AN *IN VITRO* ANALYSIS OF NEURONAL SURVIVAL IN RESPONSE TO HORMONES AND PHOTOPERIOD IN THE HVc OF THE SONGBIRD *JUNCO HYEMALIS*

By Catherine Humphries

RECOMMENDED:

Advisory Committee Chair

Department Chair, Chemistry and Biochemistry

APPROVED:

Interim Dean, College of Science, Engineering, and Mathematics

Dean of Graduate School

December 8, 2003

Date

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A

THESIS

Presented to the Faculty

Of the University of Alaska Fairbanks

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Catherine Martin Humphries, B.S.

Fairbanks, AK

December 2003

Abstract

The ability of songbirds to sing is essential for their survival, proper reproductive behavior, and territorial establishment. Male and female juvenile passerine songbirds learn their song through the formation of a song template in their earliest days of life, first by listening to their parents, and then followed by auditory feedback against their own templates to crystallize their individual songs. However, in most passerine species, only the adult males actually sing on a seasonal basis with little to no singing during winter, followed by a phase of song production in the spring in correlation with increased plasma testosterone concentration and extended photoperiods. While the production of new neurons in the song system of adult males is continuous throughout the year, a counterbalancing turnover of these neurons must exist until the spring, when a three- to four-fold decrease in dying HVc (hyperstriatum pars ventralis caudale or higher vocal center) neurons in males initiates song production.

We hypothesized that testosterone, under the influence of increased photoperiod, attenuates the rate of programmed cell death (apoptosis) of newly generated neurons migrating into the HVc song nucleus in the wild arctic songbird *Junco hyemalis*. Using an organotypic culture system, we examined the effect of testosterone and β-estradiol on the degree of apoptosis in the HVc obtained from photostimulated and non-photostimulated male and female juncos. We employed a TUNEL assay and BrdU-labeling to detect and quantify apoptosis. We found that hormonal treatment with testosterone, and β-estradiol in photostimulated birds only, extends the lifespan of cells within the HVc compared to controls, as shown by BrdU labeling, and decreasing apoptosis, as shown by TUNEL assay.

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Introduction

1.1 Songbird Physiology and Ecology

1.1.1 Systematics and Comparison of Oscines

Songbirds are in the Order Passeriformes, which are generally perching birds. The true songbirds are in the Suborder Oscine, and are characterized by the presence of an oscine syrinx. There are 25 families of oscines, which include wrens, sparrows, cardinals, redpoles, finches, and juncos. Juncos are in the Family Emberizidae, Genus Junco, Species *Junco hyemalis* and are found throughout North America. They are migrant birds with a photoperiod-dependent annual breeding cycle. In addition, they are known as "critical period learners," meaning that they learn their songs in a defined period in adolescence and do not modify it throughout adulthood (Gulledge and DeViche, 1995).

Most studies on development and production have been performed on canaries and zebra finches. Canaries are in the order Passeriformes, family Fringillidae, genus Serinus, species *Serinus Canaria,* and are found primarily on the Canary Islands. They are also seasonal breeders, but are "open-ended learners," meaning that they continuously modify their songs throughout adulthood (Nottebohm et al., 1986). Zebra finches are endemic to Australia, and are also in the Passeriformes order, but in the family Estrildidae, genus Taeniopygia Bichenovii, species *T.B. annulosa.* Zebra finches are

opportunistic breeders in response to rainfall (Crews, 1993), and are "critical period learners" (Bottjer et al., 1986). Therefore, to this point canaries have been used to study the seasonal responses of the song system, while zebra finches have been used to study the ontogeny of the song system and its components (Gulledge, 1997). Though all oscines have similar song nuclei in their brains, the learning patterns are very different in domesticated species compared to wild species. For instance, canaries and zebra finches both start practicing singing well before the end of the sensory phase (Nottebohm, 1993), whereas some wild species (White-crowned Sparrow) respect a winter long hiatus between song memorization and song production the following spring (Whaling et al., 1995). Furthermore, domesticated Canaries and Zebra Finches have been bred especially to enhance their singing ability, and therefore may not give an accurate indication of how the brain controls song learning and behavior in the wild.

Juncos also learn their song in the first summer they are born, but do not sing until their first breeding spring, lending to substantial separation in the learning and production periods that may lead to a better understanding of the physiology involved in song learning in general (Gulledge, 1997). In addition, Juncos are high latitude migratory breeders and are therefore an excellent prototype to study the effects of changing photoperiod. Even more so than lower 48 latitudes, northern latitudes provide distinct differences and challenges for a migratory bird to adapt to, including low temperatures and dramatic photoperiod fluctuations (Deviche, 1995). Thus, successful breeding in the arctic depends upon precise and specific timing of reproductive activities. This alone provides researchers with a very pronounced model of photoperiodism in song

production in contrast to a perhaps less dramatic reproductive phenomenon in subarctic and/or opportunistic breeders. Lastly, Juncos are abundant in Alaska, and are therefore logistically sensible birds to study.

1.1.2 Song vs. Speech

Bird song has intrigued scientists in its similarities to human speech. For instance, birds and humans must be able to hear and produce sounds (auditory feedback) to facilitate vocal learning (Marler, 1970b; Slater and Clayton, 1988; Konishi, 1965; Doupe and Solis, 1997). In contrast, most other animals do not have to hear the communicative signals that their own species uses in order to be able to reproduce them. Also, both birds and humans share very similar specialized forebrain motor and auditory nuclei in their brains that interact to control production of speech and song. Lastly, both humans and birds experience a change in their ability to learn over time, namely that it becomes harder for them to learn their song or language later in life (Doupe and Kuhl, 1999). Although there have been many studies that indicate that specific bird songs do not have the meaning that words in human language have, the study of bird song could potentially lend insight into how the language learning centers in the human brain act to control speech (Doupe and Kuhl, 1999).

1.1.3 Why Birds Sing

Although it may not seem apparent to the bird producing the song, birdsong is a quintessential behavior for the survivorship of wild oscine songbirds in general. Song is

simply a means of communication composed of the sustained and "quasi-rhythmic" sound units (Smith, 1996b). Both speech and song can be characterized as a highly ordered series of complex acoustic signals separated by brief silent intervals. Though in most passerines only the males sing, there are a few exceptions in which the females also sing, such as the Northern Cardinal and the White-throated Sparrow. The song itself has been recognized as an evolutionarily favorable adaptation for singing species such as birds and insects. For instance, it is a means of communication used to disclose the singer's location, sound alarm calls, courtship songs, and define territorial boundaries. Interestingly, while many mammals depend upon their olfactory senses to find prey, forage, or mate, birds, in contrast, depend upon their hearing, and in turn, their vocalization abilities for these purposes, thereby making use of this important niche (Hartshorn, 1973). Moreover, a further advantage of song behavior is that prey species (insects) and other food sources of songbirds (seeds) typically cannot hear, and are therefore unaffected by their predators' announcements.

As birds and other singing insects are small, their song is their sole means of providing for themselves spatial (and therefore genetic) isolation, or territorial establishment. It is interesting to note that birds typically do not mind hearing other birds in the distance, as long as they remain beyond specific boundary lines. There is reason to think that birds appreciate and recognize their singing neighbor's songs. Playing an individually different recorded birdsong typically ensues much excitement and agitation for the territorial bird, even though the song is coming from outside his territory zone (Hartshorn, 1973). Finally, it has been noted by scientists throughout time and especially

since the acceptance of Darwin's evolutionary theory that some songs are more aesthetically complex than simple evolutionary utility would deem necessary. Though a bird's idea of pleasure is unknown to humans, all of their behaviors may be a means to some utilitarian end. Therefore, it is logical to believe that animals must find their sole and chief pleasures in life in their essential activities. And so, in addition to the more practical reasons of mate-pairing and territorial establishment, birds may also sing because they like to.

1.1.4 Learning to Sing

Song learning and production in birds occurs in two stages known as the 'sensory' phase and the 'sensorimotor' phase, the timing of which differs among many species (Nottebohm, 1993). In the 'sensory' phase, both male and female juvenile birds listen to the sounds of the adult songs to which they are exposed and consequently form a song template in the telencephalic song centers of their brains (Marler, 1970a; Slater and Clayton, 1988). Deafening or acoustic isolation of the juvenile bird before song memorization results in an abnormal song in adulthood (Konishi, 1965). In the 'sensorimotor' phase, commonly referred to as song crystallization, birds gradually refine their cacophonous vocalizations using auditory feedback against themselves until their song matches the template song with some distinctions of their own unique song added (Konishi, 1965). This phase is often compared to the babbling phase a baby goes through in human speech development. As in the sensory phase, deafening prior to song crystallization inhibits the development of song into a recognizable melody, indicating

that the memory of a song serves as a permanent template for the reproduction of that song (Konishi, 1965). In addition, when adult passerines are deafened, their song quickly deteriorates until it is no longer recognizable, thereby emphasizing the necessity of continuous auditory feedback for 'proper' song production (Nottebohm et al., 1976; Price, 1979; Nordeen and Nordeen, 1992). Specific regions of the telencephalic vocal control system are essential in the learning, memorization, and production of song. Also, it appears that there are crucial periods when hormones are necessary, as well as periods when hormones are harmful to the learning process. For instance, although elevated testosterone levels typically increase song production and trigger the growth of central and peripheral nuclei (vocal control regions in the brain and syringeal musculature in throat) in the adult, administering testosterone prior to song crystallization inhibits singing behavior in captive yearling dark-eyed juncos (Titus et al., 1997). Hormone regulation of song learning and production has been an area of extensive study among researchers for over thirty years.

1.1.5 Song Centers in the Brain

The song control nuclei of the oscine brain have been grouped into two distinct pathways: the motor pathway and the anterior forebrain pathway. The motor pathway controls song production and includes the hyperstriatum pars ventralis caudale (HVc), which projects to the robust nucleus of the archistriatum (RA). Projections then lead from the RA to the dorso-medial subdivision of the intercollicular nucleus (DM) and on to the neuromuscular centers including the syrinx, avian vocal organs, and respiratory

motor neurons (Gulledge, 1997; Nottebohm et al., 1976; Vicario, 1991). The anterior forebrain pathway is directly associated with song learning and also includes the HVc, which projects to Area X (X), then to the dorso-lateral division of the medial thalamus (DLM), the lateral magnocellular nucleus of the anterior neostriatum (LMAN), and back to the RA (Nottebohm et al., 1976; Bottjer et al., 1989; Okuhata and Saito, 1987). The medial nucleus magnocelularis of the anterior neostriatum (mMAN) projects to the HVc, but its function is not largely understood. The subventricular zone (SVZ) overlays the HVc and is the site from which stem cells divide and migrate out into the telencephalon.

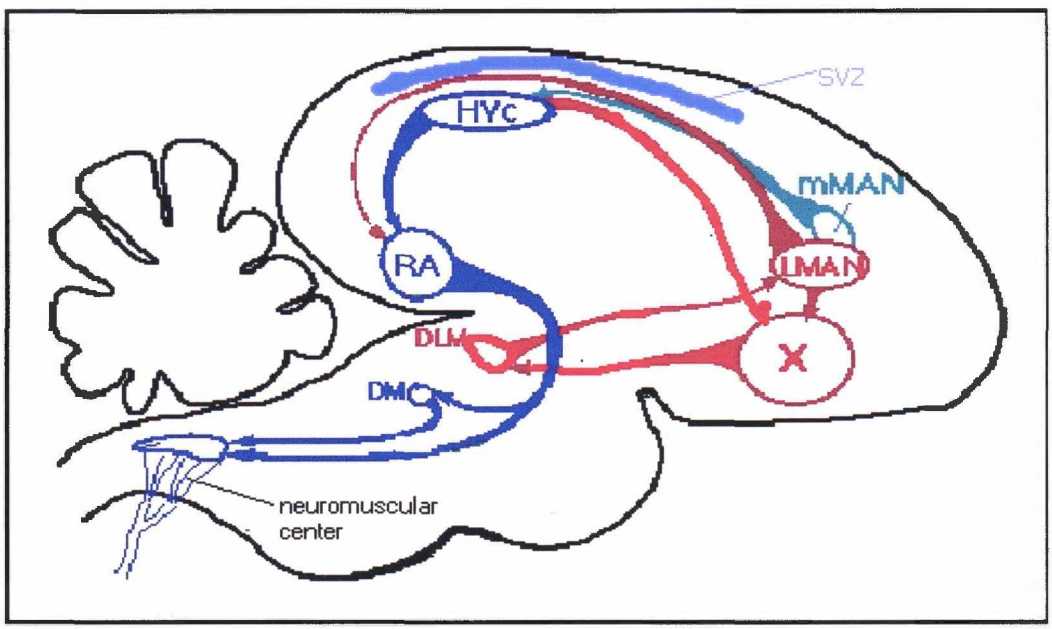

Figure 1.1 Schematic view of the song circuitry of the male songbird brain. Blue lines indicate the production pathway; red lines indicate the learning pathway.

The motor pathway must be intact in both adults and juveniles for a normal song to be produced; however, interruptions in the adult anterior forebrain (AF) (red) pathway do not affect song production (Nottebohm et al., 1976; Morrison and Nottebohm, 1993) (Figure 1.1.1). On the contrary, lesion of the AF pathway in juveniles causes abnormal song production, indicating that these nuclei aid primarily in song learning (Bottjer et al., 1984; Nottebohm et al., 1990; Scharff and Nottebohm, 1991). In addition to these forebrain nuclei being responsible for song learning and production, some are also involved in song recognition. Lesions of either or both the HVc and LMAN disrupt the song recognition abilities of female canaries (Brenowitz et al., 1991; Burt et al., 1997). Moreover, both juvenile and adult HVc, Area X, LMAN, and RA contain sensory neurons that respond to auditory stimuli; and in adults only, the responses are often selective for the bird's own song (McCasland and Konishi, 1981; Doupe and Konishi, 1991; Margoliash and Fortune, 1992; Margoliash et al., 1994; Lewicki and Konishi, 1995). Although these nuclei have specific functions, they remain dynamic throughout the birds' life.

1.1.6 Song Control Nuclei Change Seasonally

The brain nuclei that control song behavior in seasonally breeding adult songbirds are plastic. A subset of these brain regions grow in volume and cell number every spring in anticipation of reproductive activities (Nottebohm et al., 1986; Smith et al., 1997a; Brenowitz et al., 1998). The most dramatic growth is seen in the HVc with subsequent, but less pronounced, growth in the RA and Area X in some species after the HVc has

reached its full capacity. The LMAN does not increase in volume (Smith et al., 1997a; Smith, 1996a; Brenowitz et al., 1998).

Accompanying the increase in cell number in the HVc song nucleus are new neuronal projections from the HVc to the RA, which appears to directly initiate the singing behavior (Holloway and Clayton, 2001). The formation of these new, long distance connections is unusual in adult higher vertebrates, especially in the presence of myelin-producing oligodendrocytes. The degree of volume change in spring differs among species. Whereas the HVc nearly triples in Spotted Towhees and doubles in White-crowned Sparrows, only a 1.6 times increase was determined for the Dark Eyed Juncos (Tramontin and Brenowitz, 2000). Similarly, Nottebohm found that the HVc in male canaries was twice as large in both HVc volume (1981) and cell number (1987) in the spring compared to the autumn when photoperiod lengths are comparable (Nottebohm et al., 1981; Nottebohm et al., 1987). Though some wild oscine songbirds sing only one song and others have large repertoires of songs they sing, the sizes of the HVc and RA do not correspond with the size of their song repertoire (Bernard et al., 1996). The HVc and RA volumes of European Starlings do positively correspond with song bout length when compared to juvenile nuclei volumes and song bout length (Bernard et al., 1996). The seasonal volumetric increase of song nuclei is thought to be induced by a consortium of environmental and physiological signals. Increased photoperiod, increased levels of plasma testosterone, and social cues from sexually receptive females are all thought to contribute.

1.1.7 Photoperiodic Effects

Avian physiology is contingent upon the changing photoperiod for appropriate timing of seasonal events, including singing, breeding, and molting. Photoperiod clearly influences the receptiveness of avian song nuclei to gonadal hormones. Vernal photoperiods stimulate the production of gonadotropin-releasing hormone (GnRH), which in turn initiates gonadal maturation and increases in gonadal hormones (Dawson et al., 2001). However, extended exposure of songbirds to long photoperiods beyond 8 weeks results in a state of photorefractoriness (Bernard and Ball, 1997). Male juncos are the first to arrive on summer breeding grounds, and are singing from late May, when testosterone levels are high and before females arrive, until mid-late July, after fledglings flee the nest and testosterone levels decline, but while the photoperiod is still long. Thus, photoperiod appears to be the governing signal for the initial physiological changes associated with spring. It is currently unknown whether male Juncos start singing before, during, or after they migrate to summer breeding grounds. However, we do know that long-day exposure treatment to castrated male Juncos causes an increase in the size of the HVc that is not additionally increased when given testosterone treatments (Dloniak and Deviche, 2001). In addition, long days increased the volume of the HVc in castrated White-crowned Sparrows (Greenlee et al. 1997) and the volume of the HVc, RA, and Area X in castrated American Tree Sparrows (Bernard et al., 1997). Although the size of the HVc increased, castrated birds did not sing until testosterone was administered. In fact, in this same experiment, testosterone administration to photorefractory, photosensitive, or photostimulated juncos induced singing in all three groups, and caused

an increase in HVc size in the photosensitive males. In another study on European Starlings by Bernard and Ball (1997), HVc volumes were larger in T-implanted photosensitive castrates than in both T-implanted photorefractory castrates and control photosensitive males, indicating that increased photoperiod renders the song control center more sensitive to the effects of testosterone (Bernard and Ball, 1997). Curiously, although females do not sing in the wild, administration of testosterone to female juncos induces singing (Gulledge and Deviche, 1998). This finding suggests that female brains stay primed to sing, even after the sensory and sensorimotor phases of song learning, yet lack the signals to sing as adults. In conjunction with these findings, both photorefractory and photostimulated castrated male juncos resume singing when given testosterone implants. Therefore, it appears that photoperiod and testosterone are concomitantly dictating the brain signals necessary for production of song.

1.2 Hormonal Effects

1.2.1 Receptors

The location of gonadal hormone receptors can give great insight into the action sites and role of sex hormones in the song system. RA-projecting HVC cells contain either androgen or estrogen receptors (Arnold et al., 1976; Gahr, 1990; Balthazart et al., 1992; Johnson and Bottjer, 1995;Metzdorf et al., 1999). The quantity of the androgen-receptor positive cells is notably greater in the spring when the HVc is larger in size (Soma et al., 1999a). The HVc contains cells with intracellular androgen receptors, indicating that androgens may influence gene expression of the HVc cells (Smith et al.,

1996; Johnson and Bottjer, 1995). Estrogen has two receptors, ERα and ERβ. It has been suggested by Kuiper et al. (1998) that ERα is important for reproductive activities while ERß is more active in non-reproductive events (Kuiper et al., 1998). Ball et al. (1999) studied the localization of these two receptor types in the brains of the Japanese Quail (*Coturnix japonica*) and the European Starling (*Sturnus vulgaris*) and found that while ERß was detected in telencephalic areas consistent with a role for cognitive function, as well as areas believed to be involved in reproductive function (preoptic nucleus, stria terminalis, and nucleus taeniae), only ERα was detected in the HVc (Ball et al., 1999).

Another study by Bernard et al. (1999) further defined the location of ERα to be confined to the paraHVc (pHVc), an area lying medial to the HVc that primarily contains X-projecting neurons, and an overlapping expression of ERß and aromatase-producing cells in the NCM (Bernard et al., 1999). Aromatase is an enzyme known to convert testosterone into estradiol. Aromatase receptor mRNA has not been detected in the HVc, although aromatase itself has been detected in the HVC (Silverin et al., 2000). ERs are present, but not co-expressed in the HVc with aromatase receptors. However, aromatase and androgen receptors are co-expressed in the NCM, which lies adjacent to the HVc, while no ERs have been detected in the NCM (Metzdorf et al., 1999). One possible explanation for this separation of steroid receptors is that estrogen in the HVc is not resulting from a conversion of testosterone by aromatase, but travels to the HVc from the NCM in response to increased testosterone levels. Notably, the canary and house

sparrow have a much higher degree of ERs in the HVc than the zebra finch (Gahr et al., 1993).

1.2.2 Testosterone

Although photoperiod strongly influences size fluctuations of vocal control nuclei in the avian brain, it does not act alone. Gonadal hormones play a pivotal role in the migration and survival of neuronal precursors within the HVC and are the primary messengers ultimately coordinating song production. In several species, singing is greatly reduced or even eliminated in males by castration, and subsequent treatment with testosterone restores the behavior (Arnold, 1975; Heid, Guttinger, and Prove, 1985). Convincingly, the presence of testosterone is essential for any significant increase in song production and volumetric increase in the male HVc, but there is a discrepancy in the literature. Kirn et al. (1994) showed that peaks in cell number occur as testosterone levels rise, dramatic decreases in cell number occur at periods of low testosterone levels, and systemic testosterone administration to adult female canaries results in a significant increase in the number of newly labeled HVC neurons (Kirn et al., 1994).

Conversely, a study by Johnson and Bottjer (1993) showed that the administration of testosterone to castrated male canaries resulted in increased HVc cell density and size, increased HVc volume, and a higher percentage of androgen target cells in the HVc. It did *not* influence the overall number of cells in the HVc (Johnson and Bottjer, 1993). In that study testosterone treatment altered the distribution of RA-projection neurons and androgen target cells (Johnson and Bottjer, 1993). While it remains to be elucidated how

testosterone increases the number of cells in the HVc, it has been shown that cell death in the HVc is highest at times of the year subsequent to decreasing plasma testosterone levels (Alvarez-Buylla and Kirn, 1997). Thus, testosterone appears to provide the cellular support needed for the survival and, more importantly, the functional incorporation of neurons into the HVC. **The hypothesis that testosterone decreases apoptosis in the avian song system is the basis of my research**. There are a number of scenarios in which testosterone could affect cell survival: (1) testosterone is itself a survival signal, (2) testosterone renders the brain responsive to other survival molecules, such as growth factors, (3) testosterone is converted to estrogen, which then acts to promote cell survival, or (4) testosterone initiates an increase in the number of stem cells proliferating. To add to the complexity of this system, it has been suggested that the avian brain may be producing its own testosterone supply, unrelated to plasma testosterone concentrations. However, there is no published data supporting this hypothesis as of yet.

Photoperiod appears to influence the production of testosterone and/or androgen receptors; however, it does not affect the brain's sensitivity to the hormone. Treatment of photostimulated or photorefractory wild male song sparrows with testosterone induces song production. However, photostimulated birds sing more frequently when exposed to long days than short days (Nowicki and Ball, 1989). In castrated male Gambel's white-crowned sparrows, treatment with testosterone increased the size of several song nuclei, regardless of photoperiod (Smith et al., 1997b). Furthermore, females and castrated males do not sing; but, when given testosterone treatments *in vivo* via silastic implants, both the castrates and the females begin singing within two weeks, regardless of the

photocycle length they are maintained on (Gulledge and Deviche, 1997; Hegner and Wingfield, 1987; Nottebohm, 1980; Nowicki and Ball, 1989; Ketterson et al., 1996).

