

# **Comparative survey of adult neurogenic sites in adult avian species: an immunohistochemical study.**

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PhD Thesis

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Doctor of Philosophy.

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## DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

A handwritten signature in dark ink, appearing to read 'Pedzisai Mazenganya', with a horizontal line extending from the middle of the signature.

Pedzisai Mazenganya

28 February, 2018.

To wife Doris, my sons Beracah and Malcolm, and my mother,  
your unwavering support is forever cherished

## **Publications arising from this thesis:**

- Mazenganya, P., Bhagwandin, A., Nkomozepe, P., Manger, P.R, Ihunwo, A.O., 2017. Putative adult neurogenesis in two domestic pigeon breeds (*Columba livia domestica*): Racing homer versus utility carneau pigeons. *Neural Regen Res.* 12(7): 1086-1096.
- Mazenganya, P., Bhagwandin, A., Manger, P.R., Ihunwo, A.O., 2018. Putative adult neurogenesis in old world parrots: the Congo African grey parrot (*Psittacus erithacus*) and Timneh grey parrot (*Psittacus timneh*). *Frontiers in Neuroanatomy* 12: 7.
- Mazenganya, P., Bhagwandin, A., Manger, P.R., Ihunwo, A.O., 2017. The distribution of cells immunoreactive to proliferating cell nuclear antigen and doublecortin in the brains of adult pied crows (*Corvus albus*): widespread potential adult neurogenesis. *Neuroscience*. Submitted.

## **Presentations arising from this thesis:**

- Mazenganya, P., Bhagwandin, A., Nkomozepe, P., Manger, P.R, Ihunwo, A.O., 2015. Adult neurogenesis in the subspecies of domestic pigeons *Columba Livia domesticus*: The Racing Homer and Utility Carneau Pigeons.
- The above paper was presented as a poster at:
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  - ❖ 44th Annual Anatomical Society of Southern Africa (ASSA) Conference, Bloemfontein, Free State, South Africa, May 2016
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## **Abstract:**

Adult neurogenesis is a widely accepted and preserved phenomenon throughout the animal kingdom occurring in both invertebrates and vertebrates, including humans. The process has been extensively studied in mammalian vertebrates and comparably less in the class aves. In birds, the process has been studied more so in the song bird brain. The current thesis, investigated putative adult neurogenesis in the brains of seven species of birds from different taxonomic orders exhibiting different behavioural repertoires, particularly determining and localizing the active neurogenic regions, migratory routes of migrating neuroblasts and regions of integration of new neurons. The birds examined in the current thesis belonged to the following taxonomic orders: Columbiformes (domestic pigeons; racing homer and utility carneau pigeons); Psittaciformes (Congo African grey and Timneh grey parrots); Passeriformes (pied crow); Struthioniformes (common ostrich) and Casuariiformes (emu). Free floating immunohistochemical methods against proliferating cell nuclear antigen (PCNA) which labels proliferating cells and doublecortin (DCX) which stains immature neurons were used to determine the regions of cells proliferation, migratory routes of immature neurons and recruitment sites as final destination in the brains of the birds to see if these processes are associated with variations in behavioural repertoires and/or show phylogenetic relations. Results from the current thesis showed that cell proliferation is widespread throughout the brains of all the birds examined, with high levels of cell proliferation in the olfactory bulbs and the subventricular zones (SVZ) of the lateral ventricles in the telencephalon; the SVZ of the lateral ventricle is the primary neurogenic zone contributing new neurons in the adult telencephalon; immature neurons are localised in high density in the olfactory bulb and the subdivisions of the telencephalon, but in low densities in the subtelencephalic regions except in the ratite birds (common ostrich and emu) where moderate densities were observed in the cortex of the cerebellum; the distribution of the immature neurons showed region specificity within the

subdivisions of the telencephalon across all species with high densities in the medial and lateral regions of the pallial and subpallial regions, but devoid in the core areas; species-specific distribution of immature neurons was also observed in the hippocampal formations and song control systems of the pied crow and cerebellum of the ratite birds indicating behavioural specialisations and migratory routes of immature neurons were not clearly defined across species except in the ratites birds where three distinct bundles of migrating neuroblasts were observed in the telencephalon. In the majority of the studied birds neuroblasts migrated from the dorsal and ventral poles of the SVZ of the lateral ventricle into the hyperpallium and medial striatum respectively. The wide spread distribution of the proliferating cells and immature neurones in the brains of adult from various taxonomic orders, although in varied extents indicates that adult neurogenesis is a conserved trait in all species of birds and might be important for environmental and behavioural adaptations. In addition, the process might also help to explain phylogenetic relationships of birds along the evolutionary scale. Thus this thesis provides clues to the presence and extent of adult neurogenesis in various species of birds highlighting on possible behavioural repertoires impacted by adult neuronal plasticity.

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# **CHAPTER 1: Introduction, study design and chapter outline**

## **1.1. Introduction**

Adult neurogenesis is defined as the ability to generate new neurons in the brain of adult animals. The process of adult neurogenesis encompasses the division of neural progenitors, differentiation and migration of neuroblasts and subsequent integration of mature neurons into existing circuits (Lindsey and Tropepe, 2006). Adult neurogenesis is a widely accepted and preserved phenomenon throughout the animal kingdom occurring in both invertebrates and vertebrates, including humans (Polenov and Chetverukhin, 1993; Alvarez-Buylla and Kirn, 1997; Eriksson et al., 1998; Goldman, 1998; Cayre et al., 2002). Its discovery has contributed to the demise of the long held dogma that the adult brain is incapable of cell renewal and plasticity (Altman and Das, 1965; Gross, 2000). Most of the information on adult neurogenesis is based on the studies from mammals, especially rodents; and from birds (Barnea and Pravosudov, 2011). In birds, the best understood model of adult neurogenesis and perhaps the first to be discovered is that of the adult songbird forebrain (Goldman and Nottebohm, 1983). New neurons in the adult songbirds are generated in the subventricular zone (SVZ) of the lateral ventricle; migrate and become incorporated into various areas of the telencephalon including the high vocal center (HVC), hippocampus (Hp), area X, nidopallium caudale (NC), the hyperpallium apicale (HA) and the striatum complex (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1990b; Alvarez-Buylla et al., 1994; Alvarez-Buylla et al., 1998). However, in adult mammals new neurons are generated in the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus and migrate, differentiate and incorporate into local circuitry of the olfactory bulb and the dentate gyrus respectively (Kempermann et al., 1997; Goldman, 1998; Gould et al., 1999b; Kornack and Rakic, 1999).

Growing evidence suggests that adult neurogenesis in birds is not limited only to the songbird brain but it is also present in the brain of adult non-song birds that have been examined to date

including domestic chicken (*Gallus gallus domesticus*), parakeets (*Melopsittacus undulatus*), rock pigeons (*Columba livia*), ring doves (*Streptopelia risoria*) and domestic pigeons (*Columba livia domestica*) (Nottebohm, 1985; Ling et al., 1997; Capes-Davis et al., 2005; Mezey et al., 2012; Melleu et al., 2013; Mazenganya et al., 2017). Considering the diversity of bird species studied to date, adult neurogenesis may be a common feature to all 10 000 living species of birds and not only a peculiarity of birds with specialized brain circuits for learned vocal behaviour (Alvarez-Buylla and Kirn, 1997). Birds exhibit distinct behaviours ranging from well-known song learning, food caching, migration, and social interactions, which have possible influences on adult neurogenesis (Barnea and Pravosudov, 2011). Taking these behaviours as ‘models’ to study adult neurogenesis, more information can be revealed on the extent and possible functions of adult neurogenesis in various species of birds. Songbirds in particular display remarkable changes in the structure of the song control nucleus (HVC) during different times of the year reaching larger volumes in spring in the canaries and spotted towhee (*Pipilo maculatus*) (Nottebohm, 1981). This increase in the volume of the HVC was associated with addition of neurons and also production of a stable song during spring (Nottebohm, 1981).

With the current information on adult neurogenesis in songbirds and a few on non-songbirds, it is of interest to study adult neurogenesis in various species of birds which show distinct behavioural repertoires in their life so as to further understand the basic biological processes and various factors fundamental to adult neurogenesis. Considering that the various behaviours exhibited by different species of birds are controlled by different brain regions, for example song learning manifests in HVC, while migration and food caching involve the hippocampal formation, it is therefore possible that the active neurogenic sites vary in location and extent in different species of birds.

Based on this premise, we investigated putative adult neurogenesis in various species of birds exhibiting different behavioural repertoires, particularly determining and localizing



the active neurogenic regions, migratory routes of migrating neuroblasts and regions of integration of new neurons in the brains of seven bird species from different taxonomic orders.

#### Aim of the thesis

This thesis aims to report on the survey for active neurogenic sites, regions in the brain where these neurons migrate and become incorporated in the brains of seven avian species from different taxonomic orders

#### Specific objectives of the thesis

1. To determine the presence of active neurogenic sites in the brains of seven avian species from different taxonomic orders by immunolabelling for proliferating cells and immature neurons using proliferating cell nuclear antigen (PCNA) and doublecortin (DCX).
2. To determine the migration end points of new neurons in the avian brain by determining the trajectory of migrating neuroblasts that were DCX immunolabelled.

