCN regulates many physiological events in the body via a network of efferent connections to areas of the brain such as the habenula (Hb) in the epithalamus, subparaventricular zone (SPVZ) of the hypothalamus and locus coeruleus of the brainstem- areas of the brain associated with arousal and behavioral activation.

Substance P (SP) and the Neurokinin-1 (NK-1) receptor are present in the rat SCN and IGL, and SP acting via the NK-1 receptor alters SCN neuronal activity and

[1] A previous version of this chapter was published as Samuels RE, **Tavernier RJ Jr**,, Castillo MR, Bult-Ito A, Higgins HD (2006) Substance P and neurokinin-1 immunoreactivities in the neural circadian system of the Alaskan northern red-backed vole, *Clethrionomys rutilus*. Peptides 27(11): 2976-2992.

resets the circadian clock in this species. However, the distribution and role of SP and NK-1 in the circadian system of other rodent species are largely unknown. Here we use immunohistochemical techniques to map the novel distribution of SP and NK-1 in the hypothalamus, thalamus and brainstem of the Alaskan northern red-backed vole, *Clethrionomys rutilus*, a species of rodent currently being used in circadian biology research. Interestingly, the pattern of immunoreactivity for SP in the red-backed vole SCN was very different from that seen in many other nocturnal and diurnal rodents

Keywords: Tachykinin, suprachiasmatic, intergeniculate leaflet, raphe nuclei, habenula, subparafascicular, interpeduncular, rodent

A.2 Introduction

The suprachiasmatic nucleus (SCN) located in the anterior hypothalamus, houses the principal circadian pacemaker responsible for generating near 24h (circadian) physiological and behavioral rhythms in mammals (Hastings and Maywood, 2000; Rusak and Zucker, 1979; Weaver, 1998). The clock of this cell dense structure is synchronized (entrained) by sensory and behavioral cues such as light, temperature, food availability (Castillo et al., 2004), and social interactions, communicated to the SCN by well-characterized neuronal pathways (Kalsbeek and Buijs, 2002; Mrosovsky, 1996; Saper et al., 2005). Photic information is relayed directly from melanopsin synthesizing retinal ganglion cells to the SCN via the monosynaptic retinohypothalamic tract (RHT; Hannibal and Fahrenkrug, 1992; Lucas et al., 2001), and indirectly via the retinally innervated cells

of the thalamic intergeniculate leaflet (IGL) that project to the SCN form the geniculohypothalamic tract (Harrington, 1997; Morin, 1994; Reuss, 1996). Information relating to arousal state, or non-photic cues, is also transmitted to the SCN via the geniculohypothalamic tract, and from pathways originating in the raphe nuclei of the brainstem (Morin, 1999; Mrosovsky, 1996).

The SCN regulates many physiological and behavioral parameters such as feeding, sleep-wake and locomotor activity via a network of efferent connections with areas of the brain such as the subparaventricular zone of the hypothalamus (SPVZ), habenula nuclei of the epithalamus (Hb), paraventricular nucleus of the thalamus (PVT) and the locus coeruleus of the brainstem (de Vries et al., 1981; Kalsbeek and Buijs, 2002; Legoratti-Sanchez et al., 1989; Morin et al., 1994; Saper et al., 2005; Watts and Swanson, 1987).

Previous neuroanatomical studies in rat show that immunoreactivity (-ir) for the neuropeptide substance P (SP) is present in fibers and terminals in the SCN (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978). In vitro electrophysiological studies have demonstrated that application of SP peptide alters rodent SCN electrical activity and phase shifts the firing rate of rodent SCN cells in a manner resembling the effects of a light pulse on rodent behavioral rhythms (Kim et al., 2001; Norris et al., 1993; Piggins et al., 1997; Shibata et al., 1992; Shirakawa and Moore, 1994). These studies implicate SP in photic entrainment. Terminals immunopositive for SP are also localized within the IGL of rat, Syrian hamster (*Microcetus auratus*), Djungarian hamster (*Phodopus*

sungorus) and C57BL/6J mouse (*Mus domesticus*) (Piggins et al., 2001), indicating that SP may indirectly affect the circadian clock through the geniculohypothalamic tract.

In many areas of the mammalian central nervous system (CNS), SP acts via the neurokinin-1 (NK-1) receptor to modulate cellular activity. This G-protein coupled receptor has two isoforms, a 407 amino acid residue and a shorter 312 amino acid residue form. NK-1 mRNA is found localized in the dorsal and lateral areas surrounding rat SCN and in the IGL (Maeno, 1993; Mick et al., 1994). Immunohistochemical studies using antisera raised to a C-terminus peptide sequence of this receptor, demonstrate NK-1-ir in the rodent SCN region and within the IGL of rat, *M.auratus* and *M.domesticus* (Mick et al., 1995; Nakaya et al., 1994; Piggins et al., 2001). From these observations, SP appears to act via the NK-1 receptor to directly and indirectly affect SCN circadian clock function.

To date, the distribution of SP-ir and NK-1-ir in the CNS of commonly used laboratory rodents has been well characterized, but little is known about the pattern of SP and NK-1 immunostaining in the brain of wild-caught rodents. In this study we examine the distribution of SP-ir and NK-1-ir in the Alaskan northern red-backed vole (*Clethrionomys rutilus),* a non-hibernating, mainly nocturnal murid rodent (Tavernier et al., 2004). This vole inhabits forested regions of Alaska where it is exposed to extreme photoperiods depending on the time of year. *C.rutilus* has recently been the subject of circadian studies and here we report a novel pattern of SP-immunostaining in the SCN of this species.

A.3 Abbreviations

Arc, arcuate nucleus; dLGN, dorsolateral geniculate nucleus; DR, dorsal raphe; EW, Edinger-Westphal nucleus; f, fornix; GHT, geniculohypothalamic tract; IF, interfascicular nucleus; IGL, intergeniculate leaflet; IPDM, interpeduncular nucleus, dorsomedial; IPI, interpeduncular nucleus, intermediate; IPL, interpeduncular nucleus, lateral; IPN, interpeduncular nucleus; LC, locus coeruleus; LHA, lateral hypothalamic area; LHb, lateral habenula; ME, median eminence; MEE, median eminence, external capsule; MEI, median eminence, internal capsule; MHb, medial habenula; mlf, medial longitudinal fasciculus; MPN, median preoptic nucleus;

MR, median raphe; mt, mammillothalamic tract; ox, optic chiasm; Pa, paraventricular nucleus; PaLM, paraventricular nucleus, lateral magnocellular; PaMM, paraventricular nucleus, medial magnocellular; PaMP, paraventricular nucleus, medial parvicellular; PN, paranigral nucleus; PP, peripeduncular nucleus; PVT, thalamic paraventricular nucleus; Py, pyramidal layer of hippocampus; Rbd, rhabdoid nucleus; REM, rapid eye movement; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; SPFm, subparafascicular nucleus, magnocellular; SPFp, subparafascicular nucleus, parvicellular; SPVZ, subparaventricular zone; SuG, superficial gray of superior colliculus; SuM, supramammillary nucleus; vLGN, ventrolateral geniculate nucleus; ZI, zona incerta; ZIM, zona incerta medial; Zo zonal layer of superior colliculus.

A.4 Methods

Eight male northern red-backed voles (*C. rutilus*) were captured in the wild and kept in 16 hours light: 8 hours dark for 10 weeks. All animals were provided with wood shavings and cotton nesting material. Food (Lab Diet Co. Mouse Diet #5015, St. Louis, MO and Purina Mills, Inc. Rabbit Chow, St. Louis, MO) and water were available *ad libitum.* Animals were also fed one slice of carrot daily at random times. Animals were killed during the daytime by an overdose of sodium pentobarbital (100mg/kg). Animals were then perfused intracardially with a solution of 0.9% saline, pH 7.4, followed by periodate - lysine - paraformaldehyde (PLP, final pH 6.2) in PB solution. The brains were removed, post-fixed in PLP for 48 h and then cryoprotected in a 30% sucrose/PB solution. (48-72 hours, 4°C).