While both males and females will sing under the influence of testosterone, hormone sensitivity does appear to be sexually dimorphic in critical period learners. In adult zebra finch males, song control nuclei contain a much larger proportion of androgen-containing cells than do females (Arnold and Saltiel, 1979). Conversely, male and female canaries (opportunistic learners) appear to be equally predisposed to masculinization of their song system via hormones. They show remarkable plasticity in their response to varied hormonal states (Bottjer and Maier, 1991). Adult female canaries have proportionately the same number of hormone target cells than males or T-treated females do, however, males typically have more neurons in the HVc than normal females. Administration of testosterone to adult females produces an increased number of HVc neurons (Bottjer and Maier, 1991). In a study by Brenowitz and Arnold (1992), when testosterone was administered to male and female canaries it was found that both sexes have comparable proportions of T-labeled cells in the HVc, lMAN, RA, and the hypoglossal motor nucleus (nXII). Labeled cells were larger in size than unlabeled cells (Brenowitz and Arnold, 1992). It can therefore be deduced that males have more hormone-sensitive cells than females, owing to the fact that males have larger HVc and RA than females.

Increased levels of testosterone produce a marked volume and cell number increase within the HVc. It appears that testosterone affects the survival rate of neurons in the HVc that are constantly dividing and migrating from the overlying lateral ventricle.

Testosterone may decrease the removal of continuously incorporating neurons in the HVc by diminishing the occurrence of apoptosis, which could be mediated by brain-derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family that affects the survival of several types of neurons in development and adulthood (Kirschenbaum and Goldman, 1995; Rasika et al., 1999). BDNF and its receptor, TrkB, are both present in the avian brain in several areas throughout the brain, including the HVc (Rasika et al., 1999). The same study showed that infusion of BDNF *in vivo* into adult female canaries increases the survival of new neurons in the HVc, and the administration of testosterone increases BDNF levels in the HVc. In addition, treatment with αBDNF, a neutralizing antibody that competitively binds to TrkB inhibited growth of the HVc in response to testosterone. This suggested that testosterone and BDNF act on the same population of new neurons and that testosterone up-regulates the expression of BDNF. The effect of testosterone on BDNF expression could be direct or indirect results, such as metabolism of testosterone, or a testosterone-induced increase in singing. It has been shown that singing alone increases BDNF expression in the HVc (Li et al., 1997). BDNF also increases the number of androgen receptors in perineal motor neurons in mammals, and may do the same in the avian HVc (Al-Shamma and Arnold, 1997).

Several studies have disclosed the locations of androgen-metabolizing enzymes in the brain in an attempt to clarify the timing and action-site of the androgen metabolites. The enzyme 5α-reductase is known to convert testosterone to 5α-dihydrotestosterone (5α-DHT) and 5β-reductase deactivates testosterone by conversion into 5β-DHT, an inactive steroid. Aromatase is an enzyme known to convert testosterone to estradiol. 5α-

reductase and 5β-reductase, in addition to aromatase, have been detected in the telencephalon song region and show region-specific changes throughout the breeding season (Soma et al., 1999b). In this study, enzyme activation and deactivation was shown to fluctuate from courtship to incubation in non-song system areas of the brain related to territorial aggression, such as the hypothalamus. However, while all three enzymes were detected in the region of telencephalon containing the HVc, the activities of the enzymes did not change at different stages throughout the breeding season, (Soma et al., 1999b). This supports the idea that testosterone levels and HVc size do not decline until after fledgling, and also suggests that early hormone levels, initially preparing the brain to be able to sing, do not change after the reintroduction of song. Interestingly, no conclusive sex differences have been detected in forebrain levels of steroid-metabolizing enzymes (Schlinger and Arnold, 1992; Wade et al., 1995). In addition, DHT binding sites have been detected in the HVc, LMAN, and RA (Nordeen et al., 1986; Gahr, 1990; Brenowitz and Arnold, 1990) although DHT does not contribute to the masculinization of the song control nuclei (Jacobs et al., 1995).

As previously mentioned, most song system studies have been performed on zebra finches and canaries. These two species differ in their response to hormone treatments, presumably because of their opposing learning styles, i.e. critical period versus opportunistic learners. Female zebra finch chicks (critical period) treated with estradiol in the first post-hatch week followed by subsequent testosterone exposure in adulthood show pronounced growth of their song control nuclei and begin to sing (Gurney and Konishi, 1980). Conversely, treatment of normal zebra finch adult females

with testosterone does not induce any changes in song system morphology or behavior (Arnold, 1980). However, testosterone treatment of adult female canaries (opportunistic) quickly induces growth of song-control nuclei and also causes them to produce male-like song (Nottebohm, 1980).

1.2.3 Estrogen

While testosterone is clearly essential for the initiation and maintenance of song in males, estrogen, its metabolite, remains a controversial player in the avian song system of both males and females. Presumably, estrogen is the more operative hormone in the recruitment and differentiation of neurons in the song system during early song system development (Arnold, 1996). Additionally, estrogen receptors have been detected in the HVc of canaries in migrating and established neurons in the medial portion of the HVc near the NCM and along the lateral ventricle, from which stem cells divide and migrate out (Hidalgo et al., 1995; Gahr, 1990).

However, in zebra finches, very few HVc cells exhibit E binding or ER immunoreactivity, except for a group of ER cells that connects the lateral ventricle to the medial portion of the HVc (Gahr et al., 1993; Gahr and Konishi, 1988). In a study by Holloway and Clayton (2000), estrogen was found to be necessary for the completion of the HVc-RA song production pathway during development among captive male zebra finches (Holloway and Clayton, 2001). Interestingly, in the same study, co-culture of juvenile female brain slices (including HVc-RA pathway) with juvenile male slices led to increased male-like development in the female slices, meaning that there were more cells

in the masculinized brain than the feminized brain. Thus, although females contain the same proportion and type of hormone receptors in their song systems as males do, they lack the increase in plasma testosterone levels that males have, indicating that testosterone is mediating song production, either directly or indirectly.

Estrogen appears to regulate sexual dimorphism in developing juvenile song nuclei by promoting the neuronal differentiation and migration into the HVc. It has been rather difficult to block steroid synthesis and action in male songbirds in a way that prevents the masculinization of the brain, and females that have large amounts of testicular tissue have still developed masculine brains. It has therefore been proposed that testicular secretions are not the primary messengers in the masculine differentiation of the neural circuits of the brain. To be clear, sexual dimorphism or masculinization of the avian brain refers to males having larger song regions that contain more neurons, larger soma, and a greater proportion of androgen-concentrating neurons than females (Gurney, 1981; Arnold, 1980), in addition to exhibiting a pronounced difference in the structure of neural pathways within the song nuclei (Nottebohm and Arnold, 1976). It has been reported that in juvenile zebra finches no differences were apparent in the absolute number of apoptotic cells, although male and E-treated females had more neurons in the HVc and developed masculinized brains (Burek et al., 1997). Keeping in mind that ER positive cells reside in the HVc in the area adjacent to the proliferative ventricular zone, this same study also established no sex difference in the density of pyknotic cells in ER-positive cells in this region, suggesting that they do not promote

differential survival of migrating HVc neuronal precursors, and instead, may establish differences in HVc neuron number by influencing cellular differentiation.

To further support the belief that sexual dimorphism in regard to neuronal addition in the HVc is hormonally influenced, it has been shown that prior to post-hatch day 20 during sexual differentiation females have a greater absolute number of pyknotic cells in the HVc compared to males. However, the HVc subsequently loses more neurons in females compared to males (Burek et al., 1993; Kirn and DeVoogd, 1989; Burek et al., 1997). Thus, it appears the estrogen's role in the masculinization of the avian brain is in neuronal recruitment from the ventricular zone into the HVc, and it makes sense that in adults the same phenomenon would hold true. Furthermore, the absolute number of dying cells within the HVc is no longer sexually dimorphic after post hatch day 30, although there continues to be a difference in neuronal addition to this region. In this study we examined the hormonal influence on dividing cells in vitro.

The neuronal progeny of the ventricular zone migrate into the brain parenchyma along radial guide fibers in adult songbirds (Alvarez-Buylla and Nottebohm, 1988; Goldman et al., 1993). These cells migrate in response to Ng-CAM, a calcium-dependent adhesion molecule. In a study by Williams et al. (1999), it was found that juvenile HVc explant cultures raised in estrogen-supplemented media responded to NgCAM, while those raised in testosterone- or cholesterol-supplemented media did not (Williams et al., 1999). Additionally in the same study, in adult HVc cultures both estrogen and testosterone treatment significantly increased the number of neurons observed compared to controls. This suggests that while both estrogen and testosterone may regulate the

differentiation and or departure of new neurons from the HVc, estrogen also serves to regulate migration from the VZ via the NgCAM-dependent signaling pathway.

1.3 Neurogenesis

In many ways the molecular mechanisms underlying adult neurogenesis are similar to the embryonic system of neural development. Neurons, oligodendrocytes, and astrocytes are all generated in the embryo from the neuroepithelial cells of the neural tube. Neuroepithelial cells in the embryo appear to be the default developmental progeny of stem cells in the neural tube that escape numerous differentiating signals, including bone morphogenetic protein (BMP), which is known to induce epidermal differentiation. Due to the high incidence at which adult stem cells divide, they are unlikely to complement neuroepithelial cells, which terminally differentiate in the embryo. However, both cell types do undergo the same mechanism of neuronal differentiation, brought about by the activation of a series of basic helix-loop-helix (bHLH) transcription factors, which then act on generic neuronal genes within the neuroepithelial cell (Kintner, 2002). The neuroepithelial cells in the neural tube produce neurons first, then glial cells in a pattern along the neuraxis that indicates a highly regulated mechanism of development. Notch inhibits neurogenesis in these cells in the determinative phase of differentiation. It is presently unknown whether the bHLH cascade required for neurogenesis is used in adult stem cells in the same mechanism as in embryonic neuroepithelial cells or if they bypass the bHLH cascade and stimulate proneuronal genes directly (Kintner, 2002).

Area X-projecting HVc neurons are born in the embryo and facilitate song learning and appear not to be seasonally replaced. However, HVc neurons that project to the RA, which facilitate song production, as well as neurons within the HVc are continuously added throughout life.

Until recently, it was thought that adult vertebrates did not have functionally dividing stem cells in their CNS. However, proliferating neural stem cells have been identified in both mammalian and non-mammalian adult species. In songbirds, stem cells continuously divide in the subependymal zone (SEZ) and migrate into the HVc (Kirn et al., 1994; Alvarez-Buylla et al., 1987; Nottebohm, 1985). The SZ contains at least four morphologically and molecularly distinct cell progenitors, including neurons, astrocytes, highly proliferative precursors, and ependymal cells, which separate the SEZ from the ventricle cavity (Alvarez-Buylla and Garcia-Verdugo, 2002).

Growth factors appear to play an important role in stem cell proliferation in the SZ. EGF, FGF, and TGF-α all stimulate proliferation of cells in the SZ *in vivo* (Morshead et al., 1994; Kuhn et al., 1997). Astrocytes secrete factors that aide in the migration of neuronal precursors (Mason et al., 2001), and may also support survival of these cells (Alvarez-Buylla and Garcia-Verdugo, 2002). For instance, insulin-like growth factor-1 (IGF-1) is a neurotrophin associated with radial cells that is known to promote neuronal recruitment from the adult SZ of the songbird (Jiang et al., 1998). Steroid hormones also regulate the survival of new neurons (Rasika et al., 1994; Hidalgo et al., 1995). The effects of hormones, testosterone in particular, may be mediated through brain-derived neruotrophic factor (BDNF). Testosterone treatment increases BDNF

levels in the female HVc, and infusion of BDNF into the HVc triples the amount of new neurons (Rasika et al., 1999). Interestingly, only the HVc and Area X have been found to receive new neurons in adulthood, and Area X only receives interneurons, as opposed to the projection neurons the HVc receives (Nottebohm, 1993). More specifically, the RA-projecting neurons are replaced by new neurons of the same type (Kirn and Nottebohm, 1993). However, Area X-projecting neurons, when destroyed, are not replaced by the same type of cell (Scharff et al., 2000), thereby indicating that Area X neurons may be more permanent.

There is little evidence that gonadal hormones affect the rate of proliferation in the SEZ and the results are not conclusive (Brown et al., 1993; Hidalgo et al., 1995). In a study by Brown et al. (1993), *in vivo* treatment with testosterone, estrogen, or hormone-inhibiting drugs did not affect the division rate or the number of mitotically active cells (stem cells) (Brown et al., 1993). In addition, hormone treatment following ^{3}H-thymidine labeling increases the number of new neurons incorporated into the HVc (Hidalgo et al., 1995; Rasika et al., 1994; Burek et al., 1995). Therefore, the influence of hormones on the incorporation of newly integrated neurons appears to be restricted to postmitotic events (Alvarez-Buylla and Kirn, 1997). It is logical, then, that with hormonal signals dictating the addition of newly formed neurons there must also be a mechanism controlling the counterbalancing removal of neurons, although that mechanism remains to be discovered.

Although there is a constant influx of new neurons into HVc, the total number of neurons within the HVc, as well as the volume, remains constant in photorefractory birds

(Kirn et al., 1991). Thus, there must be a highly regulated equal and opposite reaction in the brain to maintain cellular equilibrium. More specifically, some cells must die in order that new cells can come in. There is compounding evidence that neuronal addition is coupled with cell loss. In fact, in one study by Kirn and Nottebohm (1993) in which HVc-RA projecting cells were labeled with a retrograde marker in early spring, 30-50% of the labeled cells had been lost and replaced by the same kind of neuron when analyzed in the fall of that same year (Kirn and Nottebohm, 1993). In addition, two-thirds of new neurons born in the adult forebrain on one day are gone 30-40 days later (Alvarez-Buylla and Nottebohm, 1988). More specifically in the canary HVc, nearly 50% of the new neurons are dead within 14-21 days after their birth (Kirn et al., 1999). This equilibrium could be regulated by a lack of survival factors for either new cells, thus leading to their own demise, or alternatively, and the more likely scenario, in which new cells replace older, short-lived cells. Regardless of the extent of their lifetime, the cells that die are not sick or injured, but undergo a very systematic death known as apoptosis.

1.4 Apoptosis

Apoptosis is common in every life stage in most living organisms. For instance, the re-absorption of the tadpole tail at the time of its metamorphosis occurs by apoptosis, as does the formation of the fingers and toes of the fetus by removal of the tissue between them and the sloughing off of the inner lining of the endometrium in menstruation. Cells are signaled to begin apoptosis by the delicate interaction of the withdrawal of positive survival signals and the receipt of negative death signals. There are 2 different

mechanisms by which a cell undergoes apoptosis: one generated by signals arising within the cell, the other induced by death activators binding to receptors at the cell surface, such as, TNF-α, Lymphotoxin, and Fas ligand (FasL). Briefly, internal damage in a healthy cell signals the mitochondrial membrane to leak cytochrome C out of the mitochondria. The released cytochrome C binds to molecules of caspase 9, forming a complex of proteins called the apoptosome, which aggregates in the cytosol. The protease caspase 9 cleaves proteins, and in doing so, activates other proteolytic caspases, which inevitably leads to the digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA, and the ultimate phagocytosis of the cell.

Fas and the TNF-α receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell. When these receptors are bound by the complementary death activator, (FasL and TNF-α respectively), a signal is transmitted to the cytoplasm that leads to the activation of caspase 8, which is similar to caspase 9. This, in turn, initiates a cascade of caspase activation, eventually leading to phagocytosis of the cell. Although it is unknown whether the cells in the song system are receiving internal or external signals to die, these cells have very characteristic apoptosis markers that act as indicators of their condition and allow for distinction between cells that die by apoptosis and cells that die a natural death (necrosis) or from crude treatment in the laboratory.

Many laboratory analyses have been created to determine apoptosis cells in comparison to necrotic cells. Because apoptotic cells undergo a highly systematic and ordered death, there are specific markers that can be used to identify them. For instance,

an apoptotic cell's DNA is cleaved in uniform segments of 100 to 250 base pairs, nuclear lamins are degraded, cytochrome C is released from the mitochondrial membrane, and the DNA repair enzyme PARP (Poly ADP-Ribose Polymerase) is cleaved by a single proteolytic cleavage. In contrast, necrotic cells typically undergo an unorganized breakdown of the cellular membrane and release their interior contents into the surroundings, potentially damaging surrounding cells.

In phase I of this study, I decided to investigate the neuronal response in regard to apoptosis in response to hormonal treatments with testosterone or 17β-estradiol *in vitro* in the wild species *Junco hyemalis.* The use of the wild bird species (*Junco hyemalis*) is noteworthy because these non-opportunistic breeders exhibit a relatively short breeding period. Therefore, the seasonality of the song system is more pronounced. In addition, all specimens were caught in the wild and have not been genetically "bottlenecked" in a laboratory breeding program. Wild birds analyzed for effects of long photoperiod were caught in mid-late May, when the breeding season is peaking. Although captivity has been found to affect experimental data in comparison to data collected from birds living in the wild, it is our hope that because these birds were reared in and formed their song template in the wild, that their song systems will more closely resemble those of typical wild birds. We found that testosterone attenuates the death of newly added neurons in the HVc *in vitro* in photosensitive birds, while both testosterone and estradiol attenuate neuronal apoptosis in the HVc in photostimulated birds *in vitro.* In addition, we found that hormone treatments have an almost immediate affect on the survival of new neurons generated in the photosensitive and photostimulated birds. Importantly, using BrdU

labeling to assess cell proliferation, our analysis provides evidence that testosterone and estrogen do not alter the rate of proliferation in the SEZ. Conclusively, our research strongly favors that the changes in the cell number in the HVc result from differences in the rate of apoptosis of newly added neurons.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals were from Sigma (St. Louis, MO) unless state otherwise. BrdU labeling and developing was done according to instructions from the 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I, developed by Roche Diagnostics GmbH (Mannheim, Germany), and distributed by Boehringer-Manheim (Cat. No. 1 296 736).

2.2 Capture and Care of Animals

All necessary permits were obtained prior to collection from the Department of Fish and Game (permit # 01-084) and the Department of the Interior (permit # MB692453-2). All protocols have been approved by the University of Alaska Fairbanks International Animal Care Use Committee. Adolescent and juvenile (approximately 2-3 month old) male and female juncos (*Junco hyemalis*) were collected after breeding season had ended (mid to late August) in the vicinity of the University of Fairbanks Alaska (65°N, 147°W) using millet seed-baited Potter traps and vocal playback. All birds received food and water *ad libitum.*

At this time of the year, the photoperiod is approximately 12h: 12h light/dark cycle and birds are photorefractory and not singing. Captured birds were quarantined for 4 weeks and held in small aviaries containing 7-10 birds both male and female on short photoperiod (8h: 16h light/dark cycle). Birds were kept on short photoperiod over the

winter (7 months) prior to experiments. For photostimulation, captive adolescent juncos were randomly separated in two groups containing both males and females. One group of birds (photostimulated) was exposed to a gradually increasing photoperiod of 1 h per day to a final 20hr: 4hr light/dark cycle, typical for the latitude of Fairbanks, AK, and maintained at long photoperiod for thirty days prior to sacrificing. The other group of birds, our control, remained at an 8hr: 16hr light/dark cycle. After 30 days, birds were sacrificed by decapitation and fresh brains were subjected to experimental analysis.

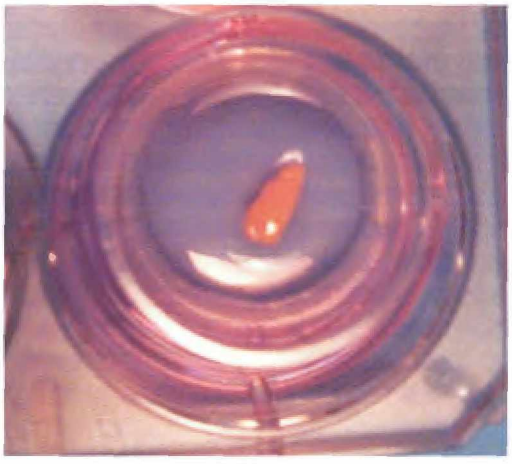

Figure 2.1 Culture Conditions for Junco HVc Slices
Junco brain HVc slice in a Millipore culture dish set within a 6 well culture plate. The well is filled with junco brain media, and the slice is exposed to the media through the Millicell filter that it sits upon. It is not submersed in media, so can get plenty of oxygen while staying moist.

2.3 *Junco hyemalis* Brain Atlas and Localization of the HVc

The published stereotactic atlas of adult songbird brain is derived from studies in the canary (Stokes et al., 1974). To account for subtle anatomical differences between distinct songbird species, we established a brain atlas based on adult brain of *Junco hyemalis*. Two freshly dissected brains obtained from photostimulated birds were fixed in 4% paraformaldehyde in sodium-phosphate buffer for 30 min at 20°C, cryoprotected in

10% sucrose solution overnight at 20°C, and frozen at -80°C in embedding medium. Brain tissue was mounted on a Cryostat and processed into 30μm thick sections. Next, sections were placed onto gelatin-treated microscope slides and Nissl stained. The HVc as well as other brain nuclei were easily identified by intense Nissl staining. We found that the *Junco hyemalis* HVC in each telencephalon hemisphere was located just below the subventricular zone 5.5mm from the most anterior position of the telencephalon with dimensions of 1mm and 3mm in medial or lateral extension, respectfully.

2.4 Organotypic HVc Slice Cultures

Following euthanization of birds, intact brains were immediately removed, placed into ice-cold sterile F-12 medium (Invitrogen, Gaithersburg, MD), and the telencephalon of each brain hemisphere dissected. Each telencephalon was trimmed to a section containing the HVc enclosed by the overlaying subependymal zone and underlying brain tissue and embedded into 2% agarose in F-12. HVc-containing telencephalon regions were glued onto Vibraslicer (World Precision Instruments, Sarasota, FL) using tissue-grade glue and sliced laterally into 300μm thick sections. These HVc slices were placed onto Millipore® tissue culture inserts (30 mm in diameter, Millicell CM, Millipore Corp., Bedford, MA) placed into 6 well plates. HVc slices were cultured for 14 days (37°C, humidified CO2 incubator) as adopted from Stoppini et al (1990) in 50% DMEM, 25% F-12 (both Invitrogen, Rockford, MA), 25% charcoal-stripped Fetal Bovine Serum (Hyclone, Logan, UT), 6.5 g/ml glucose, 4 mM L-Glutamine, 25 mM Hepes and 1% N3 supplement (Bottenstein and Sato). HVc slices were subjected to one of four treatments

in the above media: testosterone (10 ng/ml), estradiol (10 ng/ml), cholesterol (10 ng/ml) or ethanol (1 μl/ml), our carrier. Culture medium including hormonal treatments was replaced every 3 days throughout the duration of experiments.

2.5 Photostimulation of Songbirds Subjected to BrdU Labeling

Junco hyemalis birds (4 males and 10 females) were housed over the winter (7 months) on an 8h: 16h light/dark cycle (short photoperiod). For photostimulation, 2 males and 5 females were housed in a separate room and photoperiod gradually increased by 1 h/day to a 20h: 4h light/dark cycle (long photoperiod). Birds were kept on long photoperiod for 5 weeks. Birds on long and short photoperiod were sacrificed within a three-day period and organotypic HVc brain slice cultures established.