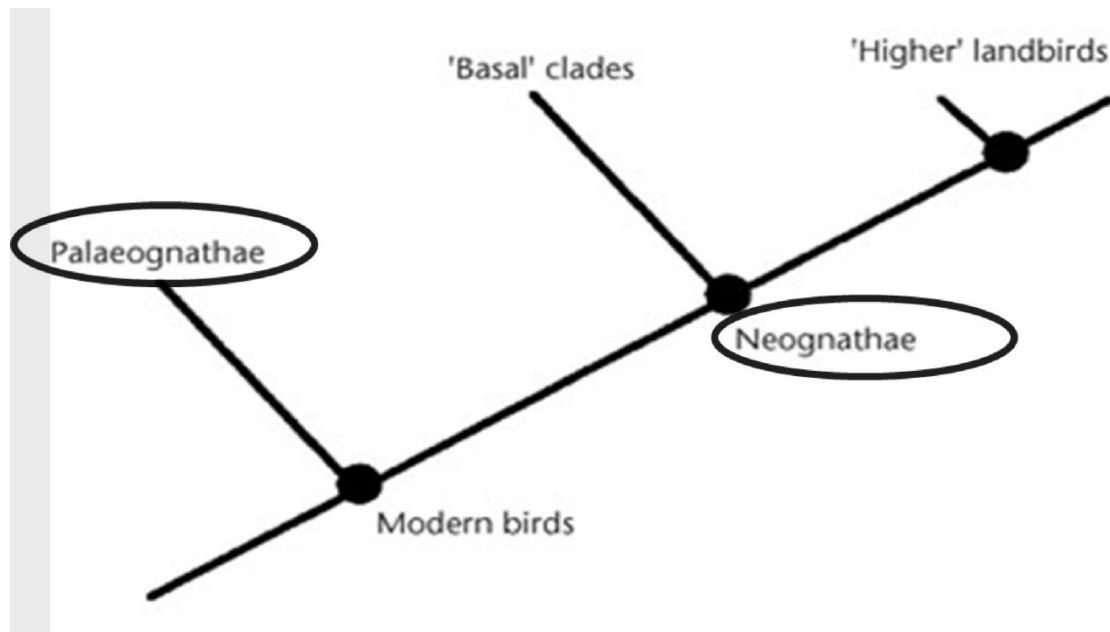
### **1.2. Study design**

The current study is divided into four projects; each project examined and compared adult neurogenesis in closely related species from the same taxonomic order (Fig. 1.1). The study utilized male subjects in order to minimise the effect of androgens on adult neurogenesis in the analysis (Barnea and Pravosudov, 2011). In addition, the birds were grouped according to similarity in behaviours.

1. This project examined the extent of adult neurogenesis in the two breeds of Columbiformes (domestic pigeons; racing homer and utility pigeons). Domestic pigeons have been selectively bred for enhanced flight and navigation; being capable

of flying long distances, but returning to same locations using sophisticated navigational cues. Some breeds are selectively bred for meat production. The study aimed to see if adult neurogenesis is affected by these different characteristics or behaviours in order.

2. This project examined the adult neurogenesis in the two subspecies of the Psittaciformes (the Congo African grey and Timneh grey parrots). The parrots are capable of continuous learning throughout life. They can recognise meanings of words and do live in large social grouping.
3. This project will focus on the Passeriformes the pied crows. These birds possess song control systems and display other characteristics like food caching, complex nest building and courtship, tool use and manufacture, dominance behaviors and complex social interaction. This study lacks a comparator animal to compare and contrast the pattern of adult neurogenesis
4. This project analysed and compared adult neurogenesis in Struthioniformes (common ostrich) and Casuariiformes (emu). The Struthioniformes and Casuariiformes are flightless birds with a distinct home range. The two taxonomic orders belong to the paleognathae clad of birds and is phylogenetical older than the neognathae clade.



**Figure 1.1.** Digramatic representation of the phylogenetic tree showing the relationships between paleognathae (evolutionary old) birds to the neognathae (evolutionary new) birds. The paleognathae birds include ostriches and emu while the neognathae birds include the pigeons, parrots and crows.

### 1.3. Chapter outline

This thesis is organized into several chapters, with each chapter analysing a specific taxonomic order of birds as outlined in the study design.

#### **Chapter two: Literature review**

This chapter presents the background information on adult neurogenesis in vertebrate species, focusing more closely on class aves and also provides the rational for the PhD thesis.

#### **Chapter three: Putative adult neurogenesis in two domestic pigeon breeds (*Columba livia domesticus*): racing homer versus utility carneau pigeons.**

**Article published:** Neural Regen Res 12(7):1086-1096.

**Author contributions:** AOI and PRM designed the study and analysed data. PM, AB and PN collected data and performed a preliminary analysis. PM wrote the paper. All authors read and approved the final version of this paper.

Generation of neurons in the brains of adult birds has been studied extensively in the telencephalon of song birds and few studies have been reported on the distribution of PCNA and DCX in the telencephalon of adult non-song learning birds. We report here on adult neurogenesis throughout the brains of two breeds of adult domestic pigeons (*Columba livia domesticus*), the racing homer and utility carneau using endogenous immunohistochemical markers proliferating cell nuclear antigen (PCNA) for proliferating cells and doublecortin (DCX) for immature and migrating neurons. The distribution of PCNA and DCX immunoreactivity was very similar in both pigeon breeds with only a few minor differences. In both pigeons, PCNA and DCX immunoreactivity was observed in the olfactory bulbs, walls of the lateral ventricle, telencephalic subdivisions of the pallium and subpallium, the diencephalon; mesencephalon and the cerebellum. Generally the olfactory bulbs and

telencephalon had more PCNA and DCX cells than other regions. Two proliferative hot spots were evident in the dorsal and ventral poles of the lateral ventricles. PCNA and DCX cells migrated radially from the walls of the lateral ventricle into the parenchyma. In most telencephalic regions the density of PCNA and DCX stained cells increased from rostral to caudal, except in the mesopallium where the density decreased from rostral to middle levels and then increased caudally. DCX immunoreactivity was more intense in fibres than in cell bodies and the shapes of DCX positive cells included small granular cells, fusiform bipolar cells to large round and or polygonal multipolar cells. The two breeds revealed similar patterns of the distribution of the PCNA and DCX immunoreactivity with minor variations. The similarity in the distribution of proliferating cell and new neurons in the telencephalon of the two breeds of pigeons may suggest that adult neurogenesis is a conserved trait as an ecological adaptation irrespective of body size.

**Chapter four: Putative adult neurogenesis in old world parrots: the Congo African grey parrot (*Psittacus erithacus*) and Timneh grey parrot (*Psittacus timneh*).**

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**Author contributions:** AOI and PRM designed the study and analysed data. PM and AB collected data and performed a preliminary analysis. PM wrote the paper. All authors read and approved the final version of this paper.

In the current study, we examined the potential for adult neurogenesis throughout the brain of the Congo African grey parrot (*Psittacus erithacus*) and Timneh grey parrot (*Psittacus timneh*) using immunohistochemistry for the endogenous markers proliferating cell nuclear antigen (PCNA), which labels proliferating cells, and doublecortin (DCX), which stains immature and migrating neurons. A similar distribution of PCNA and DCX immunoreactivity was found throughout the brain of the Congo African grey and Timneh

grey parrots, but minor differences were also observed. In both species of parrots, PCNA and DCX immunoreactivity was observed in the olfactory bulbs, subventricular zone of the lateral wall of the lateral ventricle, telencephalic subdivisions of the pallium and subpallium, diencephalon, mesencephalon and the rhombencephalon. The olfactory bulb and telencephalic subdivisions exhibited a higher density of both PCNA and DCX immunoreactive cells than any other brain region. DCX immunoreactive staining was stronger in the telencephalon than in the subtelenchalic structures. There was evidence of proliferative hot spots in the dorsal and ventral poles of the lateral ventricle in the Congo African grey parrots at rostral levels, whereas only the dorsal accumulation of proliferating cells was observed in the Timneh grey parrot. In most pallial regions the density of PCNA and DCX stained cells increased from rostral to caudal levels with the densest staining in the nidopallium caudolaterale (NCL). The widespread distribution of PCNA and DCX in the brains of both parrot species suggest the importance of adult neurogenesis and neuronal plasticity during learning and adaptation to external environmental variations.

**Chapter five: The distribution of cells immunoreactive to proliferating cell nuclear antigen and doublecortin in the brains of adult pied crows (*Corvus albus*): widespread potential adult neurogenesis**

**Article ready for resubmission:**

**Author contributions:** AOI and PRM designed the study and analysed data. PM and AB collected data and performed a preliminary analysis. PM wrote the paper. All authors read and approved the final version of this paper.