Substance P immunoreactivity (SP-ir) was detected using a previously characterized monoclonal anti-SP antibody raised in rats (1: 100; Harlan-Sera Labs, Loughborough, UK). Neurokinin-1 receptor immunoreactivity (NK-1-ir) was determined using a polyclonal antiserum raised in rabbits, against the NK-1 receptor (1:1000; Novus Laboratories, Cambridgeshire, UK). Biotinylated antisera to goat anti-rat and goat anti-rabbit (Vector Labs, Peterborough, UK) were used as secondary antibodies to SP and NK-1 antisera respectively.

Control experiments were performed on alternate sections by omission of primary or secondary antibodies. Specificity of antisera was confirmed by preadsorption of the SP peptide (10^{-6}M, 2 h preincubation at room temperature) with the SP monoclonal antibody, and a synthetic peptide to NK-1 (10^{-7}M, 2 h preincubation at room temperature)

corresponding to the sequence 393-407 (KTMTESSSFYSNMLA) of the C-terminus of the NK-1 receptor (Severn Biotech, Kidderminster, UK) with the NK-1 antibody.

In the protocol to follow, all rinses and solutions for the sections treated with the SP antibody were carried out in a solution of PB with 0.03% Triton X-100 (Sigma, Poole, UK) added (PBx), while all rinses and solutions for sections treated with the NK-1 receptor antiserum contained PB only.

Cryoprotected tissue was rapidly frozen using dry ice crushed with a mortar and pestle, and 30μm coronal sections cut using a freezing sledge microtome equipped with a Peltier Stage (Bright Instruments, Huntingdon, UK). Four sets of sections were collected into PB from the level of the diagonal band of Broca to the retrochiasmatic area (referred to as SCN sections). Another set of sections were taken from the hypothalamic arcuate nucleus to the superior colliculus, (collectively referred to as thalamic sections), tissue collected thereafter were referred to as brainstem. Floating sections were rinsed, incubated in a solution of 20% methanol, 1.5% hydrogen peroxide and PB/PBx (20 min, room temperature) to abolish endogenous peroxidase activity. Sections were then rinsed, blocked in 5% normal goat serum (Sigma) in PB/PBx (60 min, room temperature) then left to incubate in primary antiserum also diluted in blocking solution (48-72 h, 4°C). Sections were rinsed, incubated in the appropriate biotinylated secondary antibody diluted in PB/PBx (90 min, room temperature), rinsed again and incubated in an avidin-biotin immunoperoxidase complex (Vectastain ABC kit, Vector Labs, Peterborough, UK) diluted in PB/PBx (90 min, room temperature). Tissue underwent two PB washes and one wash in 0.1M sodium acetate buffer (pH 6.0) before the location of the antigens was

visualized using a 3,3'-diaminobenzidene tetrachloride (DAB) complex with nickel intensification (NiDAB) giving a purple/black stain when developed. 0.1M acetate buffer solution was used to stop further development of the reaction. Sections were rinsed to remove traces of NiDAB, mounted onto gelatin/chrome alum coated slides, and dried. Slides were then dehydrated through a series of ethanol concentrations and cleared in Histoclear (National Diagnostics, Hull; UK) before being coverslipped using Entellan (Merck, Darmstadt, Germany).

Mounted sections were examined using an Olympus BX50 microscope with an Olympus Camedia C2020Z digital camera attachment. Brain structures were described with reference to the mouse brain stereotaxic atlas by Paxinos and Franklin, 2001 as well as descriptions in Simerly, 1995. The density of immunostaining was graded according to the following criteria. A high density indicates the brain area is well defined by immunolabelled fibers and or neurons. A moderate distribution indicates an area slightly less well defined and less dense in immunopositive fiber and or neurons. A sparse or absent distribution is an area containing single or no immunoreactive fibers. Photomicrographs were taken and panels constructed using Microsoft PowerPoint.

A.5 Results

A.5.1 Substance P

A.5.1.1 Hypothalamus

In the preoptic region rostral to the SCN, SP-ir was generally absent or sparse throughout most of the medio-lateral axis. The only staining of note was detected in the

median and medial preoptic nuclei; (MPN; Fig. A.1A) where fiber and terminal immunolabelling for SP was moderate to dense, becoming sparser in the lateral zone. A moderate distribution of lightly labeled cells was present in the ventral aspect of the MPN (see Table 1).

In the periventricular zone of the anterior hypothalamic region, SP staining was differentially distributed throughout the rostrocaudal axis of the SCN. SP-ir fibers and terminals were sparse at rostral SCN and moderate in the caudal SCN. At intermediate levels, a dense, intensely labeled cluster of SP-ir fibers was readily detected in the ventral SCN, while the dorsal SCN was sparsely innervated. (Fig. A.1 A, C, E, G). The periventricular nucleus showed a moderate to dense distribution of terminals that continued across the rostrocaudal extent of the structure, similarly the SPVZ showed a sparse to moderate terminal distribution throughout (Fig. A.1G, A.2A). The paraventricular nucleus of the hypothalamus (Pa) displayed a variable distribution: the medial magnocellular paraventricular nuclei (PaMM) contained a moderate to dense distribution of fibers and terminals that increased in intensity rostrocaudally, while the lateral magnocellular paraventricular nucleus (PaLM) showed a dense fiber and terminal distribution which decreased to sparse at caudal aspects (Fig. A.2A). A small number of faintly labeled SP-ir cells were also observed in the PaMM and PaLM at caudal levels (Fig. A.3B; compare with darkly stained cells in hippocampus in Fig. A.3A or superior colliculus. Fig. A.3C). The medial parvicellular subdivision of the Pa (PaMP) showed a moderate to dense terminal and fiber distribution (Fig. A.3A).

The medial zone of the anterior hypothalamus contained a sparse to moderate distribution of fibers and terminals localized to the anterior hypothalamic and retrochiasmatic areas. Lateral zone structures, including the supraoptic nuclei and the lateral hypothalamic area (LHA), were generally sparse to moderately innervated by SP-ir fibers and terminals. For example, the perifornical area showed moderate SP-ir fiber and terminal innervation. Lightly stained SP-ir cells were sparse in this region with most detected at caudal aspects.

The periventricular zone of the tuberal region displayed intense cellular staining of moderate density in the arcuate nucleus (Arc) and a moderate fiber distribution at caudal aspects, while the median eminence (ME) contained a moderate to dense distribution of terminals in its external layer (MEE; Fig. A.4A, C). SP-ir fibers and terminals were seen in the dorsomedial hypothalamus in the area closest to the periventricular nucleus. Medial tuberal areas contained a sparse overall distribution of fibers and terminals with a slightly denser innervation at the ventral aspect of the dorsal hypothalamic nucleus. The posterior hypothalamic nucleus contained a moderate fiber distribution. The lateral hypothalamic area was again sparsely innervated with terminals and fibers.

SP-ir staining was absent from the periventricular zone of the mammillary region but present in moderate density in the medial zone structures such as the supramammillary and medial mammillary nuclei.

A.5.1.2 Epithalamus and Thalamus

The epithalamus containing the habenula nuclei displayed faintly stained cells and intensely stained fibers at the border between the medial habenula (MHb) and lateral habenula (LHb). SP-ir terminal innervation was dense at the dorsal aspect of the MHb, while SP-ir in the LHb was sparse to absent (Fig. A.4E, G).

The majority of SP-ir detected was found in the midline group of thalamic structures. The PVT contained a moderate density of fibers and terminals immunoreactive for SP, but anterior, dorsal, ventral as well as laterodorsal nuclei of the thalamus showed a paucity of SP-ir (see Table 1).