All birds' brains were sectioned into 300μm slices containing the HVc. HVc sections were cultured on Millicell 30 mm culture units (Millipore Corp., Bedford, MA) in brain slice media containing 50% DMEM, 25% F-12, 25% FBS, 1% N3, 25mM Hepes, 4 mM L-Glutamine, and 6 mg/ml Glucose. BrdU labeling solution and hormone treatments were added directly to the culture medium, and slices were cultured for 14 days.

2.6 BrdU Labeling of Organotypic HVc Brain Slices

Organotypic HVc brain slice cultures after 24 hours of culturing were labeled with BrdU (1:1000) in culture medium for 2 h, rinsed for 20 minutes at 37°C in wash

solution (Boehringer-Mannheim BrdU kit), and replenished with fresh culture medium. Sections were then cultured for an additional 13 days in the presence of either testosterone (10 ng/ml), 17 β-estradiol (10 ng/ml), or no treatment (ethanol carrier, 1:2000). Culture medium and treatments were replaced every 3 days throughout the duration of experiments. On culture day 14, all sections were relabeled with BrdU as detailed above, washed, and fixed in 70% EtOH containing 20 mM glycine (4°C, 20 min). Next, HVc brain slices were rinsed in PBS, cryoprotected in 10% sucrose solution overnight at 20°C, and frozen at -80°C in embedding medium. Slices were processed into 20 μm thin sections (cryostat) and mounted onto 10% gel-coated slides.

2.7 TUNEL Staining

Apoptotic cells in HVc slices either cultured for 14 days or obtained fresh were revealed by endlabeling single strand DNA breaks with biotinylated nucleotides using terminal deoxynucleotidyl transferase (Neurotacs II®, Trevigen, Gaithersburg, MD). 300 μm brain tissue sections containing the HVc were removed from the filter insert and fixed with 4% paraformaldehyde in sodium-phosphate buffer for 15 min at 20°C. Following cryoprotection in 4% sucrose in ddH_2O solution for six hours, all sections were frozen at -80°C in embedding medium and cyrosectioned into ten 30 μm thin slices. Two sections each from of the anterior, midsection, and posterior regions of the original 300 μm tissue section were mounted on 10% Porcine gelatin-coated microscope slices, washed in PBS, and permeabilized with 50 μl of Neuropore. Following quenching in methanol/peroxide, samples were subjected to endlabeling with biotinylated Uridine and incubated with

Streptavidin-horseradish perioxidase/diaminobenzidine. Finally, sections were quickly counterstained, washed, dehydrated, and covered. Each slide was analyzed using an inverted Zeiss microscope at 40X magnification and TUNEL positive cells determined in the HVc area.

2.8 Comet Staining

DNA laddering is a hallmark of apoptosis and can be visualized by single cell gel electrophoresis followed by fluorescent-labeling of unwound DNA fragments that have migrated out of the nucleus (Comet Assay®, Trevigen, Gaithersburg, MD). The HVC was dissected out of a 300 μm thick tissue section and either cultured for 14 days or obtained fresh and minced in PBS. After centrifugation (2 min, 10,000 rpm, RT), supernatants were discarded and pellets resuspended in homogenizing solution (0.05% Trypsin, 0.02% EDTA in PBS). The homogenate was then centrifuged (2 min, 10,000 rpm, RT), the pellets wash with DMEM and 10% FBS, and then triturated in the same medium. The resulting cell suspension was stored on ice until embedded into a molten agarose solution at a ratio of 1:10 and transferred onto specially prepared Trevigen Comet Assay® slides. After alkaline lysis in 10 mM Tris pH 10, 2.5 M NaCl, 100 mM EDTA, 1% Sodium Lauryl Sarcosinate, 0.01% Triton-X 100 followed by a 30 min incubation on ice, slides were washed with TBE prior to horizontal gel electrophoresis and placed onto a gel tray in TBE buffer (Gibco BRL Horizon 58, 1V per cm, 15 min). Finally, slides were dehydrated in 100% ethanol for 5 min, air dried, and stained with diluted SYBR Green Stain® (Trevigen Trademark). Slides were analyzed by

fluorescence microscopy at 20X magnification (494 nm excitation filter, 512nm emission filter). Cells were counted as healthy when exhibiting a round staining (intact nuclear DNA) or as apoptotic when revealing a comet-like staining (DNA degradation).

2.9 BrdU Immunostaining

Slides containing 20 μm thin HVc brain sections were air-dried, washed three times with Wash solution, and covered with 50 μl of anti-BrdU working solution (anti-BrdU antibody diluted 1:10 in provided incubation buffer, 30 min, RT, humidified chamber). Slides were rinsed three times with Wash solution and incubated with 50 μl of a fluorescein-conjugated secondary anti-mouse-IgG (1:10, humidified chamber, 30 min, RT). After three washes, slides were covered with Citifluor mounting medium and allowed to dry for 24 hours to weaken background prior to inspection by fluorescence microscopy at 20X magnification (488 nm excitation, 520 nm emission).

2.10 Cytochrome C (Cyt C) Immunochemistry

Cytochrome C is yet another well documented molecular hallmark of apoptosis that is released from the mitochondrial membrane in a cell undergoing apoptosis. We ran preliminary Cyt C immunoblotting on chick spinal cord neurons to test if the Cyt C antibody would work in avian species. Embyonic (E 7) chicken spinal cords were dissected and allowed to recover overnight in a CO_2 humidified incubator at 37°C. Cultures were plated on Poly-L-Lysine-coated dishes (10 μg/ml in Borate buffer) in cell culture media (50% DMEM, 25% F-12, 25% FBS, 1% N3, 25 mM Hepes, 4 mM L-

Glutamine, and 6 mg/ml Glucose). The next day half of the cultures were treated with UV light for 15 minutes to induce cellular stress and trigger apoptosis and controls left untreated. Both treatments were then homogenized in 0.32 M Sucrose and 20 mM Tris buffer pH 7.4. The homogenate was centrifuged (10,000 x gmax, 20 min, 20°C) and the supernatant discarded. The pellet was re-suspended in 1% Triton, 20 mM Tris buffer pH 7.4 and spun again (10,000 x gmax, 20 min, 20°C). Then, the pellet was re-suspended again in 5X Laemmli buffer and 2% SDS (Laemmli, 1970). Samples were boiled, subjected to SDS gel electrophoresis, (200V, 1 hour) and then blotted onto nitrocellulose paper (75V, 1hr 15 min). After blocking overnight in 5% milk in Tris Buffered Saline (TBS)/Tween (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween) blots were incubated with a primary anti-Cyt C antibody (1:500) in 5% milk, TBS/Tween for three hours. Following three rinses for 30 min each in TBS/Tween, blots were incubated in a horseradish peroxidase (HRP) conjugated mouse secondary antibody (1:2000) for 1 hour. Blots were developed using chemiluminescence.

2.11 Neu N Neuron Labeling

Neu N is a neuronal antibody that is used to distinguish neurons from other cell types. To clarify that the cells undergoing apoptosis were exclusively neurons or not, we used the NeuN antibody in conjunction with TUNEL staining. 13 day old brains of chick embryos were dissected and fixed in 4% PFA in sodium phosphate buffer. The brains were then cryoprotected with 10% sucrose solution. They were sliced on a cryostat (20 μm), placed

on gel-coated slides, and permeabilized either with Neuropore (Trevigen®, Gaithersburg, MD), or treated with sodium citrate solution (pH 9 for 1 hour at 37°C). All slices were washed one time in 1X PBS, then blocked in 3% HS in PBS (30 minutes at RT). Following one wash with PBS, all slices were incubated with a primary anti-Neu N antibody overnight at RT (1:500 in 3% HS, PBS), then rinsed for 10 minutes in PBS (3 changes), and incubated with a FITC-labeled goat-anti-mouse secondary antibody (1:500) diluted in 3% HS, PBS. Our positive control consisted of brain slices exposed to treatment with TACS Nuclease (50 µl, 30 min, 37°C). All slides were then TUNEL-stained as previously described. Using a Streptavidin Rhodamine for development (1:750), analysis was performed on a Zeiss inverted microscope at 40X to detect congruent and non-congruent staining.

2.12 PARP Immunodetection

PARP, or poly-ADP-ribose polymerase, is an enzyme involved in DNA repair and becomes inactivated during apoptosis. Intact PARP, with a molecular weight of 116 kD is cleaved into an 85 kD fragment and a 31 kD fragment. We subjected chick and junco brain homogenate to western blotting against cleaved PARP (85 kD fragment). Briefly, spinal cords were obtained from E7 chicken embryos. To obtain a single cell suspension, spinal cords were trypsinized (0.05% Trypsin/0.2 mg/ml EDTA, 10', 3PL), centrifuged (200 x gmax, 4 min), triterated in DMEM and10% FBS, and pre-plated for one hour in the same medium. Non-adherent spinal cords were plated in Ventral media

(DMEM/10% FBS, 1% N_3 100X, 2.5% FdUR) and incubated at 37°C (humidified incubator, 5% CO_2) for 24 hours. Next, apoptosis was induced by exposing cultures to oxidative stress or UV radiation. Two cultures were not exposed (control), two cultures were triterated with 100 μM of $FeSO_4$ and 100 μM H_2O_2 for 5 hours, two cultures were exposed to $FeSO_4$ and Ascorbic Acid for 5 hours, and two cultures were exposed to UV light for 30 minutes. After stress exposure, all cultures were washed three times with DMEM and cells recovered by centrifugation and kept on ice until solubilization in 10 μL of solubilization buffer (2% SDS Triton, 10 mM Tris-HCl, 1 mM NaF, 1 mM DTT, and 1 mM EGTA). Samples were boiled in eppendorf tubes in solubilization buffer for 5 min, then stored at -80°C until use in the SDS Page Western Blot procedure. Samples were boiled for three minutes and added to 1X SDS sample buffer and ddH_20 to make 20 μL. Samples were subjected to gel electrophoresis (175V, 1 hour) and western blotting onto nitrocellulose (100V, 1hr). Blots were blocked in 5% milk and TBS/0.02% Tween for 30 minutes (three washes) and incubated with primary anti-PARP antibody (1:1,000 in 1% milk, 0.01% Na N_3, in TBS/Tween) for 3 hours. After rinsing in TBS/Tween (3 times, 10 min), blots were incubated in secondary HRP antibody overnight (1:10,000 in 1% milk, 0.01% Na N_3, and TBS/Tween) and was developed using a chemiluminescence procedure.

2.13 Microscopic Imaging

All HVc brain section subjected to Nissl staining, TUNEL staining, and Comet analysis were analyzed on an inverted Zeiss Axiovert S100 research microscope equipped with a 10X, 20X, 40X, and 60X oil objective and a Peltier-cooled CCD camera (Photometrics, Tuscon, AZ). Brain sections stained with BrdU were analyzed on a Zeiss LSM Confocal Microscope at 20X magnification (488 nm excitation, 520 nm emission) using a stacked imaging technique.

2.14 Analysis and Statistics

In BrdU analysis, all fluorescent cells were counted per HVc section. Three 15 μm thick slices were analyzed, derived from a 300 μm thick cultured section, one from the anterior portion, one from the midsection, and one from the posterior end.

TUNEL-positive cells (apoptotic cells) were stained brown, as opposed to blue-stained controls. In ambiguous cases, cells were neither counted as positive nor negative for TUNEL. Each section of HVc surveyed was 0.3 mm^2 in area.

All cell counts were were analyzed using ANOVA statistical analysis with testosterone-treated cultures as the dependent variable. ANOVAs were performed using a SigmaStat program (SPSS Inc., © 1992-1997, version 2.3), and plotted with SigmaPlot 2000 program (SPSS Inc., © 1986-2000, version 6.10) and Microsoft Excel. Comparisons among statistically significant variables were derived from a Tukey Test

(Multiple Pairwise Comparison Procedure). Two way ANOVAs were used to determine the dependence of photoperiod and hormone treatments upon one another. Multiple Comparisons were calculated between significantly different variables. All tests were performed with power of alpha set at 0.05.

Chapter 3

Junco hyemalis Brain Atlas

3.1 Junco Brain Atlas

Figure 3.1 Male *Junco hyemalis* foraging for food.

In order to pinpoint the location of the HVc in the *Junco hyemalis*, we established a brain atlas using Nissl staining techniques. The brains of the adult juncos were dissected and fixed in a 4% paraformaldehyde solution for 15 minutes, then frozen at -80°F. Brains were sliced laterally, from anterior to posterior, into 50 μm slices and placed on 3% porcine gel-coated slides. The slices were then stained with Nissl stain following a standard procedure and examined under a light microscope at 20X magnification. In a total of four adult male (1) and female(3) brains, the HVc was found to lie slightly posterior compared to the canary HVc. The junco HVc starts 5,000 μm back toward posterior from the anterior end, and ends between 6,500 μm and 6,800 μm (anterior to posterior). The HVc is found on the medial side of each hemisphere, just below the ventricular zone, which is easily distinguished by its tendency to separate from the rest of the forebrain. It is located just beneath the region where the lateral ventricle rises and falls laterally away from the hemispherical division. The HVc begins anteriorly with a medial to lateral width of roughly 1.75 to 3.5 mm, with the medial beginning of the hemisphere at 0 mm on the following diagrams. It shifts medially and expands in

width as it extends posteriorly, to have a maximum width of 2mm (from 1 mm to 3.25 mm medially to laterally) at roughly 5,800 μm anterior to posterior). The HVc then begins to decrease in width as it extends more posterior to a final size of 1mm (1.5 mm to 2.5 mm medial to lateral) at 6,500 μm anterior to posterior.

The following figures detail the location of the Junco HVc as adapted from the stereotactic altas of the canary brain (Stokes et al., 1974).

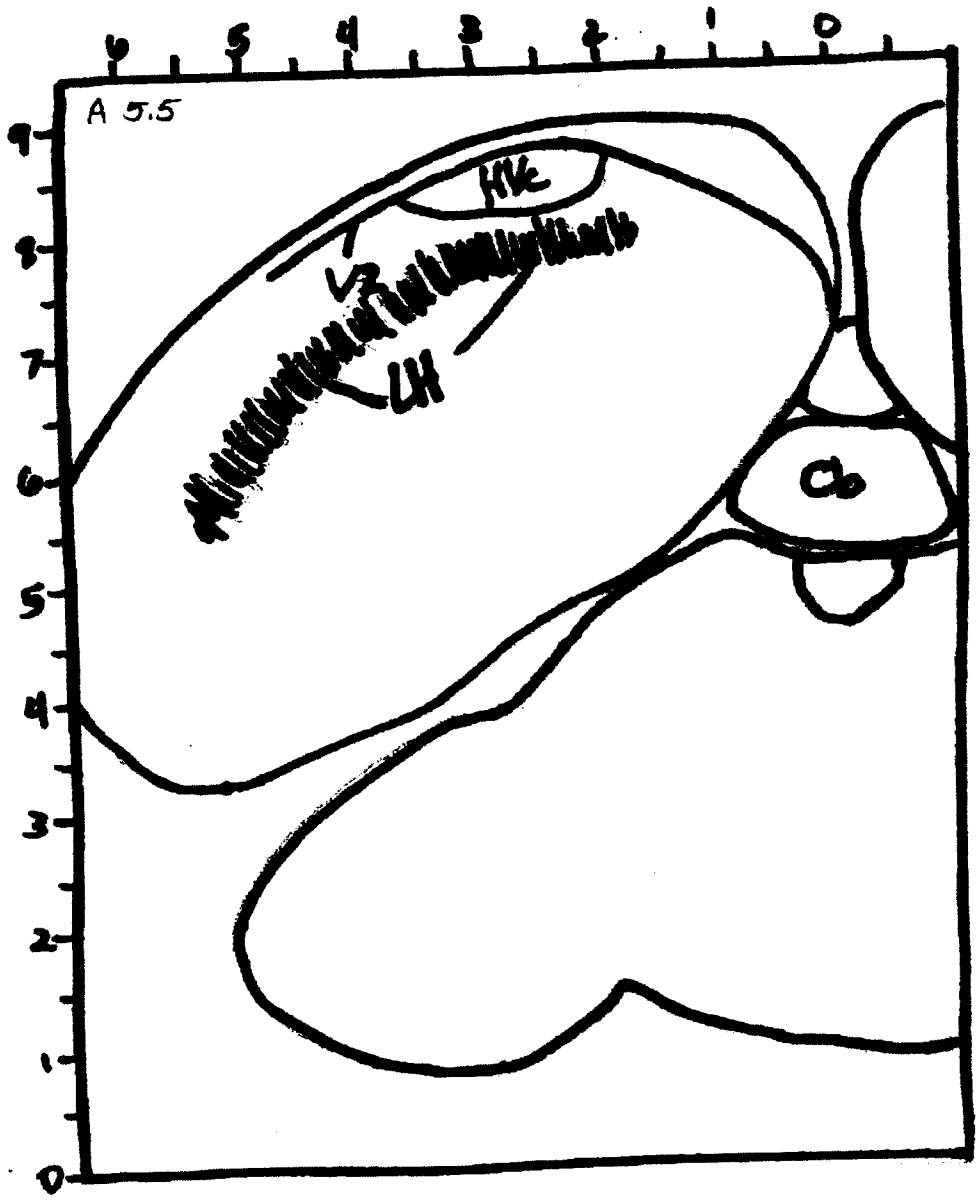

Figure 3.2.1 Junco Brain Atlas A
A 5.5 = distance in mm from most anterior point. Scale in mm.

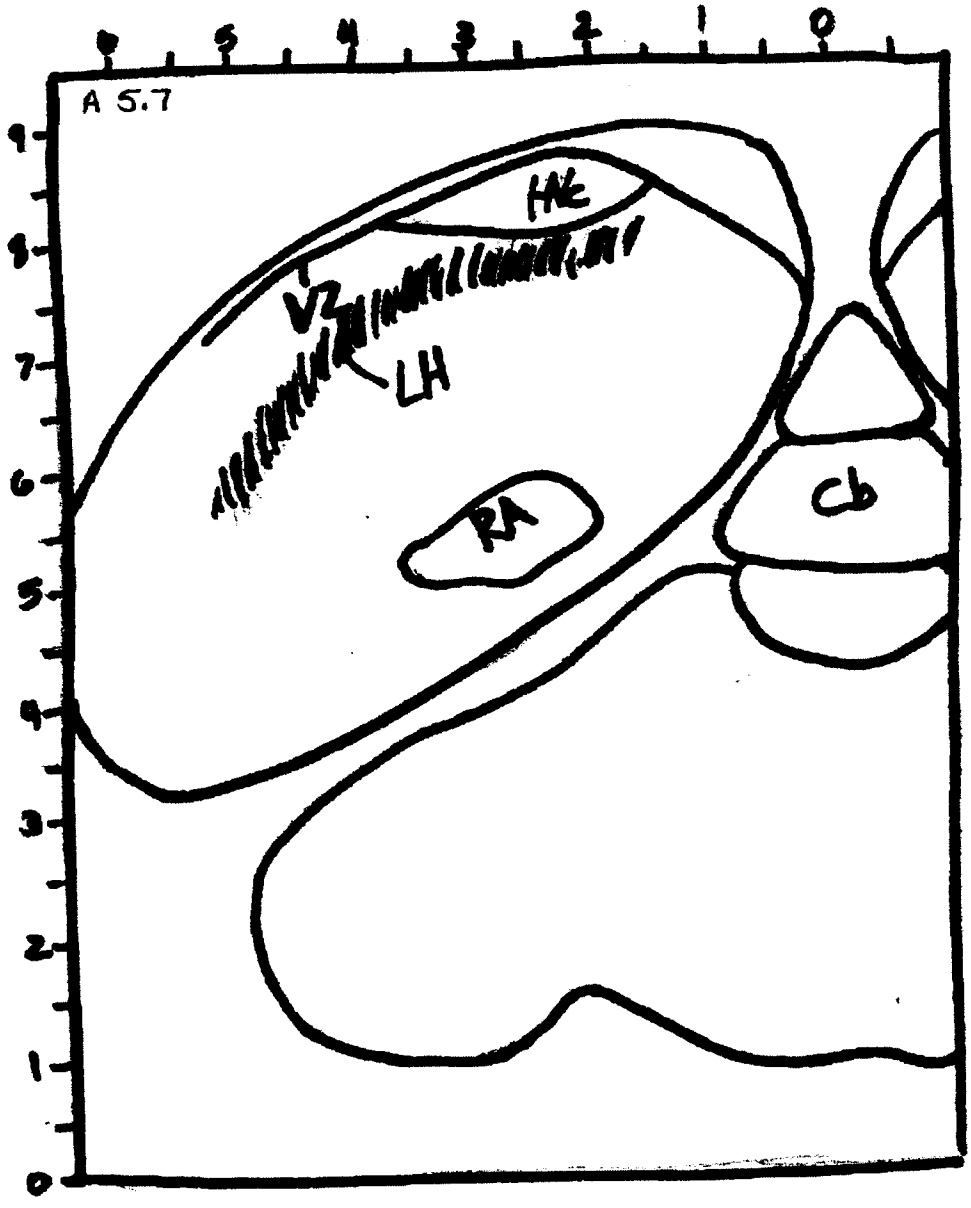

Figure 3.2.2 Junco Brain Atlas B
A 5.7 = distance in mm from most anterior point. Scale in mm.

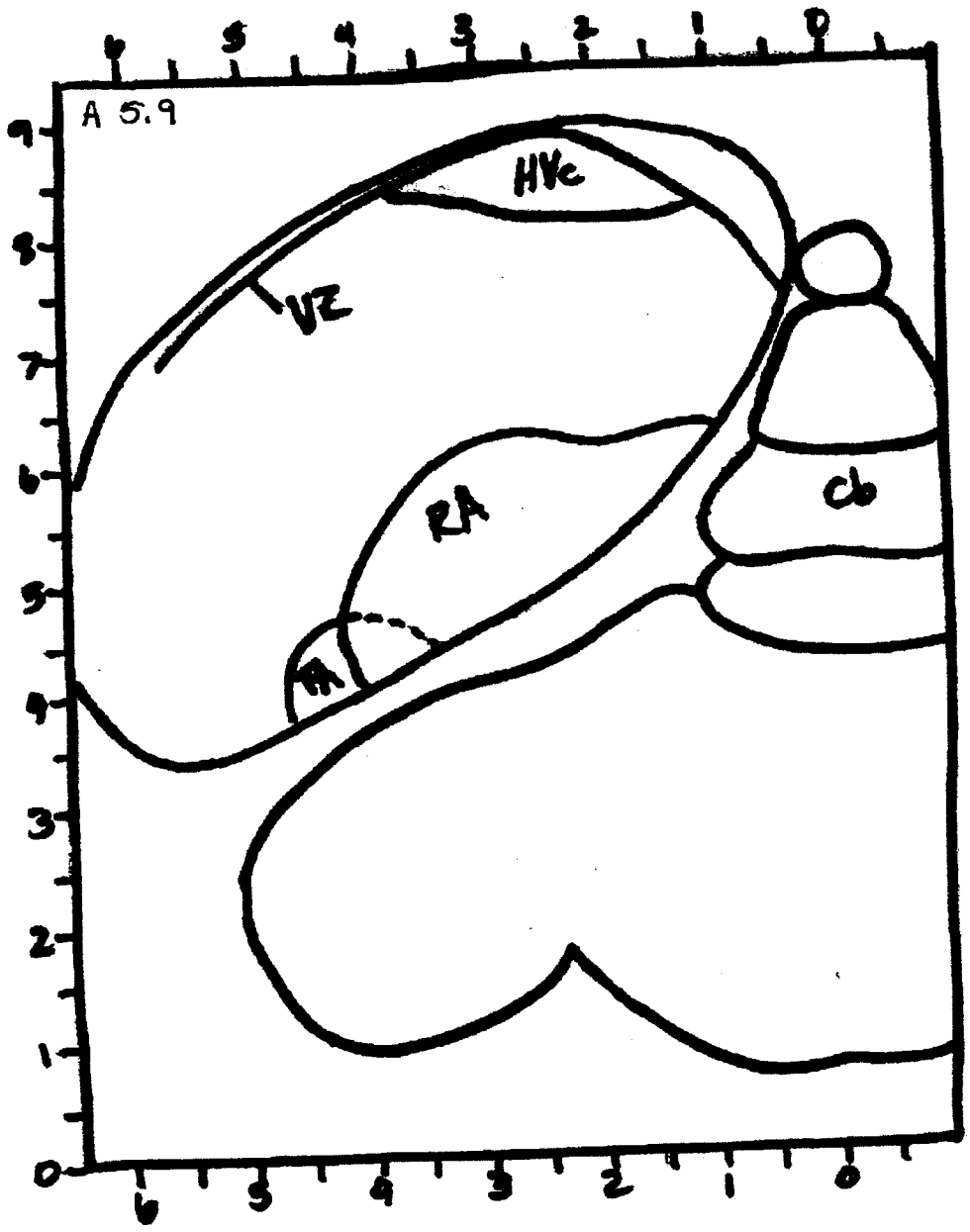

Figure 3.2.3 Junco Brain Atlas C
A 5.9 = distance in mm from most anterior point. Scale in mm.