In the current study we examined the potential for adult neurogenesis throughout the brain of the pied crow (*Corvus albus*) using immunohistochemistry for the endogenous markers proliferating cell nuclear antigen (PCNA), which labels proliferating cells, and

doublecortin (DCX), which stains immature and migrating neurons. The distribution of PCNA and DCX labelled cells was widespread throughout the brain. The highest density of cells immunoreactive to both markers was observed in the olfactory bulbs and the telencephalon, especially the song control nuclei. Proliferative hot spots, identified with dense PCNA and DCX immunolabelling of cellular components, were identified in the dorsal and ventral poles of the rostral aspects of the lateral ventricles. Substantial numbers of PCNA immunoreactive cells were observed in the diencephalon and brainstem, but DCX immunoreactivity was weaker in these regions, preferentially staining axons and dendrites over cell bodies, especially in the optic tectum. Interestingly, while PCNA immunoreactive cells were readily observed in the cerebellum, no DCX immunoreactivity was observed. The distribution of putative proliferating cells and immature neurons in the brain of the pied crow appears to be very widespread, far more so than in mammals, and suggests that brain plasticity and neuron turnover is an important aspect of normal neuronal processing in birds.

**Chapter six: Adult neurogenesis in the paleognathous birds: the common ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*)**

**Manuscript ready for submission:**

**Author contributions:** AOI and PRM designed the study and analysed data. PM and AB collected data and performed a preliminary analysis. PM wrote the paper. All authors read and approved the final version of this paper.

In the current study, we examined adult neurogenesis throughout the brain of the common ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*) using immunohistochemistry for the endogenous markers proliferating cell nuclear antigen (PCNA), which labels proliferating cells, and doublecortin (DCX), which stains immature and migrating neurons. The distribution of PCNA and DCX labelled cells was widespread

throughout the brain of both species. The highest density of cells immunoreactive to both markers was observed in the olfactory bulbs and the telencephalon, especially the subventricular zone of the lateral ventricle. Proliferative hot spots, identified with strong PCNA and DCX immunolabelling, were identified in the dorsal and ventral poles of the rostral aspects of the lateral ventricles. The density of PCNA immunoreactive cells was less exuberant in the telencephalon of the emu compared to the common ostrich. Substantial numbers of PCNA immunoreactive cells were observed in the diencephalon and brainstem, but DCX immunoreactivity was weaker in these regions, preferentially staining axons and dendrites over cell bodies, except in the medial regions of the hypothalamus where distinct DCX immunoreactive cells and fibres were observed. PCNA and DCX immunoreactive cells were readily observed in moderate density in the cortical layers of the cerebellum of both species. Columns of migrating cells were observed at three distinct points extending from the lateral wall of the lateral ventricle into parenchyma of the hyperpallium, nidopallium and medial striatum at rostral levels in both species. The distribution of putative proliferating cells and immature neurons in the brain of the common ostrich and the emu is widespread, far more so than in mammals, and compares with the neognathous birds, and suggests that brain plasticity and neuronal turnover is an important aspect of cognitive brain functions in these birds.

## **Chapter seven: Discussion**

This chapter summarizes the implications of the results obtained in the separated studies constituting this thesis and highlights on the limitations and future studies on adult neurogenesis in various species of birds.



## **CHAPTER 2: Literature review**

### **2.1. Adult neurogenesis: historical perspective and basic biological principles**

The theory of adult neurogenesis was long denied for nearly a century and scientists believed that the adult mammalian brain lacked the capacity to add new neurons once developmental processes were terminated (Rakic, 1985; Gross, 2000). The mammalian brain was thought to receive a full complement of neurons during the developmental periods before attainment of puberty (Rakic, 1985). It was believed that new neurons and neuronal plasticity might disturb the complex synaptic organization of large brained animals and subsequently interfere with the stored information, particularly in lifelong learners like primates (Rakic, 1985). In the whole of the 19<sup>th</sup> century the fixed brain philosophy prevailed and several attempts at nullifying it were carried out without success. This phenomenon was held for a longer period before it was finally overruled towards the end of 20<sup>th</sup> century and in the beginning of the 21<sup>st</sup> century (Gross, 2000). However in the early 20<sup>th</sup> century, few studies were reported on the identification of neural stem/ precursor cells in adults individuals from teleost to humans and including observations of mitotic figures in small neurons of injured brain of guinea pigs and mitotic figures along the ventricular lining in rats (Gross, 2000). These findings were objected to on the premise that they could not distinguish between a dividing neuron and or glia (Rakic, 1985; Gross, 2000). More studies in adult mammalian neurogenesis were brought up in the late 20<sup>th</sup> century with the advent of autoradiography. The method aided the identification of dividing cells and their progeny, time and place of birth neurons (Gross, 2000). During this period proliferating cells were identified in the cerebral cortex, dentate gyrus and the olfactory bulb in rats and cats (Altman, 1962; Altman and Das, 1965; Altman, 1966; Altman and Das, 1966). In addition, a first hint on the functional implications of new neurons in memory and learning was introduced (Altman, 1969). These discoveries of adult neurogenesis in rats, cats and guinea pigs from autoradiographic studies suffered the same fate as the first ones and were also rebuked (Gross, 2000). A decade and

half later after ground breaking studies by Altman and colleagues, adult mammalian neurogenesis was further confirmed by combining autoradiography and ultrastructural studies and these studies showed that  $^3\text{H}$ -Thymidine labelled cells in rats were neurons containing dendrites and synapses and not glia (Kaplan and Hinds, 1977; Kaplan and Bell, 1984). During the same period and utilizing similar methods adult neurogenesis was observed in the cerebral cortex of adult rats and in the subventricular zones of the adult macaque monkeys (Kaplan, 1981; Kaplan, 1982; Kaplan, 1985). A similar study in song birds identified and localized the proliferating cells and plasticity of neurons in the adult brains (Goldman and Nottebohm, 1983). The discoveries of cell proliferation and plasticity of neurons in song birds were more convincing and highlighted the possibility of cell proliferation in other classes of vertebrates and on the modulatory processes and functional aspects of adult neurogenesis (Gross, 2000). Similar to other past studies, adult neurogenesis was not considered a viable trait in mammals despite all the convincing evidence from birds and rodents and particularly in birds, adult neurogenesis was considered an evolutionary adaptation to flight and seasonal cycles related to song production (Gross, 2000). Studies on adult neurogenesis rose to prominence in the nineties following the development of exogenous markers to proliferating cells such as bromodeoxyuridine (BrdU), cell type specific markers and stereology. This led to the understanding of regulatory mechanisms in adult neurogenesis and confirmation of adult neurogenesis in adult animals (Gross, 2000). Regulatory factors such as enriched environment, stress, exercise and cancer were tested and positive associations with adult neurogenesis were recorded (Eriksson et al., 1998; Gould et al., 1998; van Praag et al., 1999; Kuhn et al., 2007; Kempermann, 2011). It was through its discovery in the human hippocampus of cancer patients by Eriksson et al. (1998) which led to its placement on the public interest and to date more studies have been published on the subject ranging from establishing neurogenic sites in different species to analysing the underlying factors regulating the process and its possible use in the generation of stem cell

therapies for neurodegenerative diseases (Barker et al., 2011; Barnea and Pravosudov, 2011; Bonfanti et al., 2011; Ihunwo et al., 2016).

Although there is significant progress in this field, the functional significance of adult neurogenesis is still debated. Cell proliferation is coupled by cell death in both mammals and birds (Kirn and Nottebohm, 1993; Kirn et al., 1994; Deisseroth et al., 2004). Functionally, the acquisition of new and clearance of old memories relies on the antagonistic effect of birth versus death of neurons in the hippocampus of adult species (Becker and Wojtowicz, 2007).

## **2.2. Adult neurogenesis in birds**

Adult neurogenesis in birds was first described in the high vocal center (HVC), a telencephalic nucleus unique to the brain of songbirds responsible for the production of learned song (Goldman and Nottebohm, 1983). Subsequent studies revealed that adult neurogenesis was wide spread in the telencephalon of adult songbirds, particularly the canary (*Serinus canaria*) (Alvarez-Buylla, 1990; Alvarez-Buylla, 1992). Further studies in songbirds unmasked the various basic processes of adult neurogenesis including cell birth, migration and incorporation of new neurons into existing circuitry (Paton and Nottebohm, 1984; Nottebohm, 1985; Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988; Alvarez-Buylla et al., 1990b).

Alvarez-Buylla et al. (1990b) suggested that new neurons are generated in subventricular zones in regions with a high density of proliferating cells referred to as proliferative “hotspots” located in the ventral and dorsal poles of the lateral ventricles. The proliferative hotspots also contained a high concentration of radial glia cells suggesting that radial glia cells were primary precursors (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988; Walton et al., 2012). In the adult birds, the young migrating neurons are distributed to various regions of the telencephalon (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1998). Young and migrating neurons follow two

modes of migration into the brain parenchyma, that is 1) radial migration whereby migrating neuroblasts are guided by radial glia scaffolding to their final destination and 2) tangential migration, whereby migrating neuroblasts follow a path perpendicular to that associated with radial glia near or within the walls of the lateral ventricles towards the striatum (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Doetsch and Scharff, 2001).