The centromedial nucleus showed a sparse to moderate density of fibers and terminals with none in the adjacent centrolateral nucleus. Rhomboid and reunions nuclei were devoid of SP-ir. A faintly stained cluster of SP-ir fibers was observed in the area of the midline magnocellular subparafascicular nucleus (SPFm), which continued throughout the rostrocaudal extent of this structure (Fig. A.5A,C) The parvicellular subparafascicular nucleus (SPFp) exhibited a band of moderately stained terminals as it extended laterally to fuse with the deeply stained peripeduncular nuclei (PP) at the most caudal aspect (Fig. A.6G).

The IGL of the lateral geniculate complex contained a sparse to moderate distribution of SP-ir fiber and terminals defining the intercalated structure over the rostrocaudal extent of the lateral geniculate region, with an occasional fibrous process extending into the ventrolateral geniculate nucleus (vLGN; Fig. A.6A, C, E, G). The dorsolateral

geniculate nucleus (dLGN) showed a paucity of SP staining. SP-ir staining at caudal levels of the IGL extended to become contiguous with the zona incerta (ZI).

A.5.1.3 Midbrain

Midbrain structures exhibiting SP-ir include the interpeduncular nuclei (IPN), which exhibited a moderate to dense fiber and terminal distribution. SP-ir terminals heavily innervated the interfascicular nucleus (IF), paranigral nucleus (PN), and subjacent interpeduncular dorsomedial (IPDM) and lateral nuclei (IPL). A sparse to moderate distribution of fibers and terminals was present at caudal levels of the intermediate nuclei (IPI). An occasional faintly stained SP-ir cell body was also detected at this level of the IPI (Fig. A.7A).

A.5.1.4 Brainstem

SP-ir fibers and terminals were sparse to moderate in the dorsal raphe and in the median raphe (Fig. A.10A, C, E, G), while a densely stained region of terminals was detected in the rhabdoid nucleus (Rbd; Fig. A.10A; Table 1).

A.5.2 Neurokinin-1 Receptor

A.5.2.1 Hypothalamus

In the periventricular preoptic region (Table 1), the MPN displayed a moderate to dense distribution of faintly stained NK-1-ir neurons and associated processes, as did the subjacent anteroventral periventricular nucleus. The medial preoptic nucleus displayed a more dense distribution of NK-1-ir, particularly in the ventromedial portion of the nucleus.

Immunoreactivity for NK-1 in the anterior region was moderately dense in the periventricular nucleus and areas bordering the SCN. Many of the moderate number of NK-1-ir cells bordering the SCN and in the dorsal SCN extended singular processes towards the ventral 'core' of the SCN. Sections double stained for SP-NK-1 showed SP terminals and NK-1 neurons and processes in close proximity in the dorsal SCN (Fig. A.9A, B), indicating a possible means of cellular action for SP within this area. The distribution of NK-1 within this so-called 'shell' region remained moderate throughout the rostrocaudal extent (Fig. A.1B, D, F, H). The anterior hypothalamic area exhibited a moderate neuronal distribution that increased rostrocaudally, paralleling the distribution in the lateral hypothalamic area. The supraoptic nucleus of the lateral anterior zone contained only sparse to moderate numbers of cells with very few fibers and/or terminals. The SPVZ contained a moderate distribution of fibers with sparse NK-1-ir cell bodies and associated processes (Fig. A.1H, A.2B, A.8C, D). The Pa showed a moderate to dense distribution of immunopositive cells and associated processes throughout its rostrocaudal extent. The majority of NK-1-ir cells were detected in the PaLM and in the

overlying dorsal ZI, although a sparse to moderate distribution of NK-1-ir cells was observed in the PaMP (Fig. A.2B).

Structures in the periventricular and medial zones of the tuberal region such as the Arc and at ventral aspects, the dorsomedial hypothalamic nucleus, typically contained moderate numbers of lightly stained NK-1 immunopositive neurons and processes while the ventromedial hypothalamic nucleus showed a small cluster of lightly stained NK-1-ir cells and processes (Table 1). By contrast, abundant deeply stained NK-1-ir fibers with diffuse cells were evident in the internal layer of the median eminence (MEI; Fig. A.4B and D). NK-1 immunopositive neurons were more numerous in the lateral hypothalamus of the tuberal region where a moderately dense population of NK-1-ir cells and associated processes could be seen.

Immunopositive neurons and fibers were observed in moderate density in the medial zone including the medial aspect of the supramammillary nucleus (SuM), while the lateral SuM had a denser cell population with a moderate fiber distribution. The medial mammillary nucleus displayed a moderate neuronal distribution at dorsal aspects only. Lateral nuclei of the mammillary region were densely populated by NK-1-ir neurons (Table 1).

A.5.2.2 Epithalamus and Thalamus

The epithalamus contained darkly stained neurons and associated processes clustered at the dorsomedial and ventral aspects of the MHb, along with a moderate distribution of

fibers at the MHb-LHb border. A discreet cluster of cells and fibers was also seen in the central region of the LHb with the surrounding area showing sparse NK-1 immunoreactivity (Fig. A.4F and H).

As with SP, the majority of NK-1 staining in the thalamus occurred along the midline structures. The PVT was only sparse to moderately populated with fibers and occasional cells. A distinct pattern of staining was detected in the SPFm nucleus; containing a discreet moderately stained cluster of neurons and associated processes, which persisted throughout the rostrocaudal extent of the nucleus (Fig. A.5B, C). The SPFp showed a band of faintly stained NK-1-ir fibers, the processes extended laterally, becoming indistinguishable from NK-1-ir in the more heavily stained ZI (Fig. A.6H).

Lateral structures also contained NK-1 immunopositive cells and processes but had a limited distribution (Table 1). The IGL contained a moderate to dense NK-1 fiber distribution that delineated the extent of the IGL and marked the structure out over the rostrocaudal extent. Sparse faintly stained immunopositive cells were seen at all rostrocaudal levels of the IGL. The fibers also permeated into the vLGN at caudal aspects as the IGL became contiguous with the dorsal zona incerta (Fig. A.6B, D, F, H). Anterior and mediodorsal groups of nuclei showed no significant staining for NK-1 (Table 1).

A.5.2.3 Midbrain

The IPN in the midbrain region contained intensely stained NK-1-ir fibers and processes in the IPD, IPL and in the IF nucleus (Fig. A.7B). Double

immunohistochemistry for SP-NK-1–ir revealed a mismatch between peptide and receptor in the IPI. Faintly stained SP-ir terminals could be seen in the IPI with a paucity of corresponding NK-1 receptor in this region (Fig. A.7C). NK-1-ir staining in the PN was similarly sparse.

A.5.2.4 Brainstem

The dorsal raphe contained a moderate-dense plexus of darkly stained NK-1-ir processes with lightly stained cells over the rostrocaudal extent (Fig. A.10A, D) in comparison to the median raphe which displayed only a sparse distribution of faintly stained cells and processes (Fig. A.10F, H). In the Rbd, NK-1 –ir was similarly sparse (Fig. A.10F; Table 1).

A.5.3 Controls

Preadsorption of the SP monoclonal antibody with SP peptide abolished immunostaining in the hypothalamus, thalamus, midbrain and brainstem tissue. Similarly pre-incubation of the NK-1 polyclonal antisera with the synthetic peptide (KTMTESSSFYSNMLA) corresponding to amino acid sequence at 393-407 of the C-terminus of this receptor eliminated all specific immunolabelling. Omission of primary or secondary antibodies from the protocol prevented immunostaining of the tissue (Fig. A.8A, B, C, D).

A.6 Discussion

A.6.1 *General Discussion*

SP and NK-1 immunoreactivity was observed over the rostrocaudal extent of *C. rutilus* hypothalamus, thalamus, midbrain and brainstem (Table 1), with the highest accumulation being along mid-line structures such as Arc, AHA, Pa, Hb, IPN, raphe nuclei, and in highly localized regions such as IGL, ZI and PP nuclei. These patterns of staining are generally consistent with the distribution of SP-ir and NK-1-ir reported in other rodents such as *R. norvegicus*, *M auratus*, *P. sungorus*, and *M. domesticus* (Cuello and Kanazawa, 1978; Morin et al., 1992; Piggins et al., 2001; Ribeiro-da-Silva et al., 2000; Stoeckel et al. 1982) suggesting conservation in SP-NK-1 expression in the CNS of nocturnal rodents.