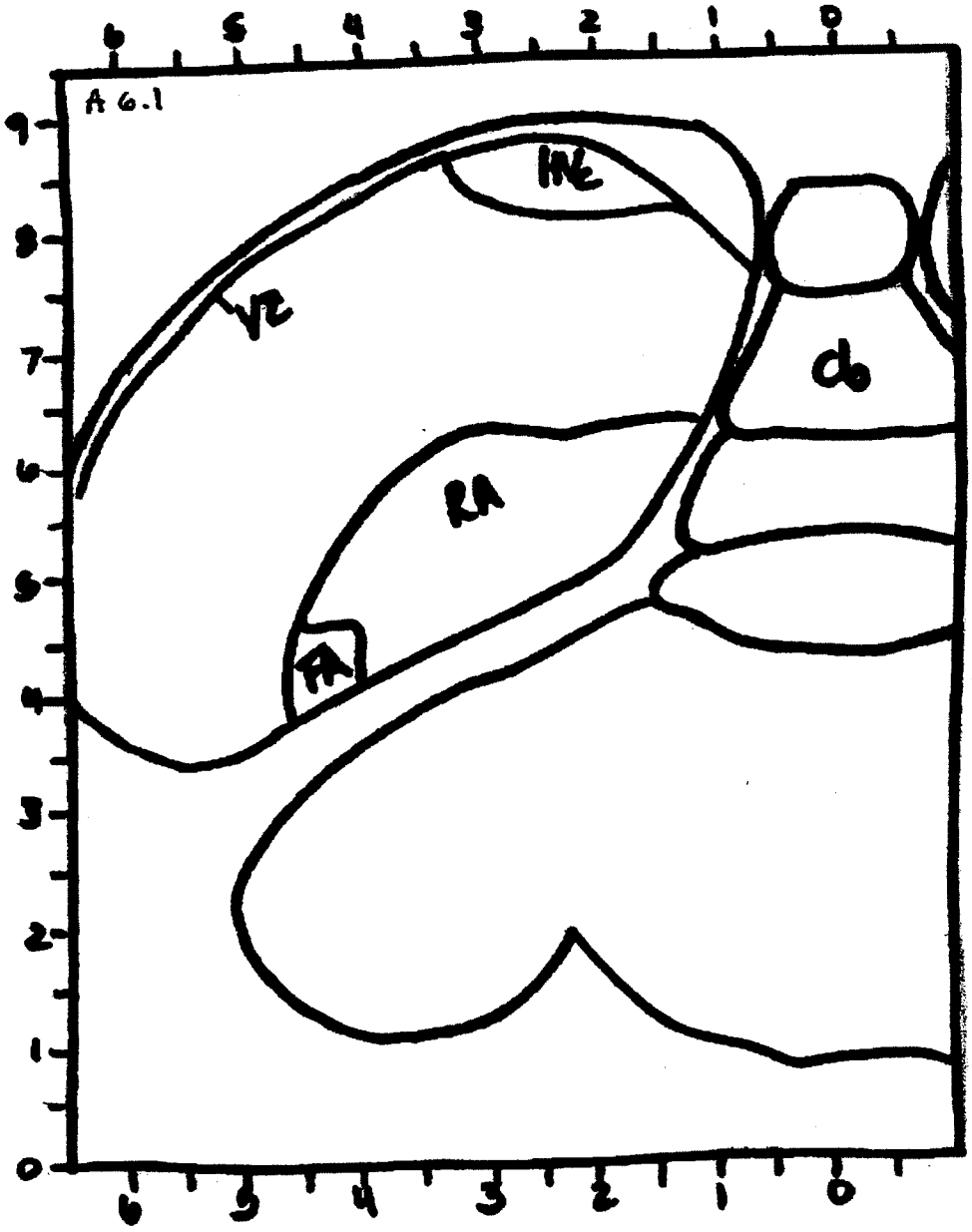

Figure 3.2.4 Junco Brain Atlas D
A 6.1 = distance in mm from most anterior point. Scale in mm.

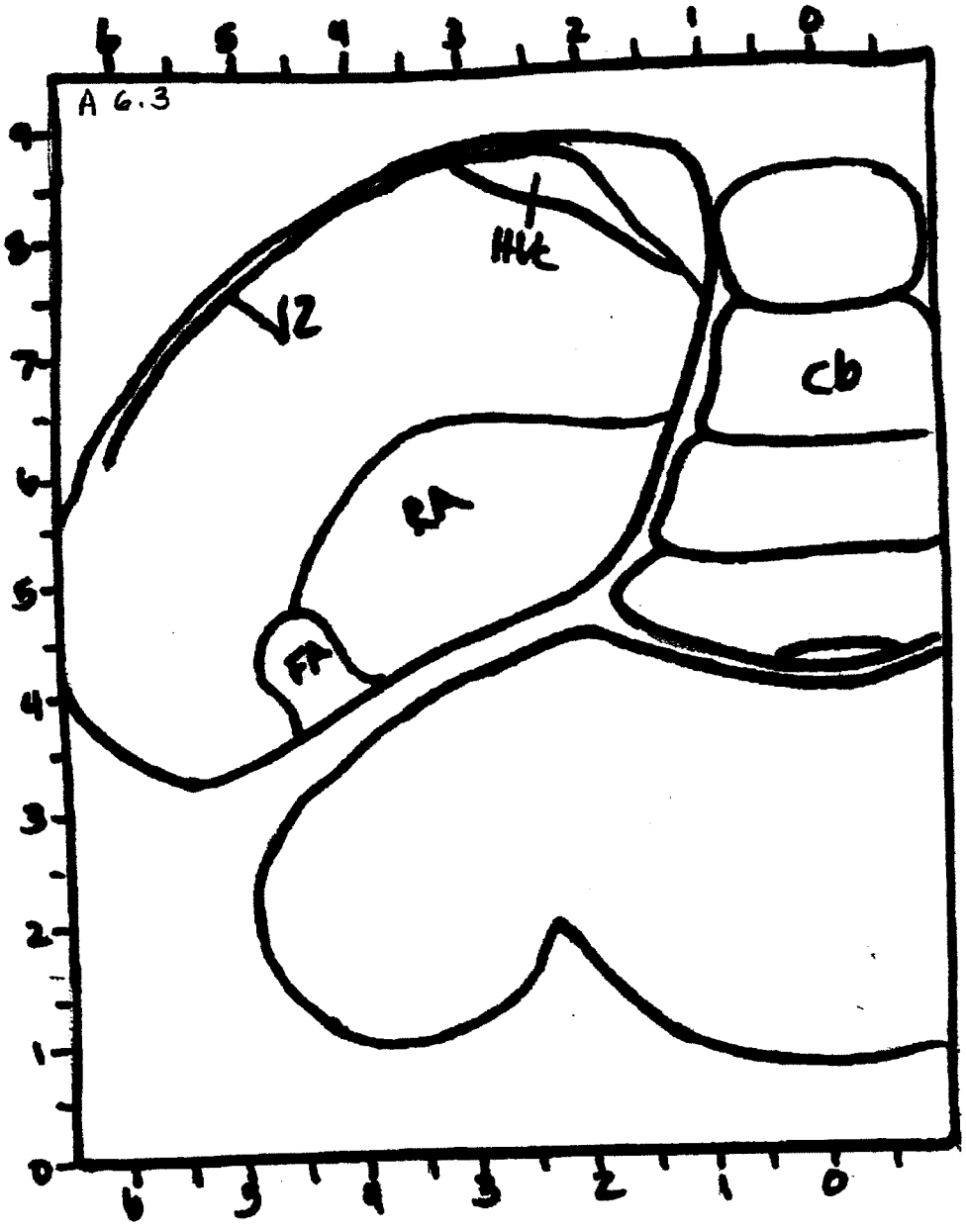

Figure 3.2.5 Junco Brain Atlas E
A 6.3 = distance in mm from most anterior point. Scale in mm.

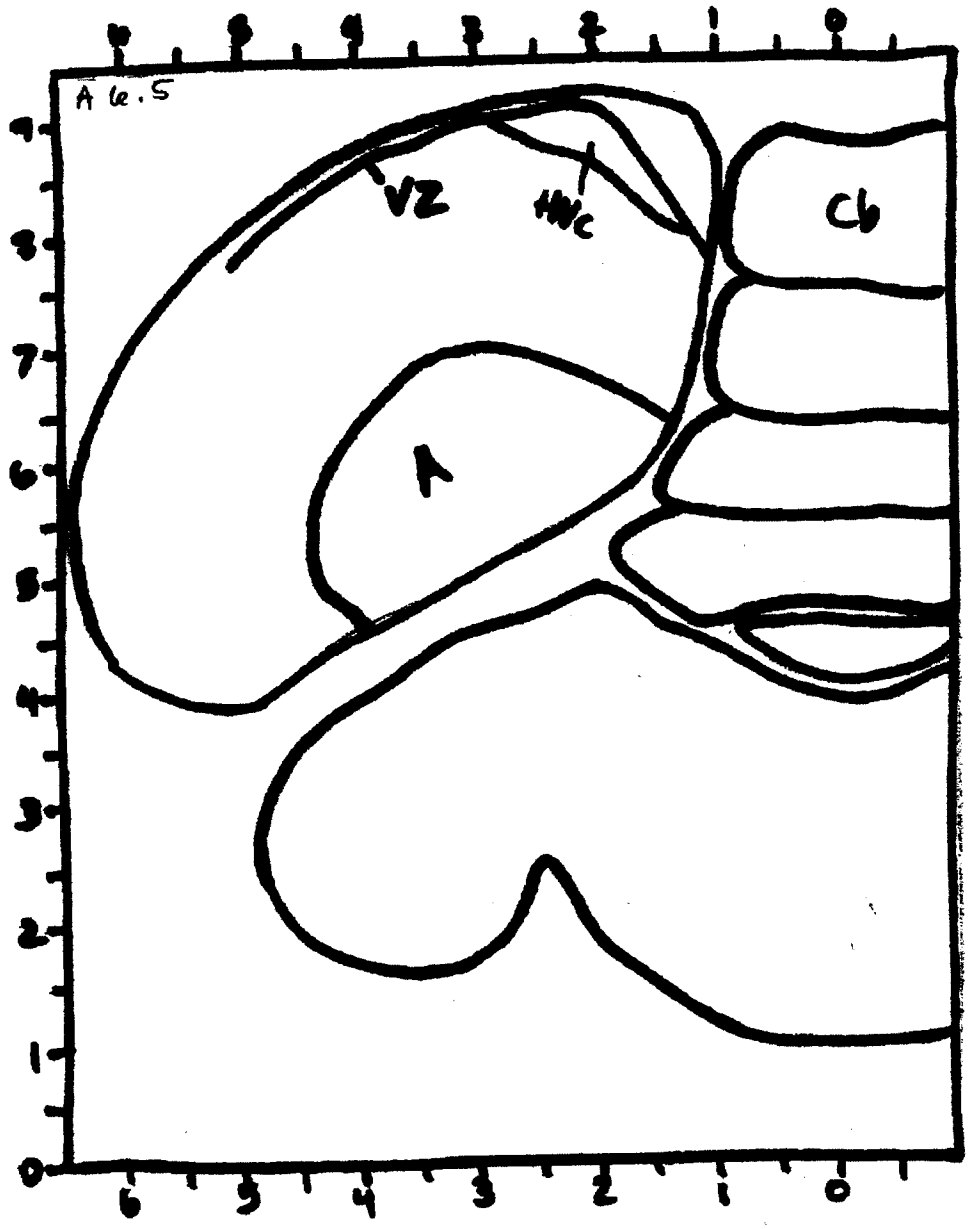

Figure 3.2.6 Junco Brain Atlas F
A 6.5 = distance in mm from most anterior point. Scale in mm.

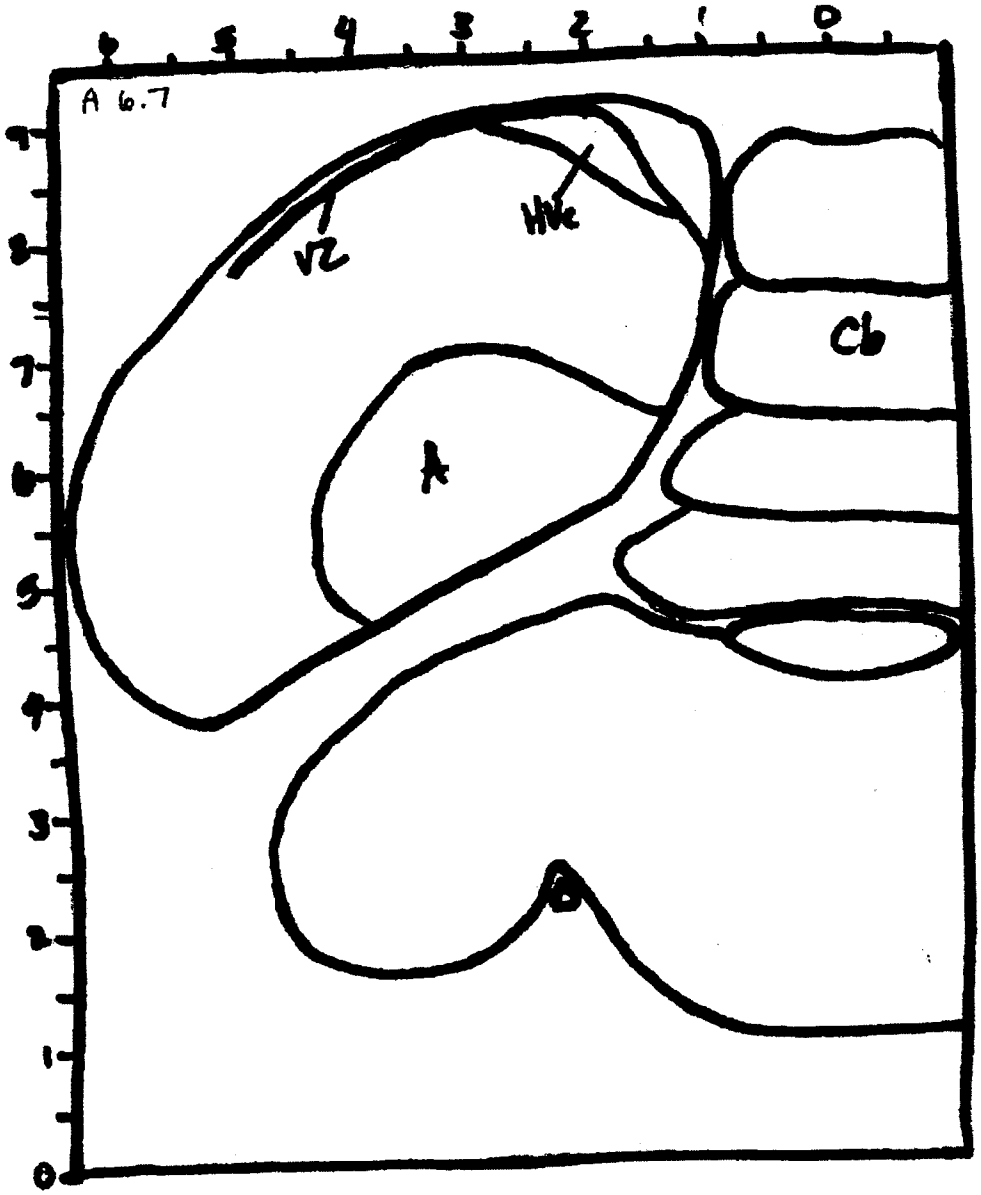

Figure 3.2.7 Junco Brain Atlas G
A 6.7 = distance in mm from most anterior point. Scale in mm.

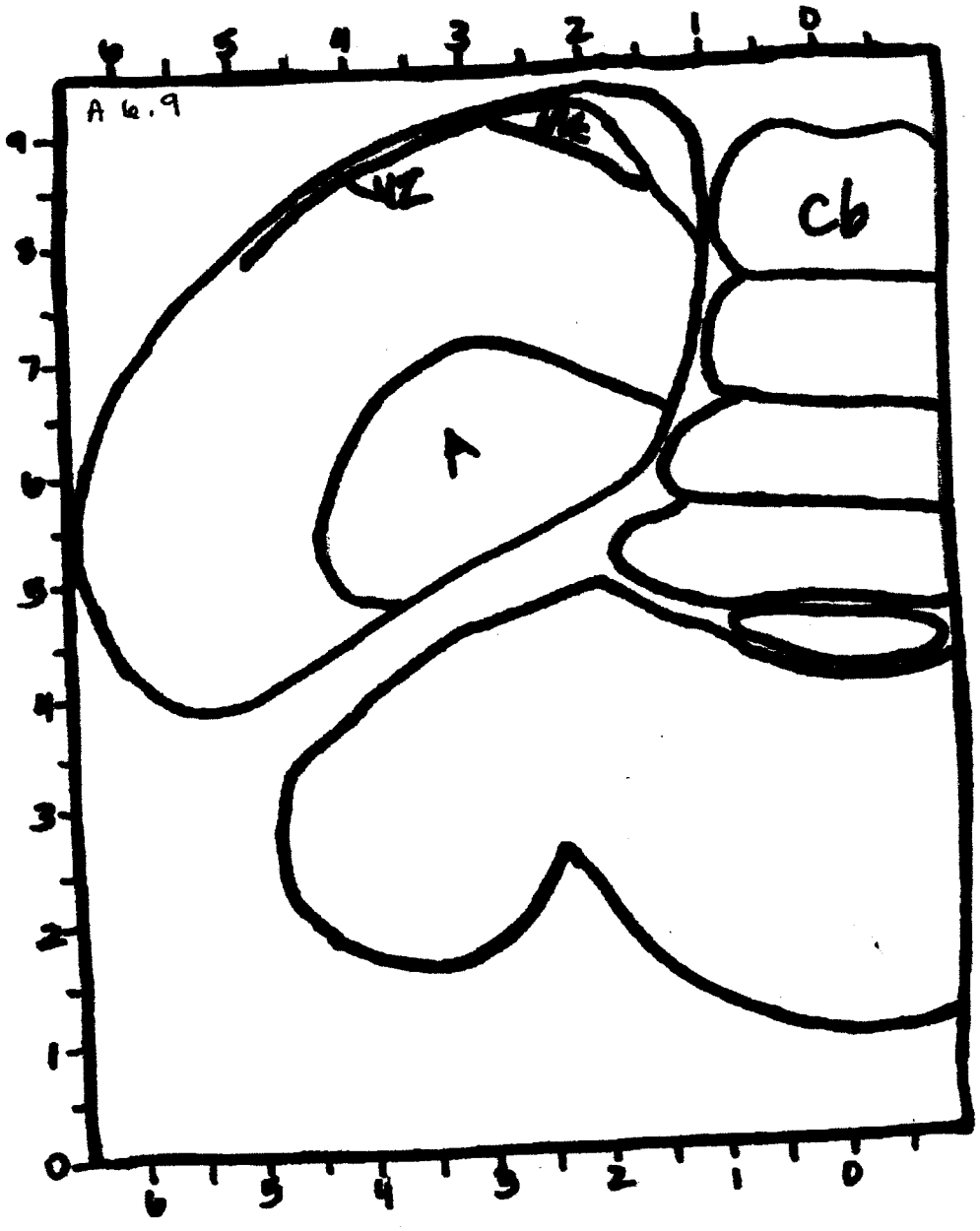

Figure 3.2.8 Junco Brain Atlas H
A 6.9 = distance in mm from most anterior point. Scale in mm.

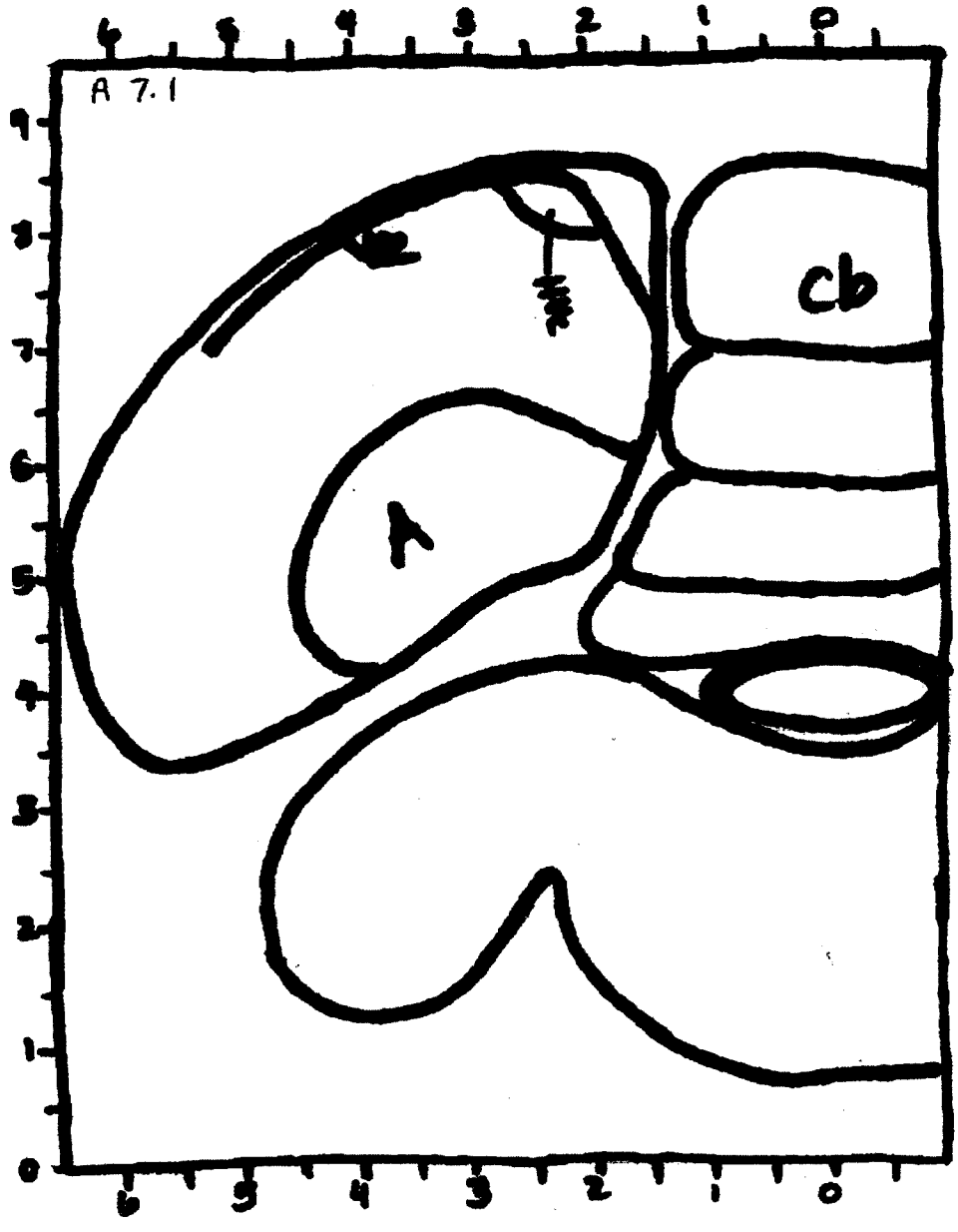

Figure 3.2.9 Junco Brain Atlas I
A 7.1 = distance in mm from most anterior point. Scale in mm.