Apart from what is known about the modes of migration of adult born neurons from the germinal zones in the subventricular zone to the various regions of incorporation in the telencephalon, the definitive routes of migration in the avian brain are not yet well characterized, for example migration towards the well-studied high vocal center in canaries (García-Verdugo et al., 2002). Unlike in mammalian species, where new adult born neurons from the subventricular zone follow the defined rostral migratory stream to the olfactory bulb (Lois and Alvarez-Buylla, 1994). In birds new neurons generally migrate rostrally and a few migrate ventro-caudally into the striatum complex (Alvarez-Buylla et al., 1990b). The final destination of adult born neurons in birds is restricted to the various nuclei and regions of the telencephalon in both song and non-song birds (Alvarez-Buylla, 1990; Alvarez-Buylla, 1992; Ling et al., 1997; Alvarez-Buylla and García-Verdugo, 2002; Melleu et al., 2013). Few studies have reported widespread recruitment of neurons in all the brain regions in adult birds (Capes-Davis et al., 2005; Kim et al., 2006; Mezey et al., 2012; Mazenganya et al., 2017). The restriction of postnatal neurogenesis to the telencephalon of birds may suggest that new neurons participate in higher associative brain functions such as perception and learning (Alvarez-Buylla and Kirn, 1997).

Incorporation of new neurons in the brain of adult songbirds was identified in the vocal control system and the hippocampus of the telencephalon. These regions are involved in the control and production of learned song for the HVC and spatial memory, and learning and memory in case of the hippocampus (Nottebohm, 1985; Alvarez-Buylla et al., 1990a; Barnea and Nottebohm, 1996; Gahr et al., 2002; Nottebohm, 2011). Within the telencephalon of

both song birds and non-song birds new neurons are added specifically in the HVC, medial and lateral striatum, medial and lateral septum, area X, the nidopallium caudale and hippocampus (see review, Barnea and Pravosudov, 2011). The area X nucleus in adult song birds is involved in the acquisition and maintenance of the learned song while the nidopallium caudale is an auditory region which indirectly project to the vocal control system and also participates in somatosensory activities (Paton and Nottebohm, 1984; Reiner et al., 2004; Güntürkün, 2005; Mello and Jarvis, 2008). The septal regions in birds are homologous to the mammalian septal complex and are involved in the modulation of complex behaviours and regulation of social behaviours such as aggression and agonistic communication (Goodson et al., 2004). The striatum (medial and lateral striatum) complex participates in learned motor movements and fine motor skills in both birds and mammals (Kuenzel et al., 2011).

The extent of adult hippocampal neurogenesis was found to vary in birds demonstrating seasonal behavioural variations such as food caching and retrieving behaviour in the black capped chickadees and in migratory species when compared to non-migratory counterparts (Hoshooley and Sherry, 2007; Sherry and Hoshooley, 2009; LaDage et al., 2010; Sherry and Hoshooley, 2010; LaDage et al., 2011). It was observed that adult hippocampal neurogenesis reached peak levels during the autumn and spring when food levels were abundant and declined in winter when food levels were low in food caching birds (Sherry and Hoshooley, 2009, 2010). In addition, the size of the hippocampus was found to be larger in migrating species than in non-migratory species and a similar trend was observed for adult hippocampal neurogenesis (LaDage et al., 2011). Bird migration is both a seasonal and hippocampal dependent task, hence investigating further adult neurogenesis in species showing this trait might provide a useful link between hippocampal neurogenesis and behaviour. Furthermore comparing adult neurogenesis in birds with different modes of survival is necessary to uncover the specific biological mechanisms responsible for distinct

proliferation activities, cell fates and also provide insight into the possible functional aspects of adult neurogenesis (Chapouton et al., 2007).

### **2.3. Adult neural stem cells/ progenitors cells in birds and mammals**

Stem cells are multipotent cells capable of generating multiple types of differentiated cells and are capable of cell division in which one daughter cell maintains stem cell potential (Morrison et al., 1997). In adult mammals including humans, neural stem cells (NSC)/ neural progenitors (NPC) are localized in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Gould et al., 1999a; Kornack and Rakic, 1999; Zhao et al., 2008). However, NSC/NPCs in the non-mammalian species including fish, reptiles and birds are located within and in the vicinity of the SVZ of the lateral ventricle (Doetsch et al., 1999a; Kaslin et al., 2008). Despite considerable advancement in characterizing and localizing the NPCs in the SVZ of both mammals and non-mammalian species the precise phenotype and location of the most primitive cells in the neurogenic niches is debatable. In adult rodents, ependymal cells were reported to have stem cell like properties (Johansson et al., 1999a; Johansson et al., 1999b). Subsequently, Doetsch et al. (1999a) provided more convincing evidence that a subset of cells in the SVZ and not ependymal cells was stem cells. These two populations of stem cells shared similar characteristics including expression of certain markers like glial fibrillary acidic protein (GFAP) and possession of cilia (Doetsch et al., 1999a; Johansson et al., 1999a; Johansson et al., 1999b). Much clearer answers to the phenotype and architecture of the SVZ stem cells came from ultrastructural and molecular characterisation experiments in both mammals and non-mammalian species (Doetsch et al., 1997; Alvarez-Buylla et al., 1998; García-Verdugo et al., 1998; Doetsch et al., 1999a; Doetsch and Scharff, 2001; Alvarez-Buylla et al., 2002). The results from these studies showed that there are both similarities and differences in the architecture and composition of SVZ between mammals, particularly in mouse and birds

(Alvarez-Buylla et al., 1998; Doetsch et al., 1999a; García-Verdugo et al., 2002). The mouse SVZ contains four principal cell types including astrocytes (type B), transit amplifying cells (type C), neuroblasts (type A) and ependymal cells (type E) (Doetsch et al., 1999a; Doetsch and Scharff, 2001). The subventricular zone astrocytes (type B cells) were found to possess neural stem cell characteristics such as the ability to self-renew, generation of both neurons and glia *in vitro*; label retention and generation of the entire subventricular zone in isolation (Doetsch et al., 1997; Doetsch et al., 1999a; Doetsch and Scharff, 2001). Consequently, type B progenitor cells generate type C and then type C cells differentiate to give rise to type A cells which then migrate and integrate into existing circuits (Doetsch and Scharff, 2001). The architecture of the SVZ in song birds shares some similarity and differences to that of mammalian species, particularly mouse species (Alvarez-Buylla et al., 1998; Doetsch and Scharff, 2001; Zhao et al., 2008). Both subventricular zones contain ependymal cells (type E cells) and young migrating neurons/ neuroblasts (type A) (Alvarez-Buylla et al., 1998; Doetsch and Scharff, 2001). However, in birds the subventricular zone lacks transit amplifying cells (type C cells) and the morphology of the glia (type B cells) has no branched processes typical of astrocytes but has a single elongated process which reaches deep into the parenchyma (Alvarez-Buylla et al., 1998; Doetsch and Scharff, 2001; Alvarez-Buylla and García-Verdugo, 2002). Thus, type B cells in mouse are astrocytes while in songbirds they resemble radial glia (Doetsch and Scharff, 2001; García-Verdugo et al., 2002). In addition, the glial cells in songbirds maintain direct contact with the ventricle whereas in mouse the cells occasionally contact the ventricle (Doetsch and Scharff, 2001). Another remarkable difference in the architecture of the subventricular zones between mammals and birds is that in birds only the radial glia cell divide while in mouse astrocytes (type B), transit amplifying cells (type C) and neuroblasts (type A) all divide (Doetsch et al., 1997; Doetsch et al., 1999a; Doetsch et al., 1999b; Doetsch and Scharff, 2001). In birds radial glia cells persist as neural stem cells from embryonic stages while in mammals embryonic radial glia cells disappear and

the astrocytes act as neural stem/progenitor cells (Alvarez-Buylla et al., 2002; Doetsch, 2003; Goldman, 2003; Alvarez-Buylla and Lim, 2004). In both mouse and songbirds the glial cells (type B) and ependymal cells (type E) maintain contact with the ventricle but the former does so with a single cilium while the latter send several cilium into the ventricle (García-Verdugo et al., 2002). Contrary to Johansson et al. (1999b), ependymal cells lack neural stem characteristics, but they are important for the maintenance of the neurogenic niche by suppressing the effects of the bone morphogenic proteins which oppose adult neurogenesis (Lim et al., 2000). In songbirds several experiments confirmed the neural progenitor capacity of radial cells utilising various methods including ultrastructural studies, autoradiography, BrdU labelling and retroviral infections injections (Alvarez-Buylla et al., 1998; Goldman, 1998; Alvarez-Buylla et al., 2002).