A.6.2 *The Suprachiasmatic Nucleus*

In contrast to the general trend noted above, the pattern of immunoreactivity for SP in the red-backed vole SCN is very different from that seen in many other nocturnal and diurnal rodents. A dense plexus of SP-ir fibers and terminals localized to the ventral SCN at mid–caudal aspects, while SP-ir was sparse or absent in more rostral and caudal levels (Figs. A.1, A.9). This is distinct to SP-ir innervation detected in the SCN of nocturnal rodents such as *M.auratus*, *P. sungorus*, and *M. domesticus*, (Hartwich et al., 1994; Morin et al., 1992; Piggins et al., 2001; Reuss and Burger, 1994) or in diurnal species such as *Octodon degus* and *Arvicanthis niloticus* (Goel et al., 1999; Smale and Boverhof, 1999). In most of these species, SP-ir fibers and terminals are sparse

throughout the rostrocaudal axis of the SCN. In rat, a dense plexus of SP-ir fibers is detected in the ventral SCN, but unlike the red-backed vole, this innervation is prevalent throughout most of the rostrocaudal axis of the SCN. These findings highlight that SP-ir in the rodent SCN shows considerably species variation.

The origin of the SP-ir fibers in the *C. rutilus* SCN is unknown. One tract tracing study in rat implicated SP-ir retinal ganglion cells as projecting to the SCN via the RHT (Takatsuji et al. 1991). Consistent with this result, optic enucleation reduced or abolished the SP-ir plexus in the retinorecipient region of rat SCN (Mikkelsen and Larsen, 1993; Takatsuji et al. 1991). However, Hannibal and Fahrenkrug, 1992 report that SP-ir is absent from anterogradely labeled retinal efferents to the rat SCN, and independently, other groups found that SP-ir fibers and terminals in the rat SCN were not diminished following optic enucleation (Hartwich et al., 1994; Otori et al., 1993). Further investigations are necessary to determine if SP is contained in the RHT of *C. rutilus*.

Cell bodies containing SP were absent from the red-backed vole SCN, which contrasts with the SCN of other nocturnal species such as *M. auratus*, *P. sungorus*, or *R. norvegicus,* where small to moderate numbers of SP-ir neurons are present (Hartwich et al., 1994; Morin et al., 1992; Piggins et al., 2001; Reuss and Burger, 1994). Our observation of SP-ir neurons in the Pa, Arc, hippocampus, and superior colliculus indicates that our immunohistochemical procedures are capable of detecting SP-ir cells in the red-backed vole brain (Fig. A.3). Pretreatment with the antineoplastic, colchicine can enhance perikaryon labeling for SP in the rodent brain and since we did not use

colchicine in this study, we cannot rule out the possibility that some SCN neurons in *C.rutilus* may contain SP.

NK-1 immunostaining was differentially distributed in the SCN. The ventral SCN contained very little NK-1-ir, whereas neurons and associated fibers immunopositive for NK-1 were concentrated around the dorsolateral borders of this structure (Figs. A.1, A.9). This pattern of NK-1-ir is consistent with those found in the SCN of other nocturnal rodents (Maeno, 1993; Mick et al., 1994; Nakaya et al., 1994; Piggins et al., 2001). The distinct SP-ir terminal/fiber plexus in the SCN shows little overlap with NK-1-ir (Fig. A.9A, B). Dendritic NK-1-ir can be seen in the immediate vicinity of this SP-ir accumulation, but the majority of NK-1-ir cell body and associated process labeling is in the dorsolateral SCN and hypothalamic region immediately supradjacent to the SCN. If SP is released into the *C. rutilus* ventromedial SCN, it presumably acts either through a volume transmission-like mechanism to alter cellular activity in the dorsolateral SCN or via uncharacterized neurokinin receptors not detected by this NK-1 antisera. Further functional and neuroanatomical studies are necessary to clarify if and how SP acts in different parts of the SCN.

A.6.3 *SCN Efferents*

SP and NK-1 immunostaining was readily observed in many hypothalamic targets of SCN efferents. In the Pa, a coordinator of a wide variety of processes including osmotic control, arousal, temperature homeostasis, food intake, hormone release and

responses to stress (for review, see Simerly, 1995), SP and NK-1 receptor activation has been implicated in mediating many of the above (Harbuz and Lightman, 1992; Jessop et al., 2000; Larsen et al., 1993; Nussdorfer and Malendowicz, 1998). In the red-backed vole, with few exceptions, SP-ir is similar to that reported in the Pa of the rat (Larsen, 1992). For example, the PaMP showed moderate SP-ir staining for terminals and fibers and NK-1-ir a limited distribution of cells (Figs. A.2, A.9), comparable with the assessment of immunoreactivity of this peptide and its NK-1 receptor in rat.

The major hypothalamic target of SCN efferents is the SPVZ (Kalsbeek et al., 1993; Moore and Danchenko, 2002; Watts and Swanson, 1987). The rodent SPVZ is split into two dorsal and ventral areas. Based on selective lesions of the rat SPVZ, these separate areas have been proposed to exert differing effects on homeostatic mechanisms. The dorsal SPVZ controls body temperature regulation, while the ventral SPVZ cells predominantly control circadian rhythms of sleep and locomotor activity (Lu et al., 2001). The SPVZ also projects to the dorsomedial hypothalamus, implicated in the circadian regulation of feeding, drinking and corticosterone release (2, Elmquist et al., 1998; Kalsbeek et al., 1996). The presence of SP-ir in this region suggests that SP can regulate output from the SCN, but since the distribution of NK-1-ir is not as dense as the SP (Fig. A.2), this putative modulation may occur via other neurokinin receptors.

In rat, the Hb is implicated in the control of rapid eye movement (REM) sleep and receives a direct projection from the SCN (Buijs, 1978). Recently, Zhao and Rusak, 2005 found many similarities in the electrical activity of the LHb and SCN in rat. For example, electrophysiological responses to light in vivo were generally greater at night and a

sustained a firing rate rhythm in vitro in the absence of a rhythmic input, with peaks in activity seen during the subjective day. These researchers also noted differences in neuronal activity between LHb and MHb nuclei. The pattern of SP immunostaining seen in the red-backed vole Hb (Fig. A.4) is similar to that seen in rat (Battaglia et al., 1992) and the uneven distribution of this peptide, and its NK-1 receptor reflects the differences in the electrophysiological activities in nuclei of the LHb and MHb. The lateral border of the MHb showed deeply stained terminals and fibers immunopositive for SP, while NK-1-ir was found only in the MHb (Fig. A.4), congregated about the border with the dorsal third ventricle. The mismatch suggests that neurokinin receptors other than NK-1 mediate the actions of SP in this region. Consistent with this idea, electrophysiological studies in the MHb of rats (Norris et al., 1993) found agonists to both neurokinin NK-1 and NK-3 excited a subpopulation of neurons.

A.6.4 *SCN Afferents*

In the vicinity of the visual thalamus, immunoreactivities for SP and NK-1 receptor were almost exclusively confined to the IGL (Fig. A.6) and adjoining structures such as the ZI. Immunohistochemical studies in other rodents including rat, *M. auratus*, *P. sungorus* and *M. domesticus* (Mick et al., 1995; Nakaya et al., 1994; Piggins et al., 2001), reported a similar distribution of SP-NK-1-ir within the IGL. The source of the SP innervation to the IGL is as yet unclear. The IGL is known to receive inputs from areas such as the superior colliculus (Pasquier and Villar, 1982; Takatsuji et al. 1990) and retina (Hickey and Spear, 1976, Speh and Moore, 1993), but it is unlikely that these

structures are the source of the SP, since lesioning these structures in rat has no significant effect on the SP-ir staining in the IGL (Hartwich et al., 1994; Miguel-Hidalgo, 1991).