Chapter 4

BrdU-Positive Cells Are Restricted to the HVc

4.1 Introduction

For over one century, neurogenesis or the generation of new neurons derived from precursor cells in higher vertebrates was thought to be restricted to embryonic development, ceasing prior to birth or soon after. Consequently, the adult nervous system represents a stable structure for the remaining life span of an individual with no replacement of neuronal loss whether naturally occurring or following injury. This dogma was removed by the discovery of neurogenesis in adult vertebrates including mammals and birds. New neurons are generated in specific locations and, following migration to their residency, become functionally incorporated into preexisting neuronal networks. Whereas neurogenesis in mammals is restricted to the dentate gyrus in the hippocampus and the subventricular zone, including its projection into the olphactory bulb, in birds it is widespread throughout the telencephalon (Lois and Alvarez-Buylla, 1994; Ling et al., 1997).

Studies on adult songbirds such as canaries and zebra finches have revealed that neurogenesis and functional incorporation of new neurons occurs in astounding numbers throughout the year into the telencephalon (Goldman and Nottebohm, 1983; Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990).

In the telencephalon, neurons are born in the ependymal and subependymal zone (SEZ) lining the cavities of the lateral ventricles. Adjacent to the SEZ is the underlying subventricular zone (SVZ) (Goldman, 1988). The SEZ contains at least four morphologically and molecularly distinct cell progenitors (Alvarez-Buylla and Garcia-Verdugo, 2002). Ependymal cells form an epithelia-like structure enclosing the ventricle cavities. The other cell types include astrocytes, highly proliferative stem cells, and bi-potential yet still mitotically active progenitor cells (Alvarez-Buylla and Garcia-Verdugo, 2002).

Stem cells are continuously dividing in the SEZ, giving rise to bi-potential progenitors throughout the year (Kirn et al., 1994; Alvarez-Buylla et al., 1987; Nottebohm, 1985). The SEZ progenitor cells predominantly migrate, guided by radial glia cells, into the SVZ and take residency in brain nuclei, such as the HVc and Area X, both associated with song learning and production (Alvarez-Buylla et al., 1988; Alvarez-Buylla and Nottebohm, 1988; Ling et al., 1997). During migration, bi-potential progenitor cells differentiate within a few hours after their last mitosis and become either radial glia cells or neuronal precursor cells, which ultimately mature into new neurons (Barami et al., 1995; Goldman et al., 1996; Ling et al., 1997).

It cannot be emphasized enough that neurogenesis during development as well as adulthood is spatially localized and distinct from the long-term residency of new neurons. Also, the fact that the neurons and their guide cells derive from the same progenitor cell perhaps assures proper migration of newly born neurons into specific brain nuclei (Goldman et al., 1996). It is noteworthy that growth factors such as EGF, FGF, and

TGF-α all play a vital role in stimulating proliferation of stem cells *in vivo* in the SVZ (Morshead et al., 1994; Kuhn et al., 1997). Astrocytes secrete factors that aide in the migration of neuronal precursors (Mason et al., 2001), and may also support survival of these cells (Alvarez-Buylla and Garcia-Verdugo, 2002). For instance, insulin-like growth factor-1 (IGF-1), a neurotrophin secreted from radial cells, promotes neuronal recruitment into the SVZ (Jiang et al., 1998). Also, steroid hormones were shown to influence the survival of new neurons (Rasika et al., 1994; Hidalgo et al., 1995). Steroid hormones also regulate the survival of new neurons (Rasika et al., 1994; Hidalgo et al., 1995). The effects of hormones, testosterone in particular, may be mediated through brain-derived neurotrophic factor (BDNF). Testosterone treatment increases BDNF levels in the female HVc, and infusion of BDNF into the HVc triples the amount of new neurons (Rasika et al., 1999).

Among several brain nuclei associated with song learning, only the HVc and Area X receive new neurons in adult songbirds. In Area X, the new neurons function as interneurons, whereas in the HVc, new neurons serve as projection neurons to the RA or Area X (Nottebohm, 1993). RA-projection neurons are involved in song production and undergo replacement in juvenile and adult life. In contrast, Area X projection neurons are not replaced, indicating that these neurons are more permanent (Kirn and Nottebohm, 1993; Scharff et al., 2000). In fact, Area X projection neurons are born during embryonic development and facilitate song learning.

In many ways the molecular mechanisms underlying adult neurogenesis are similar to the embryonic system of neural development. Both adult and embryonic stem

cells undergo the same mechanism of neuronal differentiation, requiring a series of basic helix-loop-helix (bHLH) transcription factors, which act on generic neuronal genes (Kintner, 2002). However, it is presently unknown whether the bHLH cascade required for adult neurogenesis is identical to that in embryonic neuroepithelial cells or not involved at all (Kintner, 2002).

Neurogenesis in the avian song system is particularly receptive to two physiological cues. These are increasing levels of sex hormones and seasonal increases in photoperiod. Presently, it is not clear whether changes in sex hormone levels and/or photoperiod influence the rate of neurogenesis, the differentiation of progenitors, the survival and migration of newly born neurons, or any combination of these factors. Adult avian brain plasticity is directly reflected by seasonally occurring song learning and production. Every spring due to increases in photoperiod and circulating levels of plasma testosterone, specific song nuclei including the HVc and RA in adult males increase in volume as a result of a dramatic addition of new neurons just prior to song production. Despite continuing long photoperiod for another two to three months, testosterone levels decline and the birds cease singing and enter a photorefractory phase. Concomitantly, the HVc and RA return to their pre-reproductive status and volume resulting from a loss of neurons.

It is intriguing that neurogenesis and migration of new neurons within and among the song nuclei occurs throughout the year in both male and female songbirds, regardless of photoperiod or sex hormone levels. Yet, this degree of neuronal plasticity does not result in larger brain volume unless the appropriate photoperiod and hormonal signals

coalesce with one another in the initiation of the reproductive status (recrudescence of genitals, territorial aggression, and song production). Therefore, volumetric increases of certain song nuclei likely result from an increased life span of newly incorporated neurons rather than an increase in the rate of proliferation of stem cells in the SEZ. This assumption is indirectly supported by several findings. Kirn and Schwable (1997) reported that decreased photoperiod from spring to fall-like conditions correlates with neuronal cell death. Kirn and Nottebohm (1993) and Alvarez-Buylla and Kirn (1997) provide evidence for an increase in the life span of newly incorporated neurons during long photoperiod and high plasma testosterone. No study has so far directly addressed the question as to whether increased plasma testosterone and/or photoperiod alter the rate of proliferation of stem cells in the SEZ in the avian telencephalon.

This present study examined whether changes in hormones and/or photoperiod affect the survival of newly derived neuronal progenitors or alter stem cell proliferation in *Junco hyemalis* wild song birds, an arctic breeding, migratory species. Using an organotypic cell culture system, telencephalon brain slices containing the HVc and the overlaying SEZ obtained from either long day (LD) or short day (SD) intact juncos were labeled with BrdU and cultured for 14 days in the presence of testosterone or estrogen. Our results suggest that photoperiod and increased gonadal hormone levels indeed support or promote the life span of newly incorporated cells into the HVc of LD juncos. In contrast, only testosterone provides a similar protection of cell survival in the HVc of SD juncos. Importantly, our results do not support an alteration in the rate of stem cell proliferation.

4.2 Results

Male and female *Junco hyemalis,* which were captured the previous fall and kept in captivity for 7 months on an 8hr: 16hr light/dark cycle (short days, SD) were randomly separated into two groups containing both males and females. One group (long day birds, LD) was photostimulated with a gradual increase of their photoperiod by 2 hours every other day to a final light/dark cycle of 20hr: 4hr and maintained on this photoperiod for 30 days. The SD birds remained on the 8hr: 16hr light/dark cycle. After 30 days, SD and LD birds were sacrificed by decapitation and organotypic HVc brain slice cultures established (Figure 4.1). Unfortunately, one day prior to sacrificing the LD birds, 5 of the subjects (two male, three female) disappeared from the animal holding facility at UAF. Consequently, three birds from the short-day group were subjected to long photoperiods as mentioned above. These subjects were processed and analyzed in the same manner as the other birds, however, at different times. This discrepancy must be considered and could have imposed slight deviations into the experimental procedure.

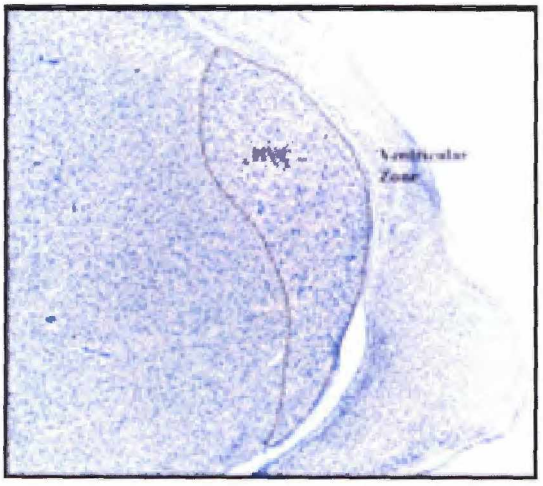

Figure 4.1. Organotypic Culture of Junco HVc
Portion of organotypic slice including the HVc and the overlying Subventricular Zone (SVZ). This 15μm slice was stained using a Nissl stain procedure immediately after sacrifice (SD bird).

Incorporation of 5-bromo-2'-deoxyuridine (BrdU) into genomic DNA is widely used to label proliferating cells *in vitro* and *in vivo*. Addition of BrdU for a short time period (pulse label) will therefore label the fraction of cells undergoing DNA synthesis during the S-phase of the cell cycle. However, any cells undergoing DNA repair will also incorporate BrdU.

To examine cell proliferation in the junco telencephalon, we pulse-labeled organotypic cultures of brain slices containing the SVZ and the HVc after one day in culture and again after 14 days. Incorporation of BrdU was revealed by indirect immunocytochemistry using a secondary fluoresceine microscopy inspection. The vast majority of BrdU-positive cells were concentrated within the subependymal zone (SEZ) and the underlying HVc (Figure 4.2). We found very few and scattered BrdU-positive cells outside of the HVc and the SEZ, and the virtual absence of BrdU-positive cells in

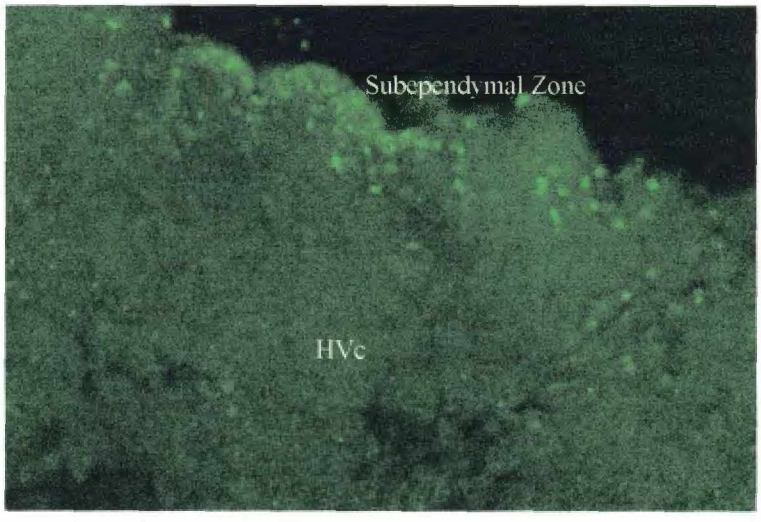

Figure 4.2 Incorporation of BrdU into the SEZ/SVZ in Organotypic HVc Cultures
Fluorescent BrdU-labeled cells are found in the SVZ and the underlying HVc. BrdU fluorescence is virtually absent in other brain regions. (20X, Confocal)

regions other than the HVc implies that the migration of bi-potential progenitor cells into the SVZ is restricted and may reflect the fact that these progenitor cells differentiate both

into radial glia cells required for neuronal guidance as well as post-mitotic neurons. Moreover, the appearance of BrdU-positive cells in other regions regardless of photoperiod or the presence of hormones is likely attributed to a proliferation of non-neuronal cells and/or DNA repair events.

4.2.1 Long day photoperiod correlates with more BrdU+ cells in the HVc

Only male juncos respond to long photoperiod with a volumetric increase in their HVc, whereas female juncos retain a constant HVc volume. This volume change results from the addition of newly generated neurons, which could result from an increased rate of stem cell proliferation in the SEZ in juncos on long day photoperiod.

We subjected organotypic telencephalon slices containing the HVc obtained from LD or SD birds to a brief BrdU pulse at the beginning and the end of the 14 day culture period and determined the number of BrdU-positive cells in the HVc under a given condition. Organotypic cultures were supplemented either with testosterone, β-estradiol, or EtOH.

Disregarding hormonal treatments, our initial assessment revealed a significant increase in BrdU-positive (BrdU+) cells in the HVc from LD juncos compared to SD juncos (Figure 4.3). The mean number of BrdU+ cells in the HVc ($0.3mm^2$) from LD juncos was 49 ± 13 cells ($n=46$, $p<0.001$), as opposed to 33 ± 12 ($n=27$) BrdU+ cell in SD juncos. This finding is consistent with numerous studies both *in vivo* and *in vitro*. It is plausible that long photoperiod increases the rate of stem cell proliferation, thus resulting in an increased presence of BrdU+ cells in the HVc.

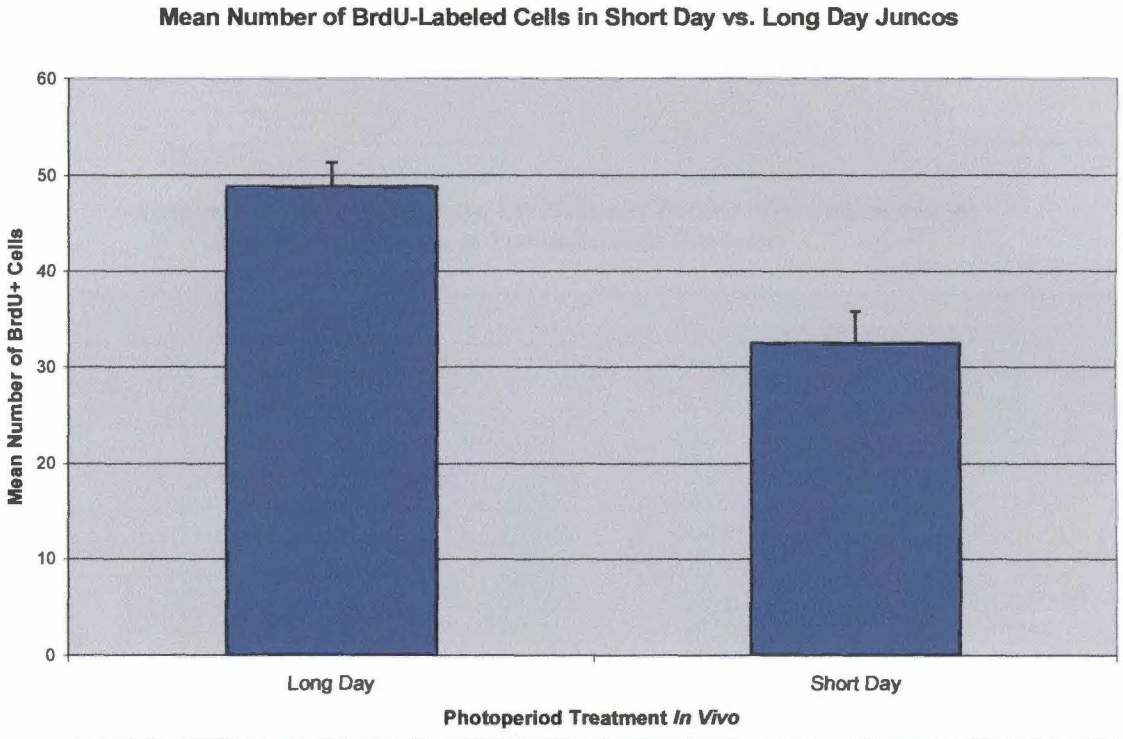

Figure 4.3. There was an increased BrdU+ cell number in the HVc of juncos kept on LD photoperiod compared to SD. HVc cultures obtained from LD or SD juncos were labeled with BrdU at day 1 and day 14 *in vitro*. BrdU incorporation was revealed by immuncytochemistry. Regardless of hormonal supplements in the HVc cultures, LD juncos incorporated significantly more BrdU+ cells in the HVc ($p<0.001$). Error bars are standard error.

4.2.2 Gonadal hormones are critical for increases in BrdU+ cells in the HVc

Under long photoperiod, male juncos also exhibit increased plasma testosterone levels. In addition, testosterone (T) supplementation of male and female juncos on short day photoperiods resulted in larger HVc volumes. Therefore, we cultured HVc slices obtained from male and female juncos in the presence of testosterone, estrogen, or carrier (EtOH) to explore the effects of gonadal hormones. Incorporation of BrdU+ cells was indistinguishable between testosterone and estrogen supplementation. Although not

statistically significant, there were fewer BrdU+ cells in our controls. The apparent difference could indicate a trend, and increasing our sample size may yield a significant difference (Figure 4.4).

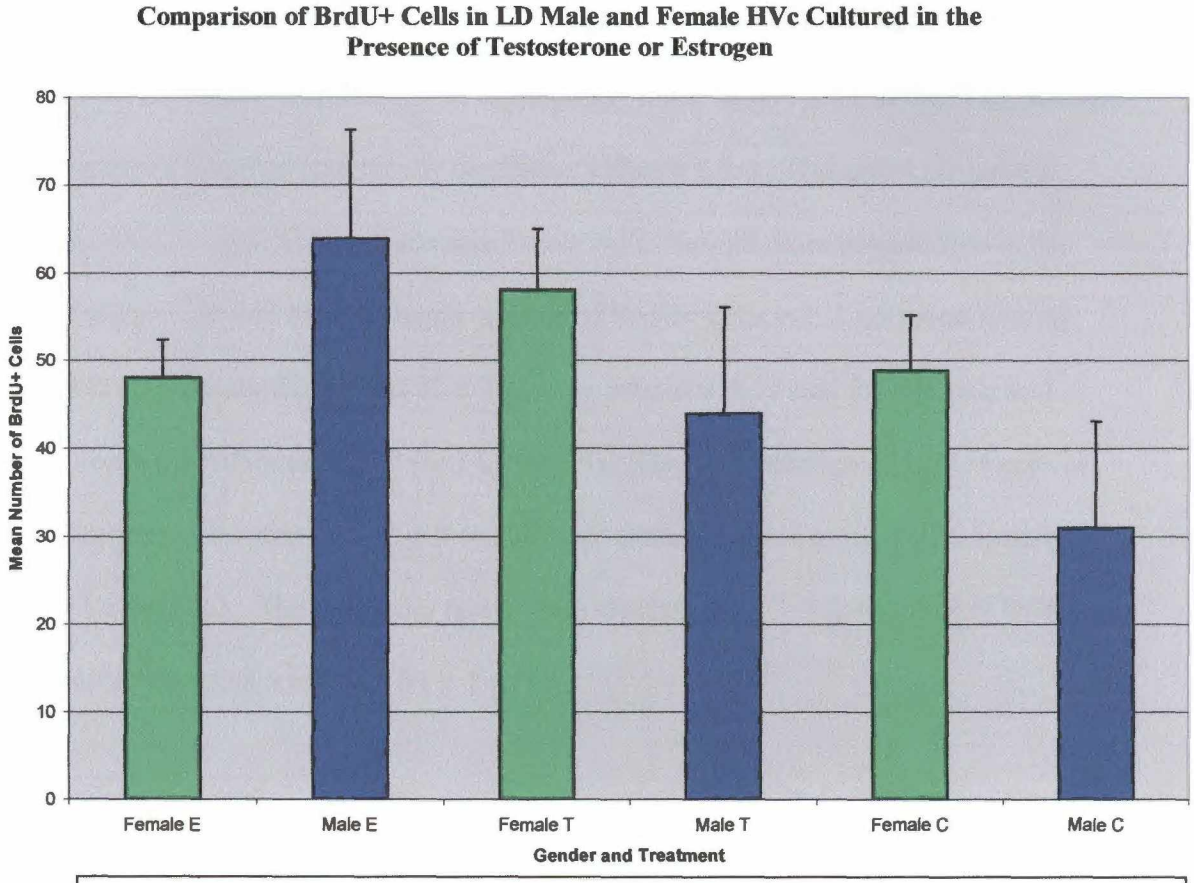

Figure 4.4. Mean number of BrdU+ cells in males and females after two weeks in culture in the presence of hormones. There was no significant difference between males and females in either the treatment or control groups.

In the presence of testosterone, we counted 44 ± 12 (n=4) and 58 ± 7 (n=12) BrdU+ cells per male or female HVc, respectively. Similarly, in the presence of estrogen, we determined 48 ± 7(n=2) and 51 ± 3 (n=16) BrdU+ cells per male or female

HVc, respectively. In our controls (EtOH), we measured 31 ± 12 (n=1) BrdU+ cells per male HVc and 49 ± 6 (n=11) BrdU+ cells per female HVc.