Apart from the SVZ progenitors, mammals possess neural progenitors in the hippocampus, particularly in the dentate gyrus which are different in morphology from the former. Fukuda et al. (2003) reported two distinct Nestin positive stem cells in the dentate gyrus of the hippocampus. Type I cells are regarded as the hippocampal progenitors and they are characterised by a triangular soma and long thick branching processes which span the granule cell layer into the molecular cell layer (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). Furthermore type 1 progenitors express molecular markers such as Nestin, glial fibrillary acidic protein (GFAP) and SRY (sex determining region Y)-box 2 (Sox2) (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). The second progenitor cell type observed in the mammalian hippocampus is the type 2 cell which is characterised by a small round soma and very thin processes; GFAP negative, but expressed polysialylated neural cell adhesion molecule (PSA-NCAM) (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). Type 2 progenitors also express Sox2 and Nestin and also show stem cell like characteristics of self-renewal (Fukuda et al., 2003; Suh et al., 2007; Zhao et al., 2008). Further observations confirmed that division of a single type 2 progenitor generates a single neuron



and an astrocyte *in vivo*, demonstrating that they are potent neural stem cells (Suh et al., 2007). Based on the morphology of type 1 progenitors, it was speculated that the morphology of stem cells was similar to radial astrocytes (Seki and Arai, 1999). Following on this premise, Fukuda et al. (2003) postulated that type I cells could generate type II cells and the phenomenon is still to be confirmed. The specific phenotype of stem cells and their conducive environment for induction needs to be established so as to facilitate the manipulation of these cells to differentiate *in vitro* or *in vivo* to develop therapies for neurodevelopmental diseases.

#### **2.4. Maturation sequence of newly generated neurons**

Maturation of adult precursor cells into mature neurons was described morphologically in rodents (Seri et al., 2004; Espósito et al., 2005) and in primates (Ngwenya et al., 2006). In these species the adult stem/progenitor cells undergo similar morphological maturation stages. Information about the morphological maturation of precursor cells into neurons or glia is lacking in birds and it is not known whether avian precursor cells undergo similar processes as observed in rodents and primates. In the monkey hippocampus, immature neurons were identified in the granule cell layer, subgranular zone and occasionally in the hilus of the dentate gyrus (Ngwenya et al., 2006). Ultrastructural and immunohistochemical studies revealed that adult mammalian precursor cells pass through three morphologically distinct maturation stages ranging from small cells with no processes to large mature cells with both axons and dendrites (Seri et al., 2004; Espósito et al., 2005; Ngwenya et al., 2006). Stage I cells showed a round or oval nucleus surrounded by a thin film of cytoplasm and small soma that lacked apical dendrites; stage II cells exhibited a large cell body and a single apical dendrite with minimum branching while stage III cells had a large soma and apical dendrites with elaborate branching (Ngwenya et al., 2006). Stage I and II cells express immature neuron markers such as doublecortin (DCX) while stage III express specific markers for mature neurons such as neuronal nuclear antigen (NeuN). In addition

stage III neurons make synaptic contacts with other mature neurons showing evidence of integration into the existing circuits (Ngwenya et al., 2006). Although studies determining the sequence of maturation of progenitors cells in the brain are lacking in birds, extensive studies have shown the location of neural regeneration, migration and integration utilizing both endogenous and exogenous markers of adult neurogenesis (see review, Barnea and Pravosudov, 2011). It is therefore important to design lineage studies utilizing both immunohistochemistry and ultrastructural methods in various bird species in order to understand the basic biological principles and stages of adult neurogenesis.

## **2.5. Factors affecting adult neurogenesis in birds**

### **2.5.1. Endogenous factors**

Various internal factors have been demonstrated to have a positive or negative influence on any of the stages of the adult neurogenesis including maintenance of neurogenic niche, proliferation of progenitors, migration of neuroblasts and integration/ recruitment into existing circuits. In birds, a few factors have been studied compared to mammals and these internal factors include but limited to steroid hormones, age and growth factors (see review, Barnea and Pravosudov, 2011). Steroid hormones (testosterone and estradiol) have been found to regulate the learning, production and maintenance of adult song in song birds (Alvarez-Buylla and Kirn, 1997). Steroid hormones act on the song control nuclei, particularly the high vocal center (HVC) and robust nucleus of arcopallium (RA) by influencing plasticity and neuronal recruitment in the adult songbirds, but not on cell proliferation in the subventricular zone (Brown et al., 1993; Gahr et al., 2002). Testosterone, in particular increases the survival and recruitment of adult HVC neurons through brain derived neurotropic factor (BDNF) (Rasika et al., 1994), while estrogen promotes the migration of neuroblasts from the subventricular zones (Lee et al., 2007). Another hormone which has direct effects on adult neurogenesis in birds is prolactin. Prolactin increases neuron

recruitment in the nidopallium caudale, a brain region involved in auditory processing (Güntürkün, 2005; Barnea and Pravosudov, 2011). Circulating levels of prolactin have been found to increase at hatching suggesting that it might be helpful in parent-offspring recognition (Barkan et al., 2007).

The effects of age on adult neurogenesis are extensively studied in mammals and it is generally agreed that adult neurogenesis declines with age (Amrein et al., 2004; Amrein and Lipp, 2009; Amrein et al., 2011). In birds, few studies have demonstrated its effects, but results correspond to those in mammals (Ling et al., 1997; Kim et al., 2006). In juvenile birds, neuronal production and recruitment was found to be higher than that of adult birds and also to exceed neuronal death (DeWulf and Bottjer, 2002; Wilbrecht and Kirn, 2004). In both song and non-song birds the rate of neuronal recruitment was found to decrease with increasing age (Ling et al., 1997; Wang et al., 2002; Kim et al., 2006). In songbirds the rate of neuronal loss was found to be unevenly distributed in the telencephalon and also to be species-specific depending on the demands for song production (Alvarez-Buylla et al., 1994; Alvarez-Borda and Nottebohm, 2002; Wang et al., 2002).

Growth factors regulate adult neurogenesis in association with sensory cues as a response to various environmental stimuli. Most of these growth factors influence adult neurogenesis by increasing the proliferation of neural progenitors in the subventricular zones and these include fibroblast growth factor-2 (FGF2); epidermal growth factor (EGF); transforming growth factor- alpha (TGF-  $\alpha$ ) and vascular endothelial growth factor (VEGF) (see review, Kaslin et al., 2008). In addition, the fibroblast and transforming growth factors increase neuronal recruitment (Craig et al., 1996; Fallon et al., 2000). The brain derived neurotrophic factor (BDNF) acts by increasing survival rates of neurons, particularly in the high vocal centre (Alvarez-Buylla and Lim, 2004). Within the SVZ, the bone morphogenetic protein (BMP) signalling inhibits adult neurogenesis and increase glial differentiation;

however its negative effects are countered by Noggin a factor released by ependymal cells which acts as an antagonist to BMP (Lim et al., 2000).

### **2.5.2. Exogenous factors**

Similarly, external factors and their effects on various stages of adult neurogenesis are extensively studied in mammals particularly rodents than in any other class of vertebrates; however, some of the factors have been found to have regulatory mechanisms in the class aves (see reviews, Barnea and Pravosudov, 2011; Aimone et al., 2014). Seasonal changes have been demonstrated in both song and non-song birds and its effects on song production and brain plasticity have been reported (e.g. Barnea and Nottebohm, 1994; Tramontin and Brenowitz, 2000; Sherry and Hoshooley, 2010; Brenowitz and Larson, 2015). Effects of seasonal changes in song production are carried out through steroid hormones which are seasonally secreted and ultimately influence adult neurogenesis as explained in previous sections. Testosterone surges just before breeding periods, increase the survival of HVC neurons and production of stable song, while reduction in testosterone post breeding is coupled with death of neurons in the HVC and production of unstable song (Kirn et al., 1994; Kirn, 2010). Surges of testosterone are also affected by the length of the day or exposure to light with more testosterone being released during the long light exposure (Balthazart et al., 2008; Balthazart et al., 2010; Yamamura et al., 2011). In the hippocampal complex, seasonal increases in the recruitment of neurons correlated with food storage and retrieval behavior in the free ranging black capped chickadees (Barnea and Nottebohm, 1994). Food caching and retrieval behavior is a spatial memory dependent task that is modulated by the dentate gyrus of the hippocampus (Barnea and Nottebohm, 1994; Hoshooley and Sherry, 2007; Sherry and Hoshooley, 2009; LaDage et al., 2010; Sherry and Hoshooley, 2010). Environmental enrichment and physical activity have similar effects in both mammals and birds. In birds, environmental enrichment is associated with increased recruitment and survival of new

neurons in the hippocampus (Barnea and Nottebohm, 1994). Species from complex environments showed increased levels of hippocampal neuronal recruitment when compared with species from less complex settings, for example, black capped chickadees from the wild showed more hippocampal neuron recruitment than captive counterparts (Barnea and Nottebohm, 1994; LaDage et al., 2010; Chancellor et al., 2011). Captive environments have negative influences on adult neurogenesis and this is mediated by stress, social isolation and loss of habitat. Stress negatively affects adult neurogenesis through the effects of glucocorticoid hormones (Lipkind et al., 2002; Barnea and Pravosudov, 2011). Separate studies have shown that complex social settings positively influence adult neurogenesis particularly in the nidopallium caudale, an auditory processing center whereby sound positively reinforces neuronal recruitment (Lipkind et al., 2002; Barnea et al., 2006; Adar et al., 2008).