Neurons expressing SP-ir were not seen within the IGL, indicating that it is improbable that the SP-ir arises from the IGL, but as none of the test animals were treated with colchicine, the possibility of SP-immunopositive soma being present cannot be ruled out. A previous study, using rats pre-treated with colchicine, found SP-ir cell bodies in the IGL (Takatsuji and Tohyama, 1989). However, other groups have also failed to find immunopositive soma in both colchicine treated and untreated animals (Moore and Card, 1994; Piggins et al., 2001).

The intensity of NK-1 staining in the IGL was light in comparison to that of SP. There was little to no staining in the dLGN and very few fibrous projections from neurons in the IGL towards the vLGN were observed. These anatomical findings are comparable to NK-1 studies in rats (Maeno, 1993; Mick et al., 1994; Mick et al., 1995; Nakaya et al., 1994; Piggins et al., 2001) and indicate that within the rodent visual thalamus, NK-1 receptor expression is confined to the IGL.

A pronounced mismatch in the distribution of SP-ir and NK-1-ir in some mid-line thalamic structures was observed. For example, the PVT, a region with inputs from the SCN and is implicated in sleep/wake function and integration of stress responses (Otake et al., 2002; Otake, 2005), was highly immunopositive for SP, but only sparsely reactive for the receptor NK-1 (Fig. A.4). Receptor mechanisms other than NK-1 may regulate SP's actions in this part of the thalamus. In rat PVT, the source of the SP-ir innervation is

from cells in the medullary raphe nucleus, the mesopontine tegmentum, and the EW (Otake, 2005). The EW is implicated in pupil dilation and oculomotor functioning (Klooster and Vrensen, 1998; Morin and Blanchard, 2005). The EW, medullary raphe and mesopontine tegmentum showed very little NK-1-ir staining in this study.

Other areas exhibiting both SP and NK-1-ir include the SPF, PP and IPN (Figs. A.4-A.7); areas thought to be associated with regulating sexual behavior and relaying sensory information to the hypothalamus (Coolen et al., 1996; Wedemeyer et al., 1999). The SPFm, for example projects to the IGL and could conceivably play a role in non-photic resetting of the circadian clock, (Vrang et al., 2003). The SPFp is further split into lateral and medial divisions. In rat, the lateral SPFp is involved with relaying auditory and nociceptive information associated with conditioned fear responses, while the medial SPFp with integrating information relating to copulation (Coolen et al., 2003a; Coolen et al., 2003b;LeDoux et al., 1987). The similar pattern of SP-NK-1-ir in the SPF across rodent species suggests a conservation of function that requires functional investigation.

The exact function of the IPN is unclear, but as it has efferent connections with structures such as the Hb and raphe nuclei, a role in the modulation of mood is suggested (Sharp et al., 2006). The rat IPN contains a distinct subpopulation of SP-containing cells that co-localize with acetylcholinesterase (Hemmendinger and Moore, 1984), raising the possibility that SP modulates cholinergic activity within the IPN. Considerable work is required to determine the precise role of SP-NK-1 signaling in these structures of the rodent CNS.

Immunopositive staining for SP was found in the DR and MR nuclei (Fig. A.10). In mammals, the raphe nuclei are a major source of most of the serotonergic projections to the forebrain (Vertes et al., 1999; Vertes, 1991) and are associated with regulation of mood and stress (Kramer et al., 1998). It is well established that SP colocalizes with serotonin-containing neurons in the rat raphe nuclei (Hökfelt et al., 1978), and this information has led to NK-1 receptor antagonists being used to treat anxiety and depression (Santarelli et al., 2001; Valentino et al., 2003). Efferents from the LHb form a major projection to the DR and can inhibit neuronal activity. Serotonergic projections from the DR can modulate stress responses from areas of the forebrain sending signals to the LHb. Areas of the forebrain showing immunoreactivity for serotonin and also SP, such as the hypothalamus (Table 1) project to the LHb and complete a feedback loop which regulates mood and anxiety using NK-1 receptors (Conley et al., 2002). The DR and MR nuclei have afferent connections to the IGL and SCN respectively, and are structures within the circadian system associated with arousal and sleep behavior (Marchant and Morin, 1999).

In summary, the distribution of SP and NK-1 receptor immunoreactivities in the *C. rutilus* hypothalamus, thalamus, midbrain and brainstem is in general agreement with that seen in other rodents. However, in the SCN, SP-ir is very different, raising the possibility that SP has a unique role in circadian timekeeping in this species. Further studies utilizing autoradiography as well as electrophysiology and behavioral investigations are required to establish the functional role of SP-NK-1 signaling in the circadian system of the northern red-backed vole.

A.7 Acknowledgements

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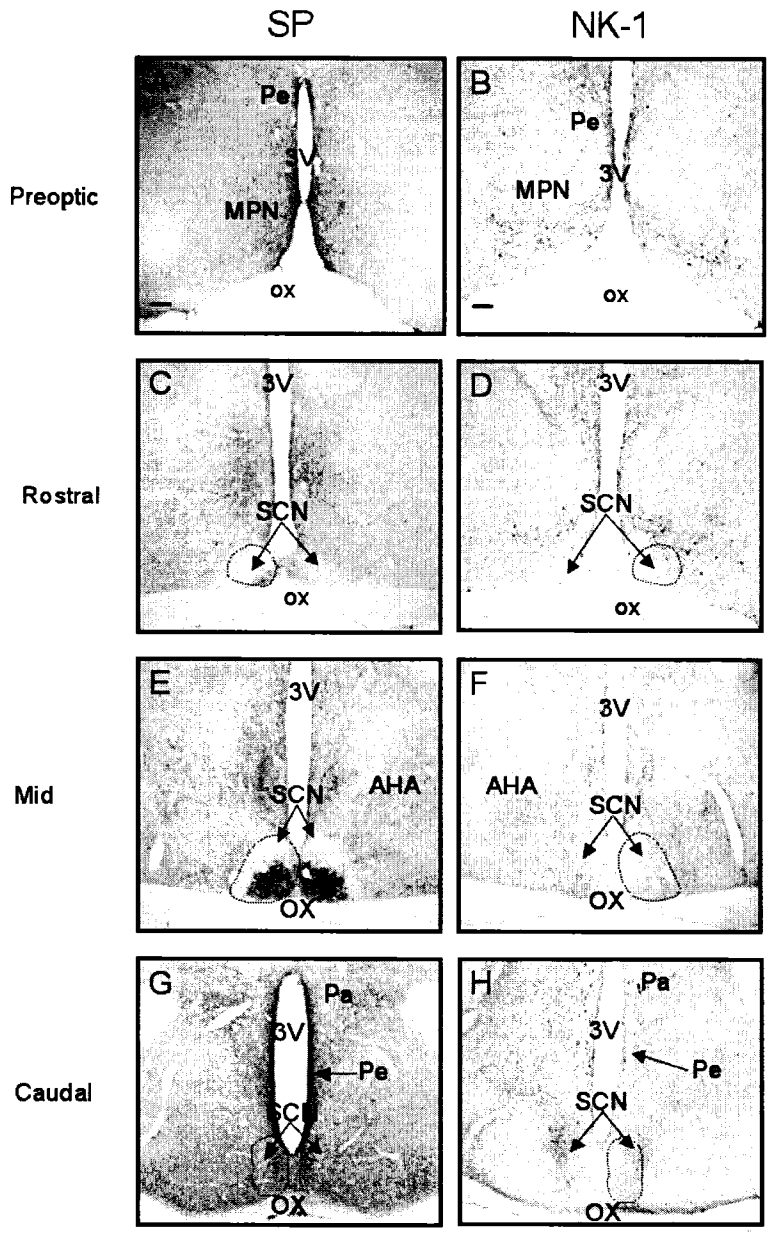

Figure A.1 Low power photomicrographs showing the distribution of SP-ir (A, C, E, G) and NK-1-ir (B, D, F, H) throughout the suprachiasmatic nucleus (SCN). 3V = Third ventricle, Pe = periventricular nucleus, MPN = medial preoptic nucleus, AHA = anterior hypothalamic area, Pa = hypothalamic paraventricular nucleus, SPVZ = subparaventricular zone, ox = optic chiasm. Scale bar 100μm.