Since estrogen and testosterone were equally potent in stimulating incorporation of BrdU+ cells into male and female HVc, we compared this effect between controls and all data from gonadal hormones combined. Clearly, supplementing HVc slices from LD juncos with hormones correlated with significantly more BrdU+ cells in the HVc, though the differences were not statistically significant (Figure 4.5a). This effect of gonadal hormones was evident in both male and female HVc, though more pronounced in the male (Figures 4.5b and c). The mean number of BrdU+ cells in LD hormone-treated males and females combined was 52 ± 3 (n=34), whereas the mean for the male and female control group was 35 ± 2 (n=14, $p<0.05$). The mean number of BrdU+ cells in hormone-treated females was 52 ± 3 (n=28), and female control group had a mean of 37 ± 2 (n=12, $p<0.05$). The LD males had a mean number of 51 ± 3 (n=8) BrdU+ cells, and the control males had a mean of 31 ± 2 (n=2).

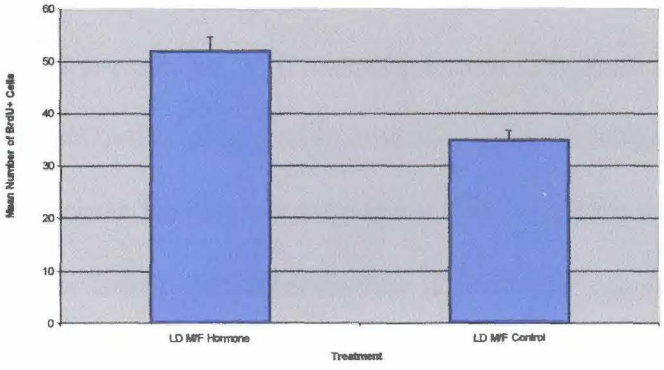

Figure 4.5a
LD Male and Female HVc

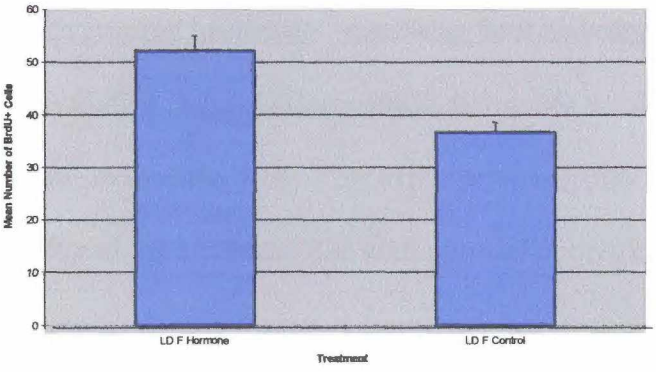

Figure 4.5b
LD Female HVc

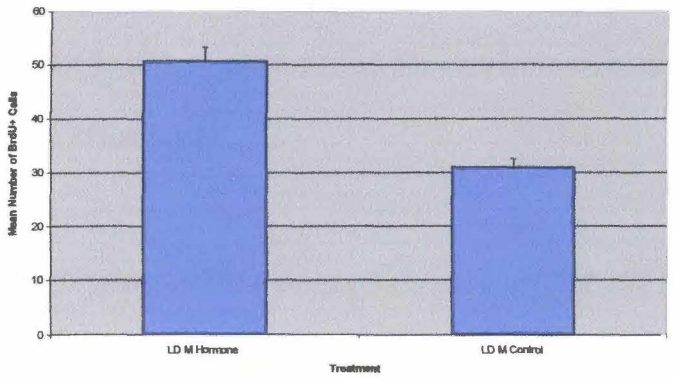

Figure 4.5c.
LD Male HVc

Figure 4.5. (a) Male and female combined hormone vs control. (b) Female hormone-treated vs. control. (c) Male hormone-treated vs. control. Results showed significant increases in BrdU+ cells in hormone-treated female and combined subjects compared to control subjects. Male juncos were not significantly different, though showed the same trend. They were probably not significant due to the low sample number as a result of disappearing subjects.

Taken together, this result provided an intriguing and novel outcome. First, the data reveals that both male and female HVc from LD juncos exhibit increased numbers of BrdU+ cells compared to controls, although insignificant in males. However, one would expect to find significantly fewer BrdU+ cells in female HVc, irrespective of treatment, since female juncos retain their HVc volume regardless of long day photoperiod. Second, increases in BrdU+ cells in male and female HVc required the supplementation with gonadal hormones. Assuming BrdU labeling reflects rates of stem cell proliferation, this finding suggests that gonadal hormones would dramatically affect cell division in the male and female SEZ. This explanation is highly unlikely, since HVc slices were cultured for less than 24hr with gonadal hormones prior to the BrdU pulse label. This outcome does not support the assumption that changes in photoperiod alter the rate of cell proliferation. Alternatively, our data favor that variation in the number of BrdU+ cells in response to long photoperiod derivers from an enhanced survival of newly incorporated cells in the HVc.

4.2.3 Gonadal Hormones Increase BrdU+ Cells in the HVc of SD Juncos

Under SD photoperiod, neuronal plasticity *in vivo* occurs at a basal rate with no change in HVc volume regardless of sex. Also, plasma levels of gonadal hormones are low in male and female juncos. Experimentally increasing plasma testosterone in SD males and females was shown to increase HVc volume, whereas estrogen was ineffective. We examined whether the number of BrdU+ cells in the HVc of SD male and female juncos varies in response to hormonal treatments. Disregarding the sex of the subjects,

HVc slices cultured juncos in the presence of testosterone exhibited a significant increase in BrdU+ cells (37 ± 3, n=11, $p<0.05$). Similarly, estrogen-treated HVc slices also showed an increase in BrdU+ cells, although it was not statistically significant (36 ± 4, n=5) compared to controls (32 ± 3, n=11) (Figure 4.6).

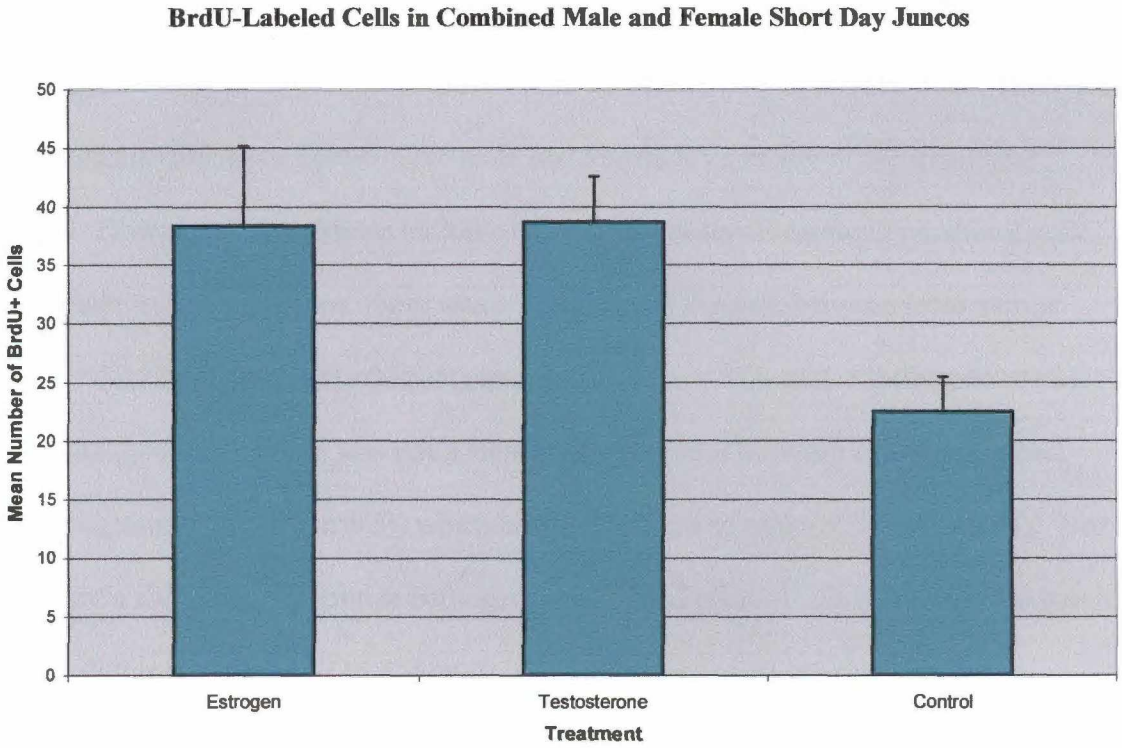

Figure 4.6. There was an Increased number of BrdU+ cells in the HVc from SD Juncos with gonadal hormone supplement compared to controls. Although the estrogen treated HVc showed the same trend as those treated with testosterone, there was only a significant difference between the testosterone-treated and control subjects ($p<0.05$).

4.2.4 Gonadal Hormones Stimulate Incorporation of BrdU+ Cells into Male and Female HVc

After two weeks in culture with hormone treatments, the ethanol-treated SD male and female HVc had means 15 ± 11 (n=3) and 27 ± 8 (n=7) labeled cells respectively (Figure 4.7). Testosterone-treated SD male and female HVc exhibited 41 ± 12 (n=8) and 51 ± 9 (n=20) BrdU+ cells per HVc respectively. Estrogen-treated male HVc had 32 ± 9 (n=4) BrdU+ cells compared to 63.00 ± 17 (n=1) BrdU+ cells in female HVc. There was no significant difference between the male and female HVc in any of the treatment groups. However, in a pairwise multiple comparison procedure among combined male and female treatment groups, there was a significant difference between testosterone (mean = 38.727, n= 11) and ethanol (mean = 22.545, n = 11), with a difference of ranks of 16.182 ($p<0.05$). There was not a significant difference between testosterone and estrogen (mean = 38.400, n = 5), which had a difference of ranks of .327 ($p>0.05$). Nor was there a significant difference between estrogen and ethanol. Nevertheless, the much greater difference of ranks (15.855, $p>0.05$) is indicative of an existing significance. Although there was no significant difference between testosterone- and estrogen-treated HVc, the close difference of ranks deems it necessary to take into consideration that the low sample number may have affected the results.

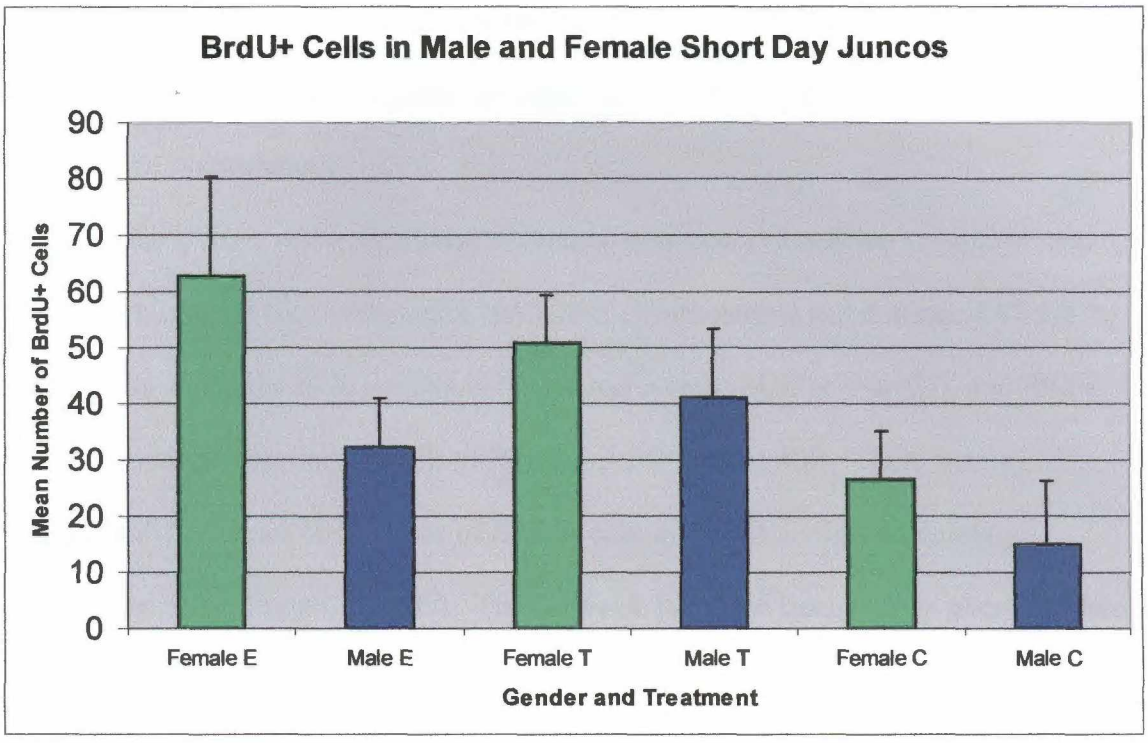

Figure 4.7 SD male and female HVc cultured in the presence of testosterone or estrogen. Testosterone and estrogen were equally potent to stimulate incorporation of BrdU+ cells into male or female HVc. Also, testosterone significantly increased the presence of BrdU+ cells in HVc compared to controls. For estrogen, a similar trend was revealed, but not statistically significant.

Taken together, our data demonstrates that gonadal hormones stimulate incorporation of BrdU+ cells into the HVc obtained from male or female juncos kept on short day photoperiod. Whereas estrogen increased BrdU label in the HVc from LD juncos, this effect was not significant in the HVc from SD juncos.

4.2.5 Incorporation of BrdU+ Cells into HVc Is Dominated by Gonadal Hormones

Previous experiments have revealed a strong influence of gonadal hormones on the incorporation of BrdU+ cells into the HVc. Moreover, effects of gonadal hormones

were similar on HVc from male as opposed to HVc from female juncos. We tested whether gonadal hormones stimulate incorporation of BrdU+ cells into the HVc regardless of photoperiod.

Indeed, there was a significant difference between testosterone vs. ethanol and estrogen vs. ethanol supplementation ($p=0.003$). Testosterone had a mean of 47 ± 3 BrdU+ cells within the HVc ($n=28$), estrogen had a mean of 43 ± 4 ($n=23$), and EtOH had a mean number of BrdU+ cells of 32 ± 3 ($n=24$) (Figure 4.8). There was no significant difference in the number of BrdU+ cells in the HVc when comparing testosterone and estrogen ($p>0.05$). Thus, overall, hormone treatment *in vitro* stimulated the incorporation of BrdU+ cells into the HVc of juncos regardless of sex and photoperiod.

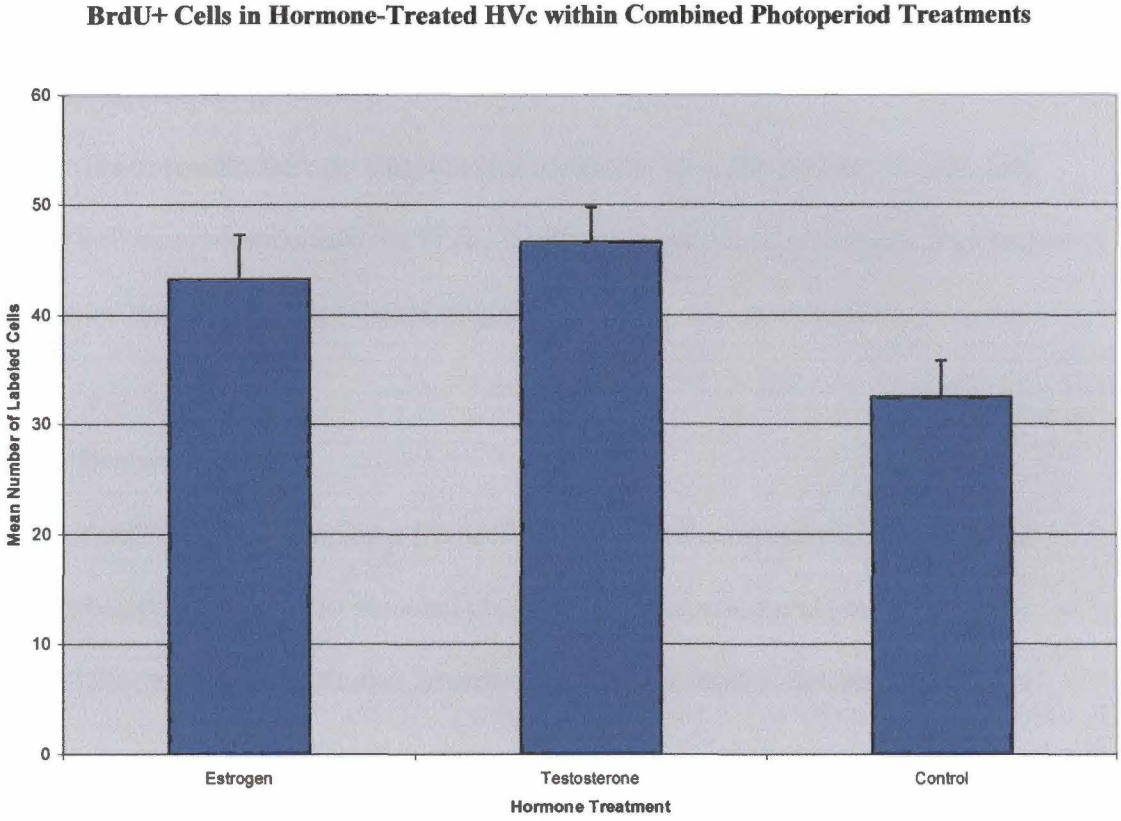

Figure 4. 8 Gonadal hormones increase BrdU label in HVc regardless of photoperiod. Testosterone and estrogen both stimulate the incorporation of BrdU+ cells into the HVc from LD and SD juncos.

A two way ANOVA was performed to test the dependence of light and hormone treatments upon one another to produce specific results. We found that there was a statistically significant difference in the mean values of photoperiod treatments compared to hormonal treatments that is greater than would be expected by chance ($p<0.001$). Similarly, the difference in means among hormone treatments is greater than would be expected by chance after allowing for the effects of photoperiod treatment ($p=0.009$). Therefore the effect of light treatment does not depend upon what hormonal treatment is

being applied, and there is not a statistically significant difference between light and hormone treatments ($p=0.627$).

These results indicate that gonadal hormones have the potency to stimulate BrdU+ cell incorporation into the HVc. Neither the gender of juncos nor their exposure to short or long day photoperiod has significance.

4.3 Discussion

Adult songbirds display a tremendous degree of neuronal plasticity in their telencephalon in response to seasonal changes in photoperiod and plasma levels of gonadal hormones. In particular, neuronal plasticity embodies the addition of new neurons derived from proliferating stem cells in the SEZ into brain nuclei associated with song learning and production. Functional incorporation of new neurons is demonstrated in the physiological phenotype of singing. Although neuronal plasticity occurs in both female and male songbirds, only males exhibit seasonal changes in song brain nuclei due to neuronal addition. Neuronal plasticity could arise from seasonal changes in the rate of stem cell proliferation, the speed of precursor migration, the fate of precursor differentiation, the survival of new neurons, or a combination of any of the these factors.

This study explored the effect of photoperiod and gonadal hormones on newly generated cells in SEZ using BrdU-incorporation in the genomic DNA of proliferating cells.

4.3.1 Distinction between Dividing and DNA Repairing Cells

Organotypic telencephalon slices containing the HVc and SEZ were cultured in the presence of testosterone, estrogen, or ethanol for 14 days. Both male and female juncos kept on long or short photoperiod were used for the production of organotypic telencephalon HVc slices. A one-hour BrdU pulse was employed to label proliferating cells at day one and day 14 in culture. Importantly, BrdU is not only incorporated into proliferating cells during DNA replication, but also into cells undergoing DNA repair. Generation of bi-potential precursors from stem cells to the appearance of post-mitotic cells in the HVc occurs over 5-7 days, estimated from data in the literature. A one hour BrdU pulse of HVc brain slices at day 1 in culture will label dividing cells in the SEZ (Figure 3.2) and DNA repairing cells in the HVc and elsewhere in the telencephalon. Over a 14-day culture period, a fraction of labeled cells will migrate from the SEZ into the HVc and thus increase the presence of BrdU+ cells. A second one-hour BrdU pulse at the end of the culture period labels all dividing and DNA repairing cells. Yet, there is no contribution to BrdU+ cells in the HVc with the exception of DNA repairing cells. Therefore, this BrdU-labeling paradigm takes into account basal occurrence of DNA repair. Any change in the number of BrdU+ cells is thus reflecting newly generated cells migrated into the HVc from the SEZ.

We found strong BrdU-labeling in the SEZ, the location of dividing stem cells, and in the adjacent HVc. The virtual absence of BrdU+ cells in other brain regions supports a very low fraction of DNA repairing cells. The junco HVc spans approximately 3 mm anterior to posterior, and we cultured 300 μm thick sections exposed

to BrdU labeling. These 300 μm thick sections were further partitioned into 20 μm slices for confocal analysis of BrdU+ cells. In the absence of hormone supplementation, we counted approximately 25 BrdU+ cells per HVc section regardless of photoperiod and gender of juncos. BrdU+ cells derived from a one-hour pulse labeling represent an approximation for a daily addition of newly generated cells to the HVc. Therefore, we estimate 3,500-4,000 BrdU+ cells per entire HVc per day. Since the male HVc reaches a maximum volume over less than 14 days, a total of 45,000 to 55,000 cells are added to the HVc using our estimated daily addition. In male juncos, the total cell number in the HVc on short days is around 100,000 and in the long day HVc around 150,000. Conclusively, our data is in close agreement and likely represents an adequate estimate, even while neglecting a small background of DNA repairing cells. Kirn et al. (1991) reported a daily addition of new neurons to the zebra finch HVc of 30% with regard to 120,000 neurons per zebra finch HVc of similar size as a junco HVc, which translates to 27 BrdU+ cells in a 20 μm slice.

4.3.2 Stem Cell Proliferation and Physiological Stimuli

Prior to this study, there was no conclusive evidence whether gonadal hormones do affect the rate of proliferation in the SEZ (Brown et. al, 1993; Hidalgo et. al, 1995). Brown et al. (1993) reported that *in vivo* treatment with testosterone, estrogen, or hormone-inhibiting drugs did not alter the division rate or the number of mitotically active cells (stem cells). In addition, hormone treatment following ^{3}H-thymidine labeling increases the number of new neurons incorporated into the HVc (Hidalgo et al., 1995;

Rasika et al., 1994; Burek et al., 1995). Therefore, the influence of hormones on the incorporation of newly integrated neurons appears to be restricted to postmitotic events (Alvarez-Buylla, Kirn, 1997). In a study by Holloway and Clayton (2000), estrogen was found to be necessary for the completion of the HVc-RA song production pathway during development among captive male zebra finches. Using a BrdU-labeling paradigm, a change in BrdU+ cells in the HVc could reflect a change in the rate of proliferation in the SEZ, a change in the migratory rates, a change in cell fate upon differentiation, a change in survival of new cells or a combination of these factors. Nevertheless, this approach should distinguish whether photoperiod and gonadal hormones affect pre- or post-mitotic events.

LD juncos did exhibit significantly more BrdU+ cells within the HVc compared to SD juncos, regardless of hormone treatments. Presently, no experiments have addressed whether photoperiod affects the migration, differentiation, or lifespan of new cells in the HVc. It is plausible that the LD juncos have a higher rate of neuronal stem cell proliferation than do SD juncos. Biologically speaking, it makes sense that the SD junco would not go to great lengths to produce a high number of new neurons during a time in which these are not needed and would only undergo apoptosis shortly after birth. However, captive male and female juncos were not gonadectomized prior to photostimulation *in vivo*. Thus, intrinsically produced hormones may have had a residual effect throughout the *in vitro* portion of the experiment. Our initial experiment reveals an increase in BrdU+ cells in the HVc of LD juncos, which could be attributed to changes of either mitotic or post-mitotic events.