## **2.6. Functional correlations of adult neurogenesis in birds**

Despite the great advances into the study of adult neurogenesis to date; its functions are yet to be fully understood. Researchers agree that new neurons born in the adult species contribute to the plasticity of neuronal circuits (Kempermann et al., 2004; Barnea and Pravosudov, 2011; Aimone et al., 2014; Paredes et al., 2016). In birds the correlative functions of adult neurogenesis were studied in the vocal control system of song birds and in the hippocampus of species which rely on spatial memory to execute their behaviours (see reviews, Gahr et al., 2002; Barnea and Pravosudov, 2011; Paredes et al., 2016). In the hippocampus, adult neurogenesis is associated with memory and learning (Nottebohm, 1981, 2011). It is assumed that seasonal changes brings in new sensory cues which needs to be learnt and promote survivals of species, hence species have to adapt by producing and recruiting hippocampal neurons to assume the new task (Barnea and Nottebohm, 1994; Barnea and Pravosudov, 2011; Aimone et al., 2014). Studies have shown that seasonal

environmental changes, for example, prompted an increase in the recruitment of hippocampal neurons (Barnea and Nottebohm, 1994; Chancellor et al., 2011; Roth et al., 2011). In food caching birds, hippocampal neurogenesis is profound during periods of food hoarding and retrieval which is a spatial memory dependent task which is also affected seasonal changes (Barnea and Nottebohm, 1994; Sherry and Hoshoooley, 2009; LaDage et al., 2010; Sherry and Hoshoooley, 2010). In a study conducted to confirm if food caching and retrieval behavior depends on hippocampal neurogenesis, results indicated that adult neurogenesis is abundant in food hoarding species than non-food caching species (Hoshoooley and Sherry, 2007). A separate study on migratory birds also revealed that adult hippocampal neurogenesis is more pronounced in migratory than in non-migratory species, although bird migration involves other sensory cues apart from spatial memory (LaDage et al., 2011). Environmental complexity exerts pressure on the adult hippocampal neurogenesis whereby species living in complex environments exhibit increased hippocampal neurogenesis than species in less complex environments (LaDage et al., 2009; LaDage et al., 2010; Chancellor et al., 2011; Roth et al., 2011). In summary, adult hippocampal neurogenesis facilitates learning, but learning has also a reciprocal effect on adult neurogenesis (Wilbrecht and Kirn, 2004; Aimone et al., 2014; Paredes et al., 2016). Adult hippocampal neurogenesis is also implicated in memory consolidation, particularly in pattern separation whereby different neurons are utilized to perform different functions (Aimone et al., 2014). This function is assumed to prevent interference between old and new memories (Kempermann et al., 2004; Kempermann, 2008; Barnea and Pravosudov, 2011). For example food caching birds would need separate neurons to store information related to old and new caches avoiding interference between the two (Pravosudov and Smulders, 2010).

In the song control systems of song birds, adult neurogenesis is associated with replacement of damaged projection neuronal circuits between the high vocal center and robust nucleus of arcopallium (HVC–RA) in order to maintain a stable song (Scharff et al.,

2000). Additionally, new neurons are required for learning of new songs (Kempermann, 2008). This function was confirmed in a study between open ended vocal learners (continuous learners) such as the canaries and closed vocal learners such as the zebra finches and it was found that open ended learners had high rates of neuronal turnover in the HVC–RA circuits than in closed learners (Alvarez-Buylla et al., 1990a). Apart from learning new syllables, new neurons are also necessary for the maintenance of a stable song by replacing damaged and worn-out neurons (Wilbrecht and Kirn, 2004). Another notable system in birds which showed the importance of adult neurogenesis is the vocal reception region in the hypothalamus in Columbidae species. In a study where the hypothalamus was lesioned, doves lost the ability to respond to vocal stimulation but after a short period the doves recovered the lost function (Cheng et al., 2004). Examination of the brain of the recovered birds showed increased adult neurogenesis towards the lesioned area indicating that new neurons were responsible for the restoration of function (Cheng et al., 2004). The olfactory bulbs also show some neuronal plasticity, particularly of the replaceable granule cells. Olfactory deprivation was associated with neuronal death and loss of neuronal recruitment; but odor enrichment had a positive effect on neuronal recruitment in the olfactory bulb (Petreanu and Alvarez-Buylla, 2002).

## **2.7. Comparative vertebrate adult neurogenesis and some evolutionary perspectives**

To date there is considerable growth in the understanding of the basic biological principles of adult neurogenesis, particularly in vertebrate species. Despite these progresses towards understanding vertebrate adult neurogenesis, the number of species examined to date is insignificant to warrant comparative conclusions considering that there are about 1.5 million vertebrate species (Lindsey and Tropepe, 2006). Hence there is growing need to examine more species in order to understand the anatomical organization, species diversity, functional significance and evolutionary history of adult neurogenesis. Adult neurogenesis

and cell proliferation occurs in numerous neurogenic niches in phylogenetically older vertebrates than in modern vertebrate species, but this phylogenetic reduction is not yet understood (see reviews, Cayre et al., 2002; Lindsey and Tropepe, 2006; Kaslin et al., 2008; Barnea and Pravosudov, 2011). In addition, adult neurogenesis varies in closely related species indicating species- specific variations (Kempermann et al., 1997; Kempermann et al., 2006; Amrein et al., 2007; Chawana et al., 2013) and interclass variations (Cayre et al., 2002). The distribution of proliferation centers and adult neurogenesis in the subphylum vertebrate shows some evolutionary trends in line with brain complexity and also with regenerative capacity (Kaslin et al., 2008).

### **2.7.1. Fishes and amphibians**

In fishes, the most studied group is the teleost fishes and in this group there are numerous proliferation and neurogenic centers in the brain of adult species throughout the rostro- caudal axis (Zupanc and Horschke, 1995; Zupanc et al., 2005). Active neurogenic areas and proliferation centers are distributed in all major brain regions including the olfactory bulb, telencephalon, thalamus, epithalamus, preoptic region, hypothalamus, tectum, cerebellum, rhombencephalon and spinal cord (Zupanc and Horschke, 1995; Lindsey and Tropepe, 2006; Kaslin et al., 2008). Of these regions, the cerebellum is the major neurogenic niche and contributes about 75% of newly generated neurons in the teleost fishes (Zupanc and Horschke, 1995; Zupanc et al., 2005). In the brown ghost knife fish (*Apteronotus leptorhynchus*) the neurogenic niche in the cerebellum contributes approximately 100 000 new cells per 2 hour period indicating high intensity of cell proliferation (Zupanc and Horschke, 1995; Kaslin et al., 2008). About half of these new cells survive and integrate to existing circuitry (Zupanc, 2006). The new neurons produced in adult fishes contribute to different types of neurons depending on the brain region; in the telencephalon they differentiate into glutamic acid decarboxylase, tyrosine hydroxylase and parvalbumin neurons



whereas in the diencephalon they form the serotonin and tyrosine hydroxylase neurons (Grandel et al., 2006).

Neurogenic niches in amphibians are well studied, but in the few species examined, active proliferation and adult neurogenesis occurs in the following brain regions including telencephalon, preoptic region, thalamus, hypothalamus, midbrain and the cerebellum (see reviews, Lindsey and Tropepe, 2006; Kaslin et al., 2008). Some species of amphibians demonstrated adult neurogenesis in the olfactory epithelium and in the retina (Kaslin et al., 2008).

### **2.7.2. Reptiles**

Adult cell proliferation and neurogenesis in the brains of reptiles is distributed in various brain regions including the olfactory bulb, striatum, subventricular zone of lateral ventricle, dorsal ventricular ridge, cortex, nucleus sphericuls and the cerebellum (Font et al., 2001; García-Verdugo et al., 2002). The subventricular zone (SVZ) and the medial cortex, particularly in regions abutting the SVZ generate the majority of neurons in most adult reptilian species (Font et al., 2001). The medial cortex is equivalent to the dentate gyrus in mammals and is analogous to regions with high proliferative capacity in birds (hot spots) (Cayre et al., 2002; García-Verdugo et al., 2002). In reptiles, the majority of the adult generated neurons survive and become integrated into local circuits (Font et al., 2001). Migration of neuroblasts from the proliferative centers to the cortex happens with or without the assistance of radial glia scaffolding (see reviews, Font et al., 2001; Kaslin et al., 2008).