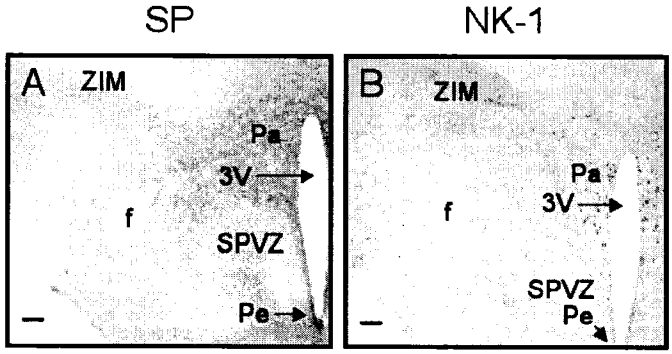

Figure A.2 Low power photomicrographs showing SP-ir (A) and NK-1-ir staining (B) around the area of the third ventricle (3V) at the anterior hypothalamic level. ZIM = Zona incerta medial, Pa = hypothalamic paraventricular nucleus, 3V = third ventricle, SPVZ = subparaventricular zone, Pe = periventricular nucleus, f = fornix. Scale bar 50μm.

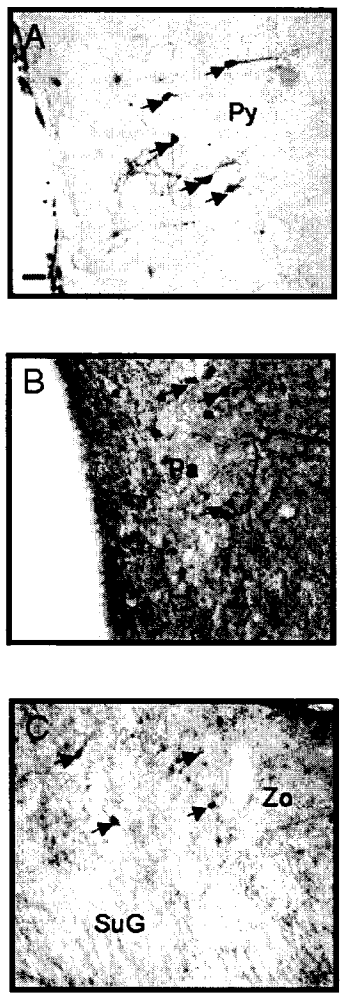

Figure A.3 High power photomicrographs showing SP-ir staining in the the hippocampus (A), paraventricular nucleus of the hypothalamus (B) and superior colliculus (C). Py = Pyramidal cell layer of the hippocampus, Pa = hypothalamic paraventricular nucleus, SuG = superficial grey layer of superior colliculus, Zo = zonal layer of superior colliculus. Scale bars 25μm.

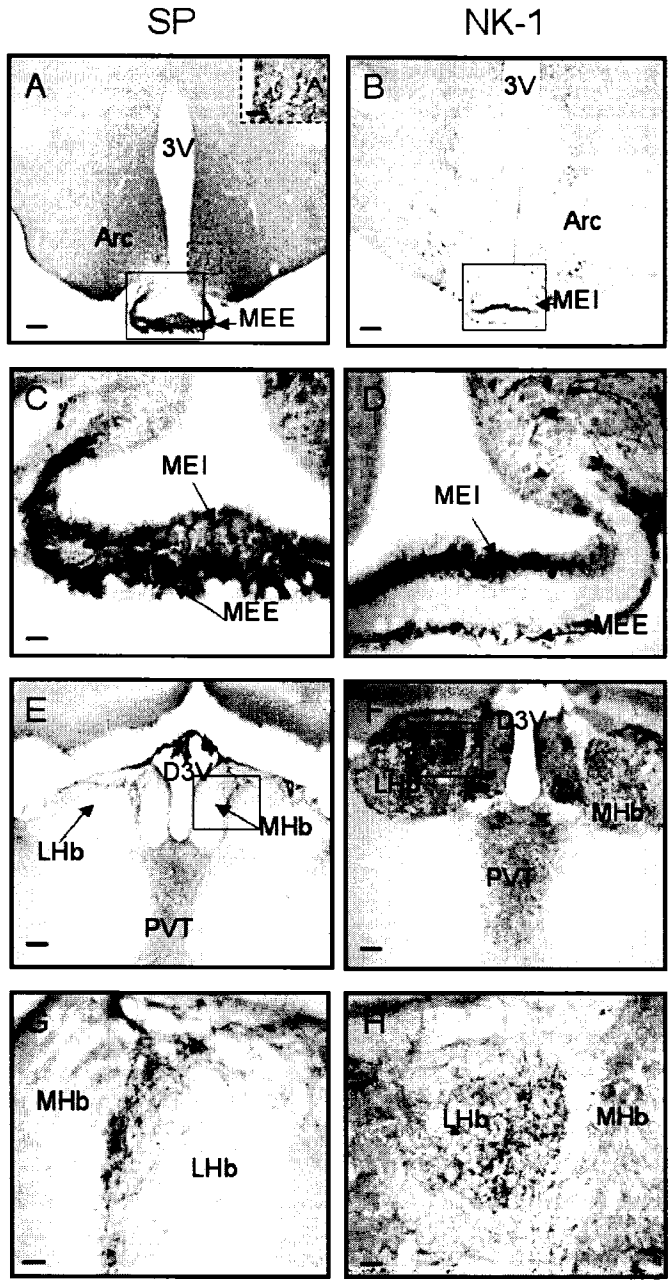

Figure A.4 Low and high power photomicrographs showing SP-ir (A, C) and NK-1-ir (B, D) staining in the arcuate nucleus (Arc) and SP (E, G), NK-1 (F, H) staining in the habenula (Hb). 3V = third ventricle, MEE = median eminence external layer, MEI = median eminence internal layer, D3V = dorsal third ventricle, LHb = lateral habenula nucleus, MHb = medial habenular nucleus, PVT = thalamic paraventricular nucleus. Scale bars 100μm (A, B, E, F); 25μm (C, D); 50μm (G, H).