Female juncos on LD photoperiod retain an HVc volume identical to that under SD photoperiod conditions and gonadal hormone levels. Regardless of our experimental conditions, LD females should only show basal levels of BrdU+ cells. However, we measured an increase in BrdU+ cells in the presence of testosterone and estrogen. This implies that hormones would indeed produce an abrupt change in physiology in less than 24 hours. Since a cell cycle is around 24 hours, it is plausible that gonadal hormones stimulate an increased rate of stem cell proliferation. This finding favors that gonadal hormones support post-mitotic events rather than the rate of mitosis itself. Moreover, increased BrdU label in the HVc from LD juncos as opposed to SD juncos further implies that photoperiod renders the system susceptible to the effect of gonadal hormones. In this respect, the HVc of LD males shows increased BrdU labeling with hormones, but not without, demonstrating the importance of gonadal hormones to invoke a significant change. As shown in Figure 3.6, gonadal hormones correlate with an increased presence of BrdU+ cells in the HVc of both male and female juncos. It is noteworthy that in LD juncos, both testosterone and estrogen potentially stimulate BrdU labeling in the HVc, which would only be expected for testosterone.

In summary, increased presence of BrdU+ cells in the HVc of male and female LD juncos strongly favors a hormonal effect on post-mitotic events rather than mitosis of stem cells.

4.3.3 Gonadal Hormones and BrdU Labeling in HVc

On short day photoperiod, male and female juncos exhibit a basal HVc volume and low levels of plasma gonadal hormones. In our experiments, the HVc from SD juncos contained significantly less BrdU+ cells compared to the HVc from LD juncos. Assuming gonadal hormones alter mitosis of stem cells, exposure of the HVc slices to gonadal hormones for less than 24 hours, i.e. less than only cell cycle, should have no effect on BrdU labeling in the HVc. In sharp contrast, gonadal hormones dramatically increased BrdU+ cell presence in male and female HVc from SD juncos. This finding further implies that gonadal hormones alter post-mitotic events and not mitosis of stem cells in the SEZ. The dominant effect of gonadal hormones is demonstrated when neglecting both photoperiod and gender of juncos (Figure 3.8).

Long day females show more BrdU+ cells with testosterone compared to estrogen, but the difference is not significant. In contrast, LD males show more BrdU+ cells with testosterone and estrogen alike.

4.3.4 Testosterone, Estrogen, and BrdU+ Cells in the HVc

Short day males and females show higher cell labeling only under the influence of testosterone treatment. When hormone treatments are compared without regard to photoperiod groups, estrogen has a significant effect on the number of BrdU+ cells. However, when hormone treatments are compared separately within LD or SD photoperiod, estrogen only affects the photosensitive group. We also found that photoperiod and hormone treatments do not have a statistically significant interaction

with each other. Thus, we are unable to state that estrogen only influences the presence of BrdU+ cells in the HVc of LD juncos. This ambiguity must be attributed to either the low number of specimens (due to loss of subjects) in the photorefractive group, human error, or simply chance.

In summary, our investigations reveal a novel and unexpected conclusion. Photoperiod and hormones are most likely altering post-mitotic events, including speed of migration to the HVc from the SEZ, the fate of differentiating precursors, or the survival of new post-mitotic neurons in the HVc. In addition, photoperiod seems to render the junco telencephalon receptive for estrogen as a regulator of post-mitotic events, resulting in cell incorporation into the HVc. These studies have addressed for the first time, to our knowledge, the effects of photoperiod and gonadal hormones on cellular proliferation and incorporation into the HVc of songbirds.

Chapter 5

Analysis of Apoptosis Using TUNEL and Comet Assay

5.1 Introduction

There is a constant influx of new cells migrating into the HVc regardless of photoperiod and plasma hormone levels. Since the HVc does not increase in size and volume until the spring, there must be an equal number of cells being removed to counterbalance the ones coming in. Apoptosis is a common method of organized cell removal that transcends all animal species. It allows cells to die without releasing its' contents onto the surrounding cells and thereby poisoning neighboring cells. Because the presence of gonadal hormones in culture appeared to increase cell survival in the HVc of long and short day juncos, we tested whether the same conditions would decrease cellular apoptosis in the HVc of either or both SD and LD birds. We cultured LD and SD junco HVc and overlying SEZ for 14 days in the presence of testosterone or estrogen. Apoptosis was analyzed using a TUNEL stain, which tags apoptosis-specific breaks in DNA and allows for the maintenance of cellular architecture throughout the assay. We also used a Comet Assay, which disrupts cellular architecture. Cell suspensions are exposed to an electric field causing DNA fragments to migrate out of the cell nucleus. In particular, apoptotic cells have short broken pieces of DNA that is pulled out into a comet tail-like formation, while healthy cells maintain a spherical shape. Necrotic cells take on a "blob-like" structure.

5.2 TUNEL Assay Results

5.2.1 Photosensitive (Short Day) Juncos

Adolescent Male and female juncos were captured in early September and held in captivity until the spring allowing ample time to reach breeding maturity. Throughout their captive time, the juncos were held on a short day light cycle (8hrs light: 16hrs dark) to ensure a photosensitive state before sacrifice. Among the SD juncos, we did find that there is a significant difference in the number of apoptotic cells under testosterone exposure compared to controls ($p<0.05$), or estrogen ($p<0.05$). The mean number of apoptotic cells in each $0.3mm^2$ section of HVc exposed to testosterone was 15 ± 2. Estrogen-treated slices revealed 37 ± 2 apoptotic cells, and controls had 40 ± 2 apoptotic cells per $0.3mm^2$ section of HVc (Figure 5.1). This result demonstrates that testosterone attenuates apoptosis in the HVc of SD male and female birds. These means were calculated with no distinction between the sexes of the specimens.

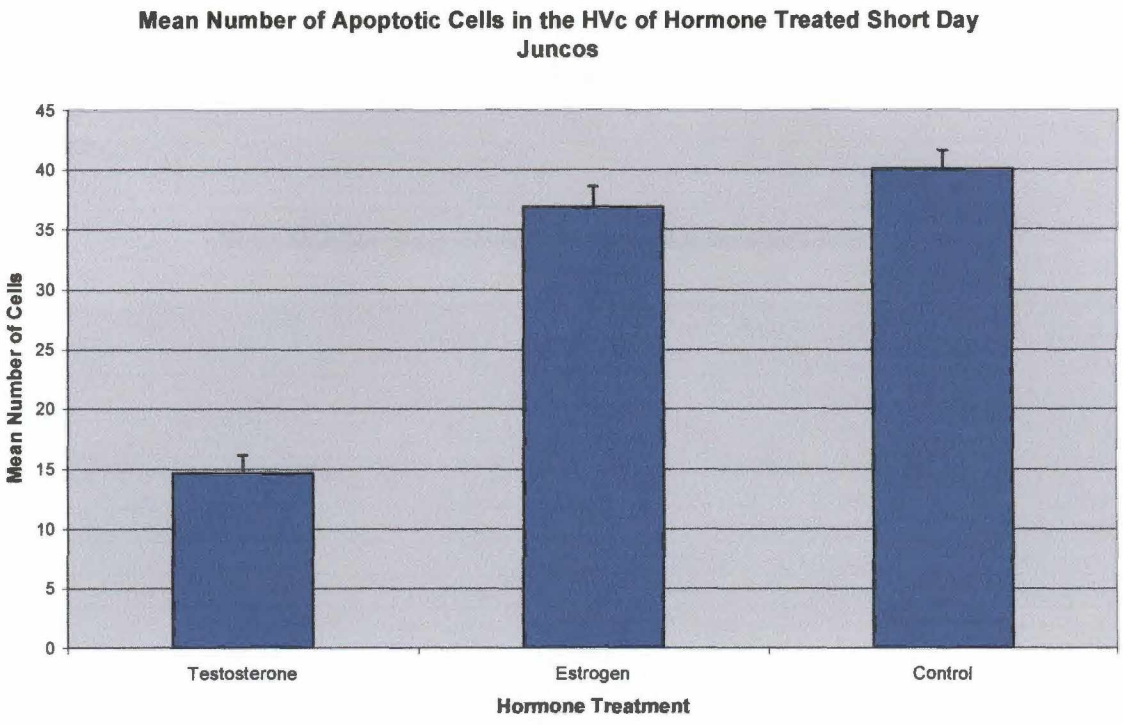

Figure 5.1 There was a significant difference between testosterone and estrogen and testosterone and control ($p<0.05$). There was not a significant difference between estrogen and the control group ($p>0.05$).

5.2.2 Photostimulated (Long Day) Juncos

Captive male and female juncos were held on an 8hr light: 16hr dark light cycle throughout the winter. In early April their light cycle was increased by 1 hour per day until reaching 20 hr light: 4 hr dark, similar to the arctic summer photoperiod. Juncos were maintained on this long day cycle for four weeks. All males were singing prior to sacrifice. In these photostimulated juncos, we found that both testosterone and estrogen attenuate apoptosis. There was a significant difference in the number of apoptotic cells upon estrogen exposure compared to control ($p<0.05$) and the testosterone exposure compared to control group ($p<0.05$). The mean numbers of apoptotic cells per $0.3mm^2$

section of HVc were 19 ± 1 (testosterone), 22 ± 1 (estrogen), and 43 ± 1 (control) (Figure 5.2). These means were calculated with no distinction between the sexes of the specimens.

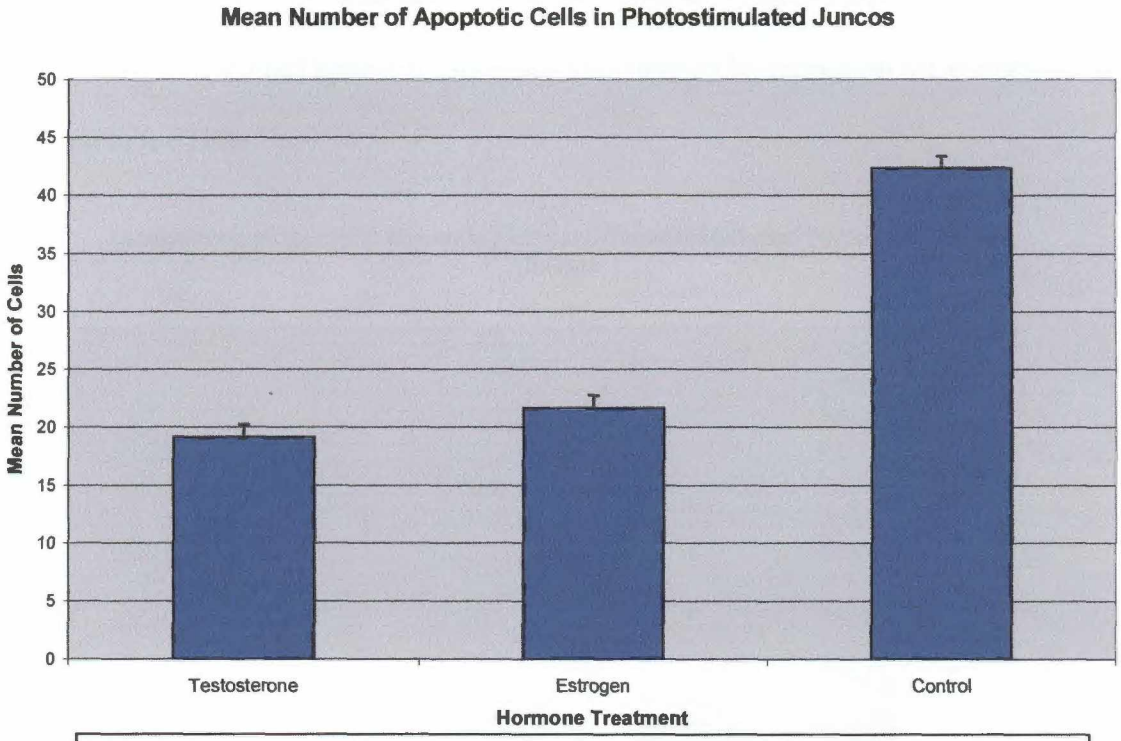

Figure 5.2. Mean number of apoptotic cells in male and female hormone-treated photostimulated juncos. There is a significant difference in the average number of apoptotic cells in testosterone vs. control group ($p<0.05$) and estrogen vs. control group ($p<0.05$).

5.2.3 Influence of Hormones and Gender in SD Birds

We used one-way ANOVA tests on the photosensitive (SD) sample data to determine if there was a distinction between the effect of *in vitro* hormone treatment in male and female HVc. We found no statistically significant difference between the males and females in any of the three conditions ($p>0.05$). Testosterone-treated males had

means of 15 ± 2 apoptotic cells (n=17), and the females had 14 ± 2 apoptotic cells (n=21, p=0.284) per 0.3mm^2. Estrogen-treatment revealed 36 ± 2 (n=15) in males, and 37 ± 2 (n=15, p=0.930) in females, compared to 41 ± 2 (n=19) in EtOH-treated males and 40 ± 2 (n=19, p=0.730), apoptotic cells in the HVc (per 0.3mm^2) (Figure 5.3). Conclusively, gender, therefore, did not appear to influence the effect of hormones on the occurrence of apoptosis in the HVc.

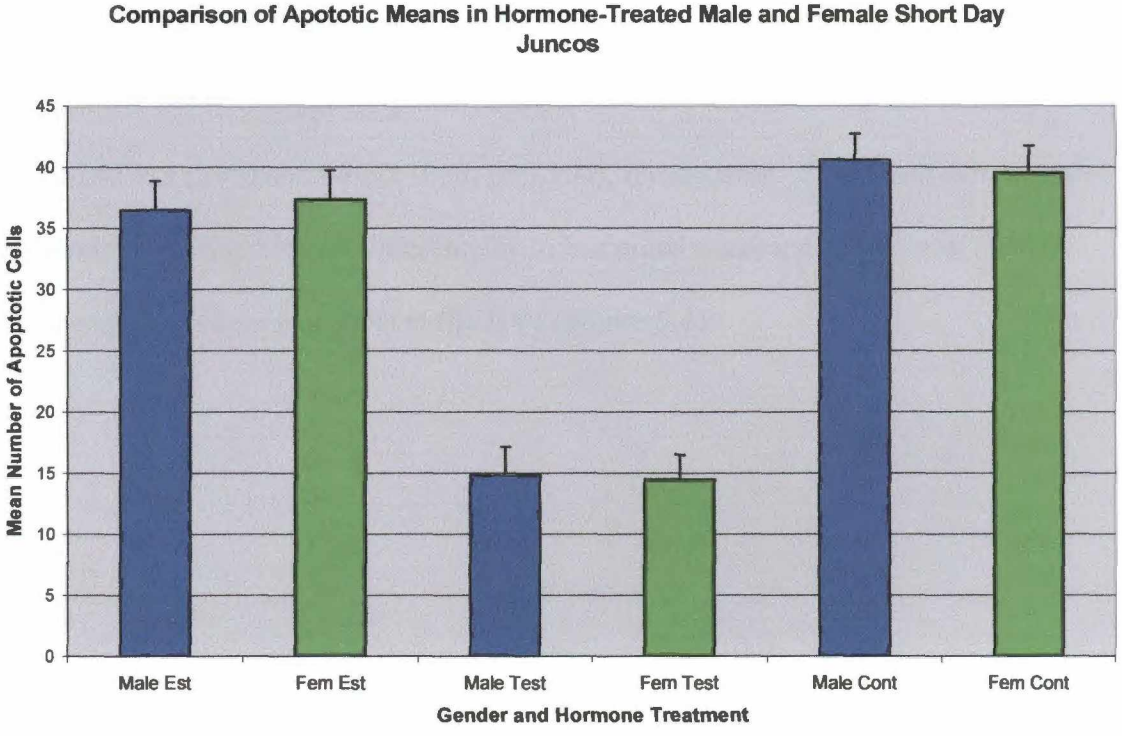

Figure 5.3. Mean apoptotic cells in 0.3mm^2 section of HVc in SD male and female hormone-treated juncos. There was no statistically significant difference between males and females in any of the treatment groups (p>0.05). (PS=Photosensitive, M=male, F=female, Test=testosterone, Est=estrogen, No T=control).

5.2.4 Influence of Hormones and Gender in LD Juncos

Similarly, we employed one way ANOVA tests to evaluate apoptosis in the HVc from photostimulated (LD) males and females to determine if hormone treatment affects genders differently. There was no significant difference in apoptotic numbers between male and female juncos in any of the treatment groups ($p>0.05$). Testosterone-treated male and female HVc exhibited 18 ± 2 ($n=8$) and 20 ± 2 ($n=8$) apoptotic cells, respectfully ($p=0.512$). Whereas, estrogen-treated males had 23 ± 2 ($n=8$), and females had 21 ± 2 ($n=8$) ($p=0.222$) apoptotic cells per $0.3mm^2$. Control male and female HVc revealed 41 ± 2 ($n=7$) and 44 ± 2 ($n=9$, $p=0.194$), respectively. This result demonstrates that gender does not respond differentially to hormonal treatments and reveal an indistinguishable neuron survival in the HVc (Figure 5.4).

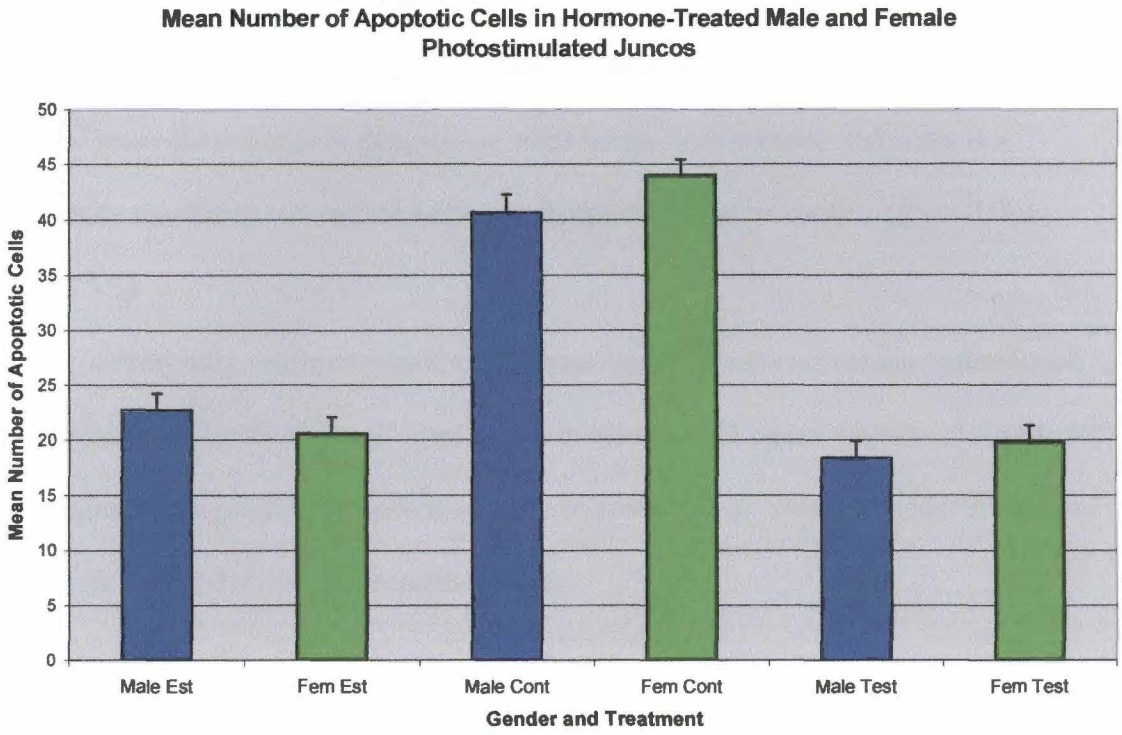

Figure 5.4. Mean number of apoptotic cells in 0.3mm^2 sections of HVc in LD male and female hormone-treated samples. There was no statistically significant difference between male and female specimens in any of the treatment groups.

5.2.5 Two-way ANOVA for Hormone vs. Photoperiod Treatment

We employed a two-way ANOVA to determine whether hormonal effect *in vitro* is dependent upon photoperiod treatment *in vivo*. We found that the difference in the mean values of apoptotic cells among different levels of photoperiod treatments is not great enough to exclude the possibility that the difference is simply due to chance or random sampling variation after allowing for the effects of hormone treatments. Although there was not a statistical significance ($p = 0.054$), the difference in the mean

numbers of apoptotic cells among the different hormone treatments is greater than expected by chance after allowing for effects of differences in photo treatment. The effect of photoperiod strictly depends on what hormone is present, and there is a statistically significant interaction between photoperiod and hormone. ($P = <0.001$) (Figure 5.5).

Interestingly, estrogen was the only hormone treatment where photostimulated birds were significantly different from photosensitive or SD birds ($p<0.05$). In contrast, the number of apoptotic cells with respect to testosterone or control in photostimulated birds is similar to that in photosensitive birds.

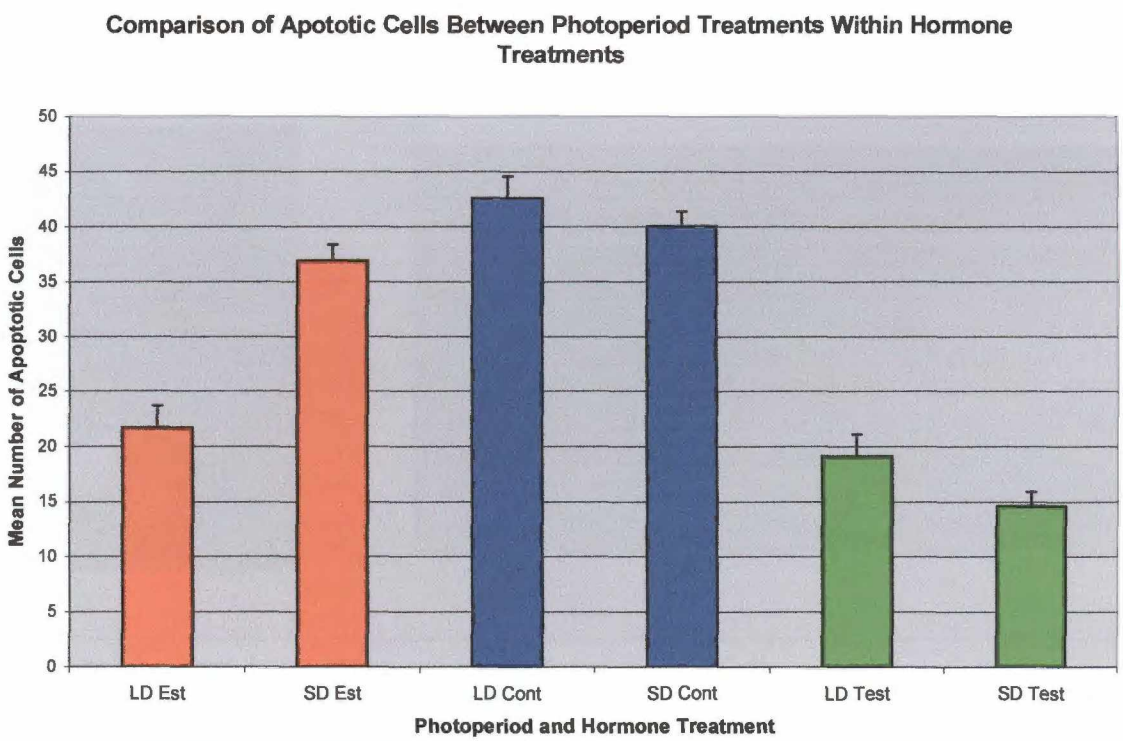

Figure 5.5. Mean number of apoptotic cells per $0.3mm^2$ section of HVc in hormone-treated, short day and long day juncos. There was a significant difference only between the SD E- and the LD E-treated birds ($p<0.05$). There was no difference in means between SD T and LD T, or the SD controls and the LD controls.