### **2.7.3. Birds**

In adult birds the active neurogenic regions and proliferation centers are concentrated in the subventricular zone of the lateral ventricles (Goldman and Nottebohm, 1983; Nottebohm, 1985; Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Barnea and

Nottebohm, 1994). Neuronal proliferation is profound in the focal areas in the SVZ of the lateral ventricle in regions referred to as hot spots located in the ventrolateral and dorsolateral walls of the lateral ventricle (Alvarez-Buylla et al., 1990b; García-Verdugo et al., 2002). The majority of new cells are generated in the ventrolateral hot spot than in the dorsolateral region (García-Verdugo et al., 2002). In this class, a few of the adult generated cells, approximately a third survive and become integrated into local circuits (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1990b). Neuroblasts migration from proliferating centers utilizes two defined modes which include tangential migration whereby migrating neuroblasts follow the walls of the lateral ventricle and radial migration whereby neuroblasts migrate away from the ventricular walls with the help of radial glia as scaffolds (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1990b). The common destination of migrating neurons from the ventral hot spot is the medial striatum (Alvarez-Buylla et al., 1994). The rostral migration stream is not well characterized in birds and migration of neurons towards the olfactory bulbs is still debatable. Other destinations of adult neurons in birds include the parenchyma of the telencephalon without cell clustering and the song control nuclei (Alvarez-Buylla et al., 1994; Alvarez-Buylla and Kirn, 1997; Ling et al., 1997; Boseret et al., 2007). The fate and identity of adult born neurons is still to be determined in birds. The only neurons characterized so far are those replaced in the song control system. Adult born neurons have been identified to form projecting neurons in the high vocal center (HVC) to robust nucleus of the arcopallium (RA) circuit (HVC – RA) and interneurons within the HVC (Nottebohm, 1985; Alvarez-Buylla et al., 1990a).

#### **2.7.4. Mammals**

In mammalian species adult neurons are generated in two canonical regions, the subventricular zone of the lateral ventricle and the subgranular zone of the dentate in the hippocampus (Lindsey and Tropepe, 2006; Kaslin et al., 2008; Patzke et al., 2013; Ihunwo

et al., 2016). Other brain regions thought to generate new neurons in the adult include the neocortex, amygdala, hypothalamus, midbrain, dorsal vagal complex and spinal but the fate of newly generated neurons from these regions and also the efficacy of these regions as neurogenic niche across mammals is still debatable (see review, Kaslin et al., 2008). The SVZ is the largest neurogenic niche of the two and produces *gamma*-Aminobutyric acid (GABA) and dopaminergic neurons which integrate in the olfactory bulb as interneurons (Lois and Alvarez-Buylla, 1994). In mammals, neuroblasts migrate tangentially through the rostral migratory stream from the SVZ to the olfactory bulb, but presence of this route in primates, particularly humans is debatable (Sanai et al., 2007). Neurons generated in the SGZ migrate short distances into the granule cell layer of the dentate gyrus and integrate as interneurons (Eriksson et al., 1998; Seri et al., 2004). In mammals neuronal birth in adult species is countered by cell death and the majority of adult born neurons die without integration into circuits (see reviews, Kaslin et al., 2008; Aimone et al., 2014).

In view of the past studies on various classes of vertebrates and the information provided so far; we investigated putative adult neurogenesis in various species of birds exhibiting different behavioural repertoires, particularly determining and localizing the active neurogenic regions, migratory routes of migrating neuroblasts and regions of integration of new neurons in the brains of seven bird species from different taxonomic orders.

# **CHAPTER THREE: Putative adult neurogenesis in two domestic pigeon breeds (*Columba livia domesticus*): racing homer versus utility carneau pigeons**

## **3.1. Introduction**

The process of adult neurogenesis occurs in both invertebrates and vertebrates, including humans (Eriksson et al., 1998; Bartkowska et al., 2010; Barnea and Pravosudov, 2011). In birds, generation of new neurons is limited to the dorsal and ventral reaches of the subventricular zone (SVZ) of the lateral ventricles (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1998). Along these areas of the SVZ, proliferating cells form aggregates referred to as proliferative hotspots (Alvarez-Buylla et al., 1990a). From the walls of the lateral ventricles, new neurons migrate to various areas of the telencephalon which includes, but are not limited to, the high vocal centre (HVC), area X, the nidopallium caudale (NC) in song birds, and the hippocampus in both non-song birds and song birds (Paton and Nottebohm, 1984; Nottebohm, 1985; Alvarez-Buylla et al., 1994; Lipkind et al., 2002; Sherry and Hoshooley, 2010; Melleu et al., 2013).

In adult song birds, such as canaries, and food caching birds, such as the black capped chickadees, seasonal variations in the recruitment of new neurons in the HVC and hippocampus, respectively, have been observed (Kirn and Nottebohm, 1993; Barnea and Nottebohm, 1994). There is evidence that adult neurogenesis varies in the members of the same species from different populations, for example in black capped chickadees (Chancellor et al., 2011) and mouse (Kempermann et al., 1997).

Adult neurogenesis has been reported in the telencephalon of song birds including canaries (Alvarez-Buylla and Nottebohm, 1988; Balthazart et al., 2008) and zebra finches

(Kim et al., 2006) and non-song birds such as chickens , black capped chickadees (Sherry and Hoshooley, 2010; Mezey et al., 2012), Japanese quails (Balthazart et al., 2010), ring doves (Ling et al., 1997) and rock pigeons (Melleu et al., 2013; Melleu et al., 2015). Domestic pigeons (*Columba livia domesticus*) of the order Columbiformes are descendants of the wild rock pigeon through domestication and selective breeding (Levi, 1986; Stringham et al., 2012). The racing homer possesses characteristics such as increased flight speed, large home ranges, improved spatial memory, and larger hippocampal formations and olfactory bulbs than other pigeon breeds (Bingman et al., 2006; Rehkämper et al., 2008; Mehlhorn and Rehkämper, 2009). Utility pigeons were selectively bred for their fast growth and large body size desirable for meat production. These different characteristics in the two breeds of domestic pigeons allows for the prospect of examining and comparing the process of adult neurogenesis in closely related species with different behaviour repertoires.

Examination of multiple species with phylogenetically diverse traits and also in closely related species, or breeds, in different ecological niches may facilitate our understanding of the functions of adult neurogenesis and the factors contributing to variances in this neural trait amongst species (Jarvis et al., 2005; Amrein and Lipp, 2009; Bartkowska et al., 2010). Based on this premise, we examined putative adult neurogenesis in the brains of two breeds of the adult domestic pigeon (*Columba livia domesticus*), the racing homer and the utility carneau pigeons using the markers proliferating nuclear cell antigen (PCNA) and doublecortin (DCX) which label proliferating cells and immature neurones respectively (Hall et al., 1990; Brown et al., 2003; Melleu et al., 2013).

## 3.2. Experimental procedures

### 3.2.1. Animals and tissue processing

Four adult male domestic pigeon brains, two of each of racing homers and utility carneau pigeons were purchased from a local breeder and used in this study. The animals were bred in isolated cages according to the breeds in large social groups consisting of both males and females and nesting sites. The animals were supplied with water food *ad libitum*. The pigeons were 8 months old and sexually mature (Ling et al., 1997) and sex was determined by inspecting gonads during dissection. The animals were purchased and sacrificed during spring (01-08 November) in 2013. Animals were transported from the aviary to the University of the Witwatersrand Central Animal Services (CAS) in well ventilated, roomy plastic boxes. Animals were housed in the CAS and allowed to acclimatise for a period of 7 days with food and water supplied *ad libitum*. All animals were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (approval no: 2013/05/02B), which parallel those of the NIH guide for the care and use of animals in scientific experimentation. Five minutes prior to being euthanized, all animals were given an intramuscular dose of Heparin (0.5 mL) to prevent blood clotting. Animals were euthanized with an intraperitoneal injection of Euthapent (0.5-1 mL/kg) and body mass recorded: racing homer pigeon (average body mass =  $316.00 \pm 23.33$  g) and utility carneau pigeon (average body mass =  $542.35 \pm 7.00$  g). Animals were transcardially perfusion-fixed, initially with a rinse of 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) solution. Brains were carefully removed from the skull, post fixed overnight in 4% paraformaldehyde in 0.1 M PB. The brains were then weighed: racing homer pigeon (average brain mass =  $2.20 \pm 0.00$  g) and utility carneau pigeon (average brain mass =  $2.80 \pm 0.14$  g) and cryoprotected in 30% sucrose in 0.1 MPB at 4°C for 3 days and then stored in an antifreeze solution at 20°C. Before sectioning, the tissue was allowed to equilibrate in 30% sucrose in 0.1M PB at 4°C. The brains were frozen in dry ice and sectioned

in the coronal plane, 50 µm thick sections, on a sliding microtome (Microm HM 430, Thermo Scientific, USA). One in three series of sections in each animal were taken and stained for Nissl substance, PCNA and DCX.

### **3.2.2. Nissl staining for histological cytoarchitecture**

The first series of sections used for Nissl staining were mounted on 0.5% gelatine coated slides, dried overnight, cleared in a 1:1 mixture of 100% ethanol and 100% chloroform and stained with a 1% Cresyl Violet solution.

### **3.2.3. Immunohistochemistry for PCNA and DCX**

The second and third series of sections from each animal were used for free floating PCNA and DCX immunohistochemistry. The sections were incubated in a 1.6% H<sub>2</sub>O<sub>2</sub>, 49.2% methanol, 49.2% 0.1 M Phosphate buffer (PB) solution, for 30 min to reduce endogenous peroxidase activity, which was followed by three 10-min rinses in 0.1 M PB. To block unspecific binding sites the sections were then pre-incubated for 2 h, at room temperature under gentle shaking, in a blocking buffer solution consisting of 3% normal horse serum for PCNA sections or 3% normal rabbit serum for DCX sections, 2% bovine serum albumin, and 0.25% Triton X-100 in 0.1 M PB. Following pre-incubation the primary antibodies were added to the blocking buffer solution and the sections were incubated for 48 h at 4°C under gentle shaking (PCNA, 1: 500 dilution of mouse anti-PCNA, NCL-L-PCNA Leica Biosystems, Newcastle, United Kingdom; DCX, 1:300 dilution of goat anti-DCX antibody, C-18, Santa Cruz Biotechnology, Dallas, TX, USA) under gentle agitation. The primary antibody incubation was followed by three 10-min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (PCNA sections, 1:1,000 dilution of biotinylated anti-mouse IgG [BA-2001, Vector Laboratories, USA] in 3% normal horse serum and 2% bovine serum albumin in 0.1 M PB; DCX sections, 1:1,000 dilution of anti-goat IgG

[BA-5000, Vector Labs] in 3% normal rabbit serum and 2% bovine serum albumin in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidinbiotin solution (1:125 in 0.1 M PB); Vector Labs, Burlingame, CA, USA), followed by three 10-min rinses in 0.1 M PB. Sections were then transferred to a solution consisting of 0.05% diaminobenzidine tetrahydrochloride in 0.1 M PB for 5 min at room temperature, after which 3.3  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub>/ml of solution was added. With the aid of a low power objective lens on a stereomicroscope (Leica MZ 7.5, Meyer instruments, Houston, USA) the progression of the staining was visually followed and allowed to continue until a level was reached where the background staining could assist in analysis by matching to architectonic borders from Nissl stained sections without obscuring the immunopositive structures. The tissue was then rinsed twice more in 0.1M PB before being mounted on glass slides coated with 0.5% gelatine and allowed to dry overnight. Once dry, the slides were placed in a solution of 70% ethanol for 2 h and then dehydrated, cleared in xylene and coverslipped with DPX mountant. To test for non-specific staining of the immunohistochemical protocol, the primary and secondary antibodies were omitted from random sections and no specific staining was evident. The observed immunostaining patterns support the specificity of the antibodies and are compatible with observations made in columbiformes and other avian species (Boseret et al., 2007; Charvet and Striedter, 2008; Melleu et al., 2013; Melleu et al., 2015).

#### **3.2.4. Analysis**

The Nissl stained sections were examined under a low power objective lens on a stereomicroscope (Leica MZ 7.5, Meyer instruments, Houston, USA) and the architectonic borders were traced using a camera Lucida fitted on the stereomicroscope (Leica MZ 7.5, Meyer instruments, Houston, USA). The PCNA and DCX immunostained sections were then matched to the drawings from the Nissl stained sections and the location of immunopositive



soma marked on the drawings. Selected drawings were then scanned (DS-50000, Epson, South Africa) and redrawn using the Canvas 8 Software (Deneba Software, Miami, FL, USA). The brain regions were identified and named in accordance with the stereotaxic atlas of the brain of the pigeon (Karten and Hodos, 1967) using the nomenclature recommended by the Avian Brain Nomenclature Forum (Reiner et al., 2004). Digital photomicrographs were captured using a digital camera (Axio Cam HRc, Zeiss South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software (Zeiss, South Africa). No pixilation adjustments or manipulation of the captured images were undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7. The distribution of PCNA and DCX-immunoreactive cells was described qualitatively using a low power (x5) objective lens on a light microscope (Axiostar plus, Zeiss, south Africa) and the densities of immunostained cells were visually compared and recorded on a scale ranging from low (+), moderate (++) to high (+++). A second observer was used to eliminate observer bias.

## **Abbreviations**

A – arcopallium

AC – anterior commissure

BSTL – lateral part of the bed nucleus of the stria terminalis

CB – cerebellum

CDL – dorsolateral cortical area

CP – posterior commissure

CPi – piriform cortex

DCX – doublecortin

DL – dorsolateral region of the hippocampus

DLL – nucleus dorsolateralis anterior thalami, pars lateralis

DLM – nucleus dorsolateralis anterior thalami, pars medialis

DM – dorsomedial region of the hippocampus

DMA – nucleus dorsomedialis anterior thalami

E – entopallium

EPL – external plexiform layer of the olfactory bulb

EW – nucleus of Edinger-Westphal

FPL – fasciculus prosencephalic lateralis

GCt – griseum centrale

GL – glomerular layer of PB

GLv – nucleus geniculatus lateralis, pars ventralis

GP – globus pallidus

HA – hyperpallium apicale

HD – hyperpallium densocellulare

HM – nucleus habenularis medialis

HP – hippocampus

HVC – high vocal centre

IGrL – internal granular layer of the olfactory bulb

IMc – nucleus isthmi, pars magnocellularis

INP – nucleus intrapeduncularis;

IPc – nucleus isthmi, pars parvocellularis

LA – nucleus lateralis anterior thalami

LHy – lateral hypothalamus

Ll – lateral layer of the ventral hippocampus

LM – nucleus lentiformis

LSt – lateral striatum

Mm – mammillary region

M – mesopallium

N – nidopallium

MCL – mitral cell layer of the olfactory bulb

MI – medial layer of ventral hippocampus

MLd – nucleus mesencephalicus lateralis, pars dorsalis

MSt – medial striatum

nBOR – nucleus of the optic root

NC – nidopallium caudale

NDB – nucleus diagonalis Broca

nIII – oculomotor nerve

OB – olfactory bulb

OC – optic chiasm

OMN – oculomotor nucleus

ON – olfactory nerve

ONL – olfactory nerve layer

OV – nucleus ovoidalis

PCNA – proliferating nuclear cell antigen

PL – nucleus pontis lateralis

PMH – nucleus medialis hypothalamic posterioris

PO – preoptic region

POM – nucleus preopticus medialis

PPC – nucleus principalis precommissuralis

PPN – pedunculopontine tegmental nucleus

PVM – nucleus periventricularis magnocellularis

RSv – nucleus reticularis superior, pars ventralis

Rt – nucleus rotundus

Ru – nucleus ruber

SCi – stratum cellular internum

SL – lateral septum

SM – medial septum

SNc – substantia nigra, pars compacta

SNr – substantia nigra, pars reticulata

SP – nucleus subpretectalis

SPC – nucleus superficialis parvocellularis

SpL – nucleus spiriformis lateralis

SRt – nucleus subrotundus

STN – subthalamic nucleus

SVZ – subventricular zone

TeO – optic tectum

TPO – temporo-parieto-occipital area

Tr – triangular area of ventral hippocampus

TrO – optic tract

TU – nucleus tuberis

V – ventricle

VP – ventral pallidum

### 3.3. Results

#### 3.3.1. General observations

We examined putative adult neurogenesis throughout the brains of the racing homer and utility carneau pigeon breeds using immunohistochemical techniques for the endogenous markers PCNA and DCX. The distribution of PCNA and DCX immunoreactivity was almost identical in both breeds, but a few minor differences were observed (Table 3.1). Due to this extensive similarity we depict only the mapping of the distribution of the PCNA and DCX-immunoreactive cells in the racing homer pigeon (Fig. 3.1, 3.2). In both pigeons, PCNA and DCX immunoreactivity was observed in the olfactory bulbs, subdivisions of the pallium (Hippocampus [Hp], hyperpallium apicalle [HA], hyperpallium intercalatum [HI], hyperpallium densocellulare [HD], mesopallium [M], nidopallium [N], entopallium [E], arcopallium [A], dorsolateral cortical area [CDL], piriform cortex [CPi] and the temporo-parieto-occipital area [TPO]), subpallium (medial striatum [MSt], lateral striatum [LSt] and the globus pallidus [GP]), diencephalon, mesencephalon (pretectum and tectum), and cerebellum. Generally, the telencephalic regions had a higher density of PCNA- and DCX-immunoreactive cells than other brain regions in both breeds of pigeons examined, while the lowest density of cells immunoreactive to both markers was observed in the cerebellum. In the majority of the telencephalic regions, the density of PCNA- and DCX-immunoreactive cells increased from rostral to caudal in both pigeon breeds, except in the mesopallium where the staining density of cells first decreased from rostral to middle levels and then increased at caudal levels. DCX immunoreactivity was more intense in fibres than in cell bodies and the shapes of DCX-immunoreactive cells included small rounded cells, fusiform bipolar cells, and large round and/or polygonal multipolar cells.

### **3.3.2. Distribution of PCNA-immunoreactive cells**

#### **3.3.2.1. Olfactory bulb**

The olfactory bulbs contained the highest density