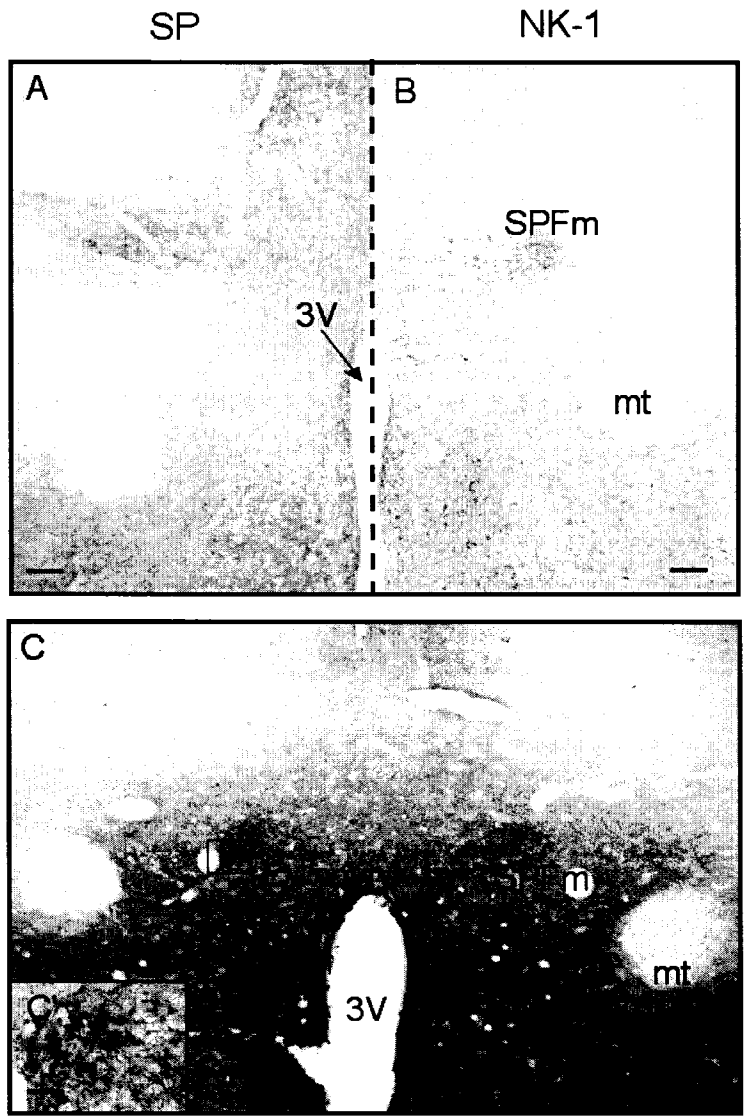

Figure A.5 Low and high power photomicrographs showing SP-ir staining (A), NK-1-ir staining (B) and SP/NK-1 double immunostaining (C) in the medial region of the subparafascicular nucleus (SPFm). mt = Mammillothalamic tract, 3V = third ventricle. Scale bars 100μm (A-C), 10μm (C').

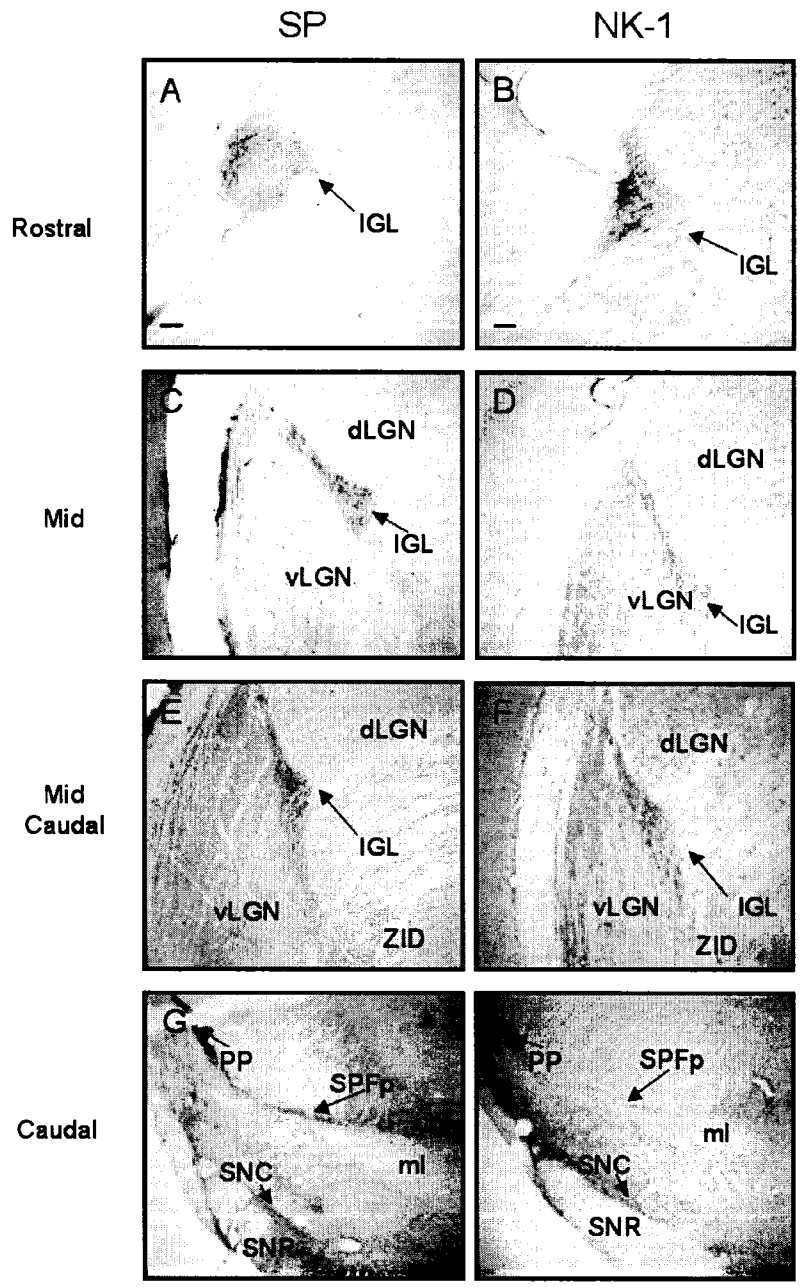

Figure A.6 Low power photomicrographs showing the distribution of SP-ir (A, C, E, G) and NK-1-ir (B, D, F, H) throughout the rostrocaudal extent of the intergeniculate leaflet (IGL). dLGN = dorsal lateral geniculate nucleus, vLGN = ventral lateral geniculate nucleus, ZID = zona incerta dorsal, PP = peripeduncular nucleus, SPFp = subparafascicular nucleus parvicellular region, SNC = substantia nigra pars compacta . Scale bar 100μm.

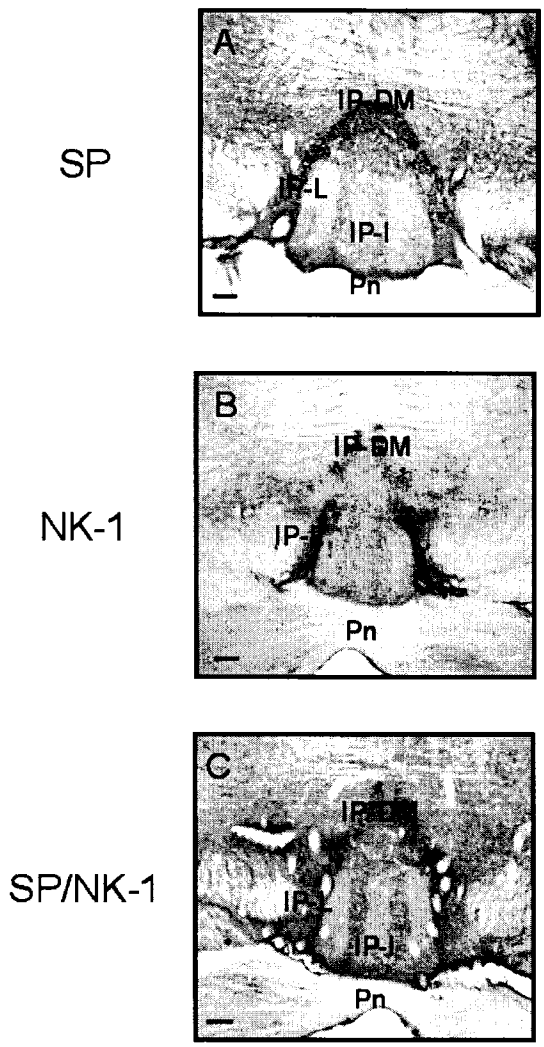

Figure A.7 Low power photomicrographs showing SP-ir (A) and NK-1-ir (B) and SP/NK-1-double immunostaining (C, D) in the interpeduncular nucleus of the midbrain. Double stained photomicrograph shows SP-ir stained purple with NiDAB and NK-1-ir labeled red with Vector NovaRed. ® IP-DM = interpeduncular dorsomedial nucleus, IP-L = interpeduncular lateral nucleus, IP-I = interpeduncular intermediate nucleus, Pn = pontine nucleus. Scale bars 50μm (A-C), 25μm (D).

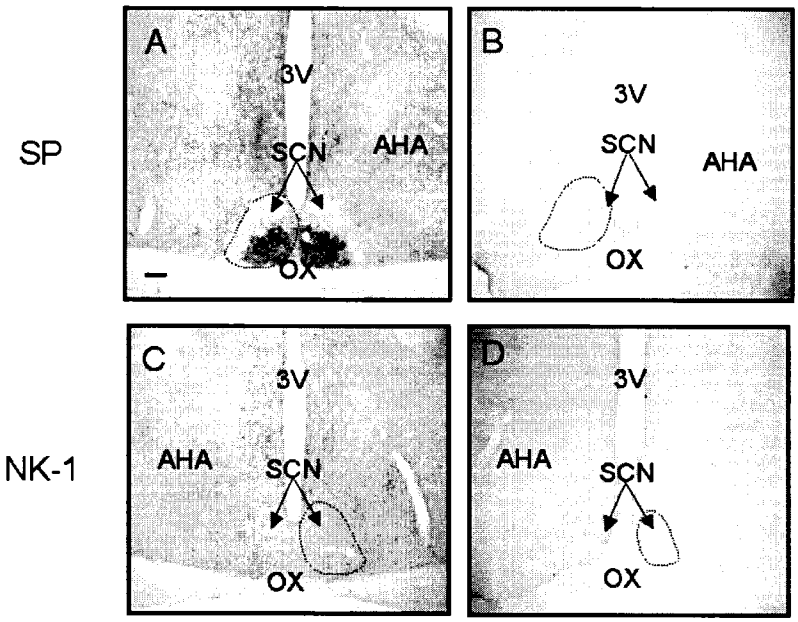

Figure A.8 Control preadsorption photomicrographs for SP-ir (A, B) and NK-1-ir (C, D). Suprachiasmatic nucleus (SCN) delineated by dotted line. 3V = Third ventricle, AHA = anterior hypothalamic area, ox = optic chiasm. Scale bars 100μm.

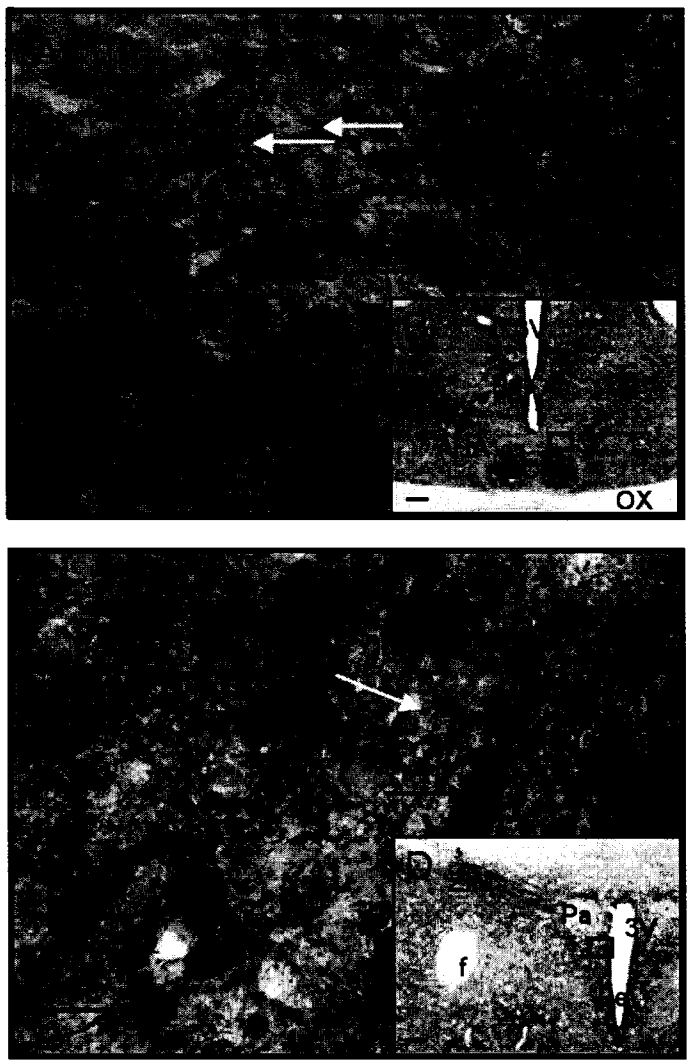

Figure A.9 Low and high power photomicrographs depicting SP/NK-1 double immunohistochemical staining in the SCN (A, B) and Pa (C, D). Black arrows indicate NK-1-ir neurons; white arrows indicate SP-ir terminals; blue arrowheads indicate an SP-ir terminal in close proximity to an NK-1-ir cell body. AHA = anterior hypothalamic area, 3V = third ventricle, ox = optic chiasm, SCN = suprachiasmatic nucleus, Pa = hypothalamic paraventricular nucleus, Pe = periventricular nucleus, ZIM = zona incerta medial. Scale bars 100μm (A, C); 10μm (B, D).

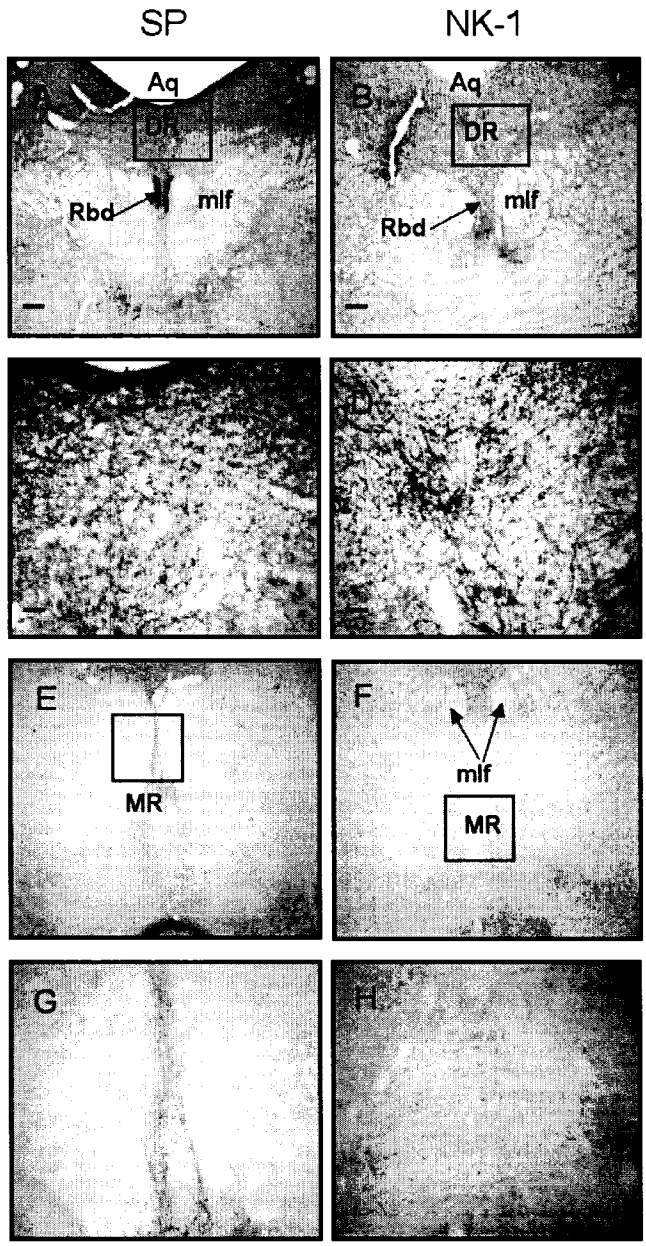

Figure A.10 Low power photomicrographs showing SP-ir staining (A, C, E, G) and NK-1-ir staining (B, D, F, H) in the dorsal raphe (A-D) and median raphe (E-H). Aq = aqueduct, DR = dorsal raphe, mlf = medial longitudinal fasciculus, MR = median raphe, Rbd = rhabdoid nuclei. Scale bars 100μm (A, B, E, F) 25μm (C, D, G, H).