5.3 Analysis of Apoptosis Using Comet Assay

5.3.1 Photosensitive (SD) Male and Female Juncos

Comet analysis allows visual distinction of apoptotic cells from healthy cells using fluorescence microscopy, yet does not maintain tissue architecture. Apoptotic cells were identified by the presence of comet-like staining of the cell nucleus resulting from the differential migration of fragmented apoptotic DNA in an electric field. Healthy cells were indicated by circular intact nuclei (Figure 5.6). Using a t-test analysis against testosterone-treated samples, we found that there was a significant difference between testosterone and estrogen (P=0.032) and testosterone and control (P=0.042). There was

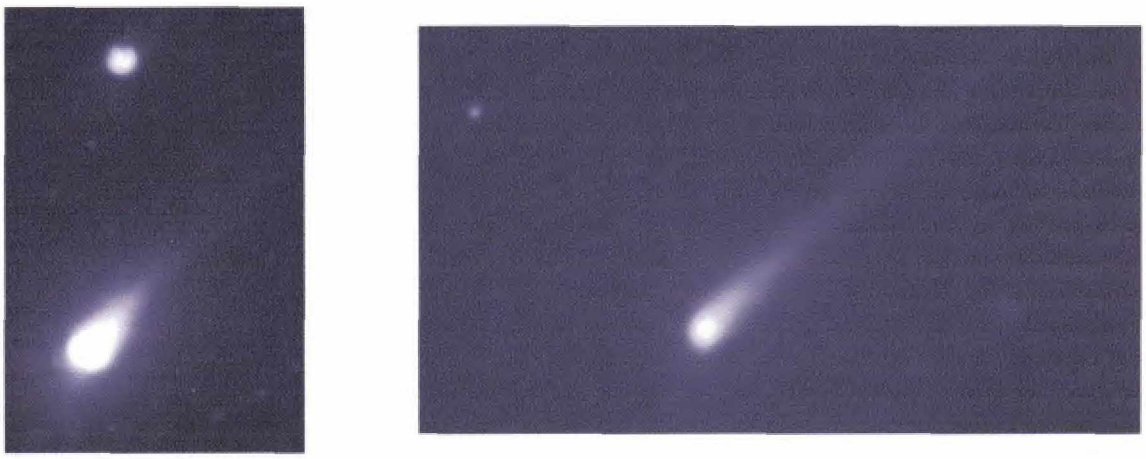

Figure 5.6. Comet Assay Analysis of Apoptotic and Healthy Cells
Apoptotic cells have a comet-like tail in varying degrees resulting from electrophoresis of fragmented DNA in cell nuclei suspended in an agarose gel. Healthy cells retain their DNA in their nuclei, and their circular shape, because it is not fragmented and does not easily move out of the nucleus despite the broken nuclear membrane.

not a significant difference between estrogen and control groups (P=0.732). Therefore, only testosterone treatment *in vitro* attenuated apoptosis (see Figure 5.7). Moreover, these results obtained with a comet assay confirmed our experimental outcomes using TUNEL staining. Testosterone treatment revealed 27.62% apoptotic cells (total cell number n=402) exhibiting the classic comet tail staining. In contrast, estrogen treatment revealed 50.70% (n=428 cells), and the control group 53.37% (n=756 cells) apoptotic cells.

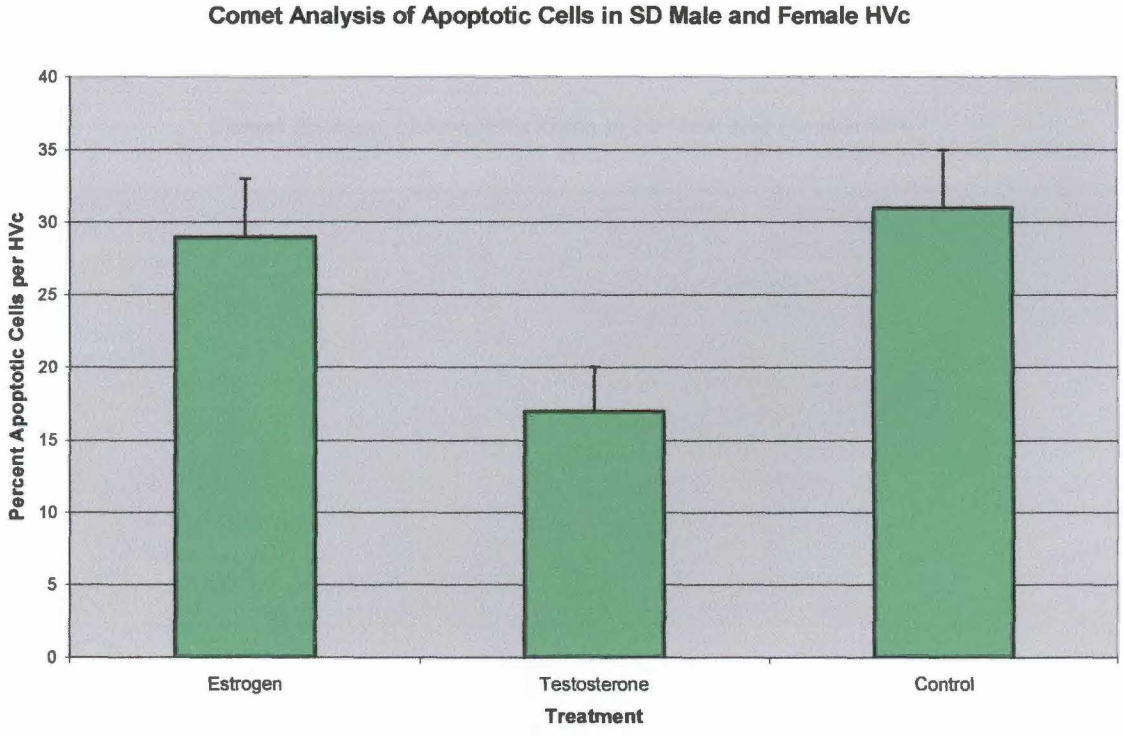

Figure 5.7 *Testosterone inhibits apoptosis in SD HVc as analyzed by comet assay.* Comet assay analysis of SD males and females in which testosterone-treated subjects had a significantly fewer number of apoptotic cells than did estrogen-treated (p=0.032) or control HVc (p=0.042).

5.3.2 Photostimulated Males and Females

In photostimulated male and female HVc, the comet assay revealed similar relations as found using TUNEL assay. However, the differences in numbers of apoptotic cells did not reach statistical significance. Testosterone-treated photostimulated HVc exhibited 17.83% apoptotic cells, while estrogen-treated had 21.30%, and control had 27.37% apoptotic cells. A t-test analysis neither revealed a statistical significance between testosterone and estrogen (P=0.615), testosterone and control (P=0.605), nor estrogen and control (P=0.919). As shown in Figure 5.8, the trend in the data is analogous to our finding in the photostimulated HVc employing TUNEL analysis.

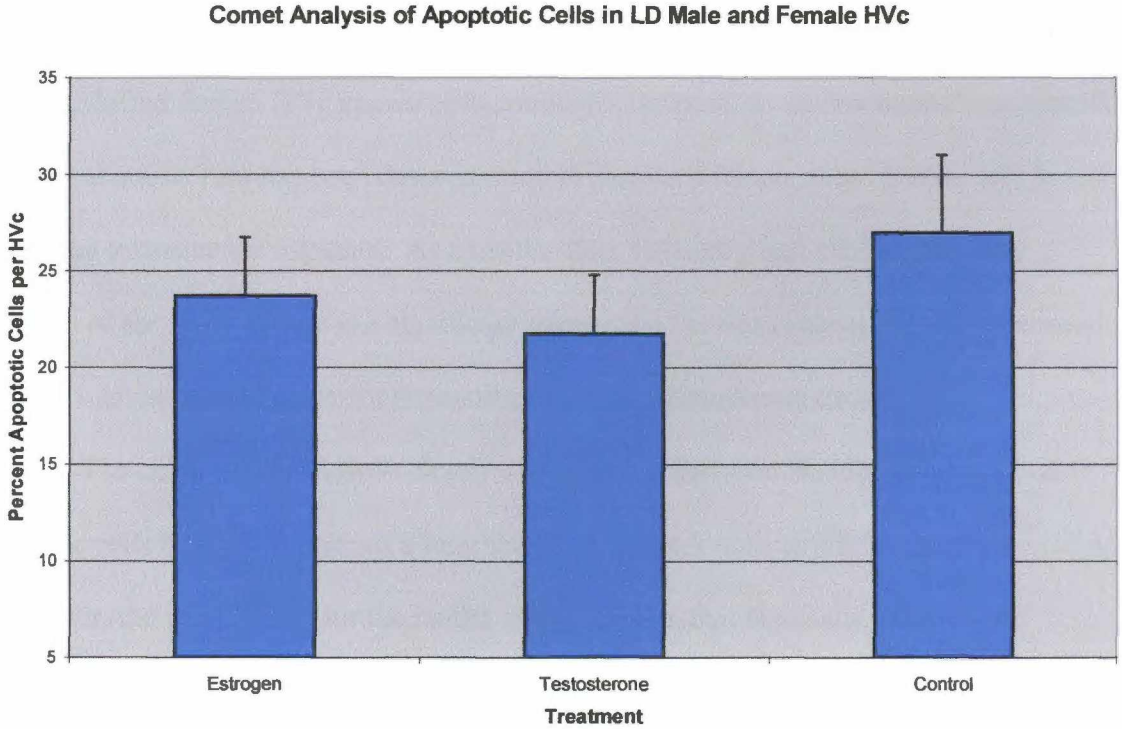

Figure 5.8. Comet assay analysis of LD male and female HVc in response to hormone treatments *in vitro*. Testosterone-treated subjects had a mean 17.83% apoptotic cells, estrogen-treated had mean 21.30%, and control group had a mean of 27.37% apoptotic cells per field. The differences were not significant.

5.4 Discussion

Cells undergoing apoptosis exhibit distinct molecular features including a defined DNA degeneration. TUNEL assay targets DNA degradation by labeling single strand DNA breaks and microscopic visualization. However, cells that repair damaged DNA might introduce false positive results. Nevertheless, TUNEL assay is widely used and, in our research, applied for the first time to assess apoptosis in HVc of songbirds.

The presence of testosterone *in vitro* in organotypic cultures of brain slices containing the HVc significantly reduces the number of cells undergoing apoptosis in both photosensitive and photostimulated male and female juncos. In comparison, estrogen also reduces apoptosis in the HVc of photosensitive male and female juncos. Both male and female HVc appear to be primed to respond to environmental and specific hormonal cues. This has been demonstrated in castrated female zebra finches and juncos receiving testosterone implants. As a result, these birds sing and exhibit increased volume of the HVc. There is a significant interaction between photoperiod and hormone effects with hormone treatment depending upon the photoperiod cycle given.

The comet assay analysis clearly distinguishes between healthy, apoptotic, and necrotic cells by the presence of a long fluorescent DNA tail extruding from the nucleus. However, the preparation for the comet assay requires that the tissue samples be dissociated in prior to electrophoresis. Therefore, it is not possible to distinguish between the HVc, SEZ, and other bordering nuclei. In addition, the cells must be diluted enough

to allow for proper visualization. Because only a small cluster of the total number of cells are being sampled, the assay is open to a lot of discrepancy.

In our results, the comet assay shows similar trends to those obtained with the TUNEL assay. In HVc of photosensitive (SD) juncos, there is a significant difference between testosterone treatment and both estrogen and control treatments in the number of apoptotic cells counted per $0.3mm^2$. Testosterone clearly diminished the number of apoptotic cells. However, in the HVc of photostimulated juncos, there was not a significant difference between any of the treatment groups. Although the trend was representative of the TUNEL data with testosterone and estrogen both decreasing the overall number of apoptotic cells.

Testosterone and estrogen appear to be attenuating apoptosis in the photostimulated juncos through post-mitotic events. They are possibly giving the survival signals necessary for the cell to live a prolonged life. This could be achieved through the activation of survival-specific growth factors, such as BDNF, which is a transcription factor that promotes neuronal survival. It has been previously shown that administration of a BDNF neutralizing antibody that competitively binds to TrkB in addition to testosterone inhibits the expected growth of the HVc in response to testosterone (Rasika et al., 1999). In addition, the infusion of BDNF *in vivo* into adult female canaries increases the survival of new neurons in the HVc, and the administration of testosterone increases BDNF levels in the HVc (Rasika et al., 1999). Therefore, it appears that T and BDNF act on the same population of new neurons and that testosterone up-regulates the expression of BDNF. The effect of testosterone on BDNF

expression could be a result of direct or indirect effects, such as metabolism of testosterone, or a testosterone-induced increase in singing. It has been shown that singing alone increases BDNF expression in the HVc (Li et al., 1994). BDNF also increases the number of androgen receptors in perineal motor neurons in mammals, and may do the same in the avian HVc (Al-Shamma and Arnold, 1997).

Chapter 6

PARP and Cytochrome C

6.1 Introduction

Apoptosis is common in every life stage of most living organisms. For instance, the re-absorption of the tadpole tail at the time of its metamorphosis occurs via apoptosis. The formation of the fingers and toes of the fetus are formed by an apoptotic tissue removal in between digits, as well as the sloughing off of the inner lining of the endometrium in menstruation. In addition, the formation of the proper synaptic neuronal network in the CNS that requires a surplus of cells be eliminated, among other examples. Cells in the immune system and those with nuclear damage readily undergo apoptosis, as well. Apoptosis is a highly controlled mechanism of cell removal that leaves no unwanted toxic byproducts. Cells that die by necrosis typically swell and burst, spilling their contents over their healthy cell neighbors. Because apoptosis is so methodical, an apoptotic cell leaves an apoptotic hallmark trail that enable researchers to distinguish between necrotic and apoptotic cells. Poly-ADP-Ribose Polymerase (PARP) and cytochrome C are two well accepted markers.

6.1.1 Apoptosis

Cells enter apoptosis due to a loss of the delicate balance between withdrawal of positive survival signals and receipt of negative death signals. There are two principal mechanisms by which a cell undergoes apoptosis: one is generated by signals arising

within the cell, and the second is triggered by death activators binding to receptors at the cell surface, such as TNF-α, Lymphotoxin, and Fas ligand (FasL). Briefly, internal damage in a healthy cell results in a loss of cytochrome C from the mitochondria leaking into the cytosol. The released cytochrome C binds to and thus activates the protease caspase 9 forming a protein complex called the apoptosome. Caspase 9 specifically cleaves numerous proteins and also activates other members of the caspase family. This activation inevitably leads to the digestion of intracellular proteins, degradation of chromosomal DNA, and the ultimate phagocytosis of the cell.

Apoptosis can also be triggered by external signals interacting with the Fas or TNF-α receptor. This causes a signal to be transmitted to the cytoplasm, which activates caspase 8, (similar to caspase 9). This event initiates a cascade of caspase activation, eventually leading to phagocytosis of the cell.

6.1.2 PARP in DNA Repair

PARP is an enzyme that is known to both repair damaged DNA and to splice DNA during apoptosis. Although PARP is a multifunctional regulatory enzyme, its role in DNA repair is still considered to be its primary biological function. However, it is still not precisely known how PARP contributes to DNA repair. By loosening chromatin structure, poly-ADP-ribosylation may render damaged DNA sequences more accessible to repair enzymes. Moreover, physical interactions between PARP and adapter and effector proteins of the base excision repair systems also have been reported. Recently, it was shown that PARP is used to generate ATP for ligases.

6.1.3 PARP in Apoptosis

The precise cleavage of PARP by caspases is now recognized as one hallmark of apoptosis and is believed to prevent support for DNA repair during late apoptosis, characterized by DNA fragmentation. Intact PARP is a 116 kD protein that is cleaved into an 85 kD fragment and a 31 kD fragment. We used an antibody against both the intact 116 kD and its 85 kD fragments combined with western blot analysis to determine apoptosis in the HVc of juncos.

6.1.4 Physiological Role of Cytochrome C

The primary role of the mitochondrion is to harness energy for the cell. The mitochondrion has two membranes, an inner and an outer enclose an interior matrix. NAD^+ accepts electrons during the oxidation of acetyl CoA to carbon dioxide by the Krebs Cycle in the matrix of the mitochondrion and is converted to NADH. NADH delivers its high energy electrons to Complex I of the electron transport chain (ETC) in the inner mitochondrial membrane, one of a series of protein complexes. The energy that is released as electrons pass through the ETC allows hydrogen ions to be pumped across the mitochondrial membrane, creating a hydrogen ion concentration gradient across the inner mitochondrial membrane. Eventually, this energy in the gradient powers ATP synthesis (oxidative phosphorylation).

In the ETC, electrons pass from Complex I onto a small lipophilic electron acceptor in the inner membrane, Coenzyme Q or Ubiquinone (Complex II). CoQ

transports electrons onto Complex III, which serves as a hydrogen ion pump. Then electrons transfer onto a small peripheral membrane protein, called cytochrome C, which transports the electrons onto Complex IV. Ultimately, electrons are accepted by oxygen, which converts to water, and hydrogen ions are pumped back across the inner mitochondrial membrane by ATPase, which generates ATP.

6.1.5 Cytochrome C in Apoptosis

Until recently, mitochondria were not considered central players in the effector phase of apoptosis since mitochondrial morphology remains intact throughout. Recent evidence indicates that mitochondria exhibit major functional and structural changes that regulate apoptosis. First, the mitochondrial inner transmembrane potential ($\Delta\Psi_m$) collapses prior to more obvious signs of apoptosis, such as PARP cleavage. Second, studies using cell-free systems suggest that certain mitochondrial proteins are rate-limiting for the activation of endonucleases and proteases of the caspase family. Third, drugs that stabilize mitochondrial membranes inhibit apoptosis (Susin, 1998). Fourth, the anti-apoptotic protein, Bcl-2, blocks the release of the intermembrane mitochondrial protein called cytochrome C, thus blocking apoptosis as well (Kluck, 1997; Yang, 1997). Mitochondria serve as key regulators of apoptosis via this cytochrome C-mediated pathway. Various apoptotic signals such as oxygen radicals lead to mitochondrial release of cytochrome C and apoptosis-inducing factor (AIF). Cytochrome C in its holo-form with its heme group attached associates with Apaf-1, caspase-9, and ATP to form a complex called an 'apoptosome'. This apoptosome proteolytically activates caspase-3,

which leads to the activation of the caspase cascade and the degradation phase of apoptosis (Li, 1997; Susin, 1999). Cytochrome C is key to apoptosis because its release from the intermembrane space is irreversible.

Either apoptosis occurs through the caspase-mediated process described above, or the cell goes through a necrosis-like death due to the collapse of electron transport. Release of cytochrome C also interrupts the transfer of electrons between respiratory chain complexes III and IV, resulting in the generation of deleterious oxygen radical species and the cessation of ATP synthesis.

In the following experiments we attempted to identify the process of apoptosis in the *Junco hyemalis* HVc. Using western blot technique and immunochemistry, we aimed at demonstrating PARP-1 hydrolysis and cytochrome C release from the mitochondria.

6.2 Results

6.2.1 PARP Results

Although the PARP antibody was supposed to recognize avian PARP, we never got consistent immunoreactivity on western blots regardless of the avian species used (chicken or junco). We performed a dilution series to test individually both the primary anitibody (1:50, 1:100, 1:300, 1:500, 1:750, 1:1,000, 1:1,500, 1:2,000, and 1:5,000) and the secondary antibody (1:1,000, 1:5,000, 1:10,000, 1:15,000, and 1:20,000). We finally got a faint signal in the combinations of primary Ab (1:1,000) and secondary Ab (1:15,000 and 1:10,000), however, these results were not consistent in the chick.

Therefore, this assay was not repeated in junco samples that had been treated *in vitro* for 14 days with testosterone (10 ng/ml), estrogen (10 ng/ml), and EtOH carrier control.

6.2.2 Cytochrome C Results

Similar outcomes resulted when assaying for cytochrome C. Since cytochrome C is released from the inner mitochondrial membrane into the cytosol upon apoptosis, we expected to reveal strong immunoreactivity in the mitochondria/nucleus fraction with a faint signal in the cytosol fraction. Conversely, strong immunoreactivity was expected in the mitochondrial fraction from UV treatment. Eventually we obtained immunoreactivity testing several antibody dilutions, but signals were inconsistent with expected results. We saw bands in the control cytosol lane and in the treated mitochondria/nucleus lane and very faint bands in the control mitochondria/nucleus lane and treated cytosol lane.

6.3 Discussion

Signals for both the PARP and Cyt C western blots were inconsistent and unreliable using neuronal tissue from both the chicken and junco. Antibodies against avian species are rather difficult to obtain. We had hoped that either one or both of these assays would allow us to detect apoptosis in junco HVc slice cultures on a mechanistic basis. However, the inconsistency of both the PARP and Cyt C assay halted further progress. The antibodies were simply not able to cross-react with the avian tissue, and therefore we did not proceed further in this direction.

Chapter 7

Future Directions

In order to truly assess the affect of hormone treatment on the rate of neuronal stem cell proliferation, gonadectomized subjects should be subjected to the same *in vivo* and *in vitro* treatments and only the subventricular zone analyzed for BrdU staining, since the HVc is know to not contain neuronal stem cells.

Neuronal migration could be assessed in two principal experiments. HVc cultures are BrdU-pulse labeled every day for up to 14 days and BrdU+ cells determined in the HVc from day 1 to day 14. Analysis of the overall number of labeled cells within the HVc and SZ combined would give a clear indication of whether one, both, or neither hormone affects the rate of proliferation, and also which, if any, treatment affects the migratory pattern of the newly divided neurons.

One effective method to evaluate photoperiod's influence on proliferation within the SZ is to pulse-label gonadectomized birds with BrdU every day *in vivo* for two weeks, then sacrifice and immediately stain and analyze the number of labeled cells within the SZ alone in the SZ and HVc combined (to account for cells that migrated during the experiment). It would also be interesting to continue the photoperiod treatment *in vitro* by fitting a timed light fixture in separated incubators to determine if the song system contains and responds to light-sensing cells. This should be done with and without hormone treatment to assess if *in vitro* light treatment affects the interdependence of the hormone and light treatments upon one another. Alternatively,

dividing cells could be labeled with a retrovirus containing green fluorescent protein or an inert dye labeling. Then, migration could be observed using live video fluorescence microscopy.

A dependence of neuronal survival on gonadal hormones and photoperiod provides the technically most feasible avenue of research. Our investigation using TUNEL staining and Comet Analysis have already demonstrated the significance of this post-mitotic effect on neuronal incorporation into the HVc. Studies of further apoptosis markers and inclusion of caspase inhibitors would provide further insight.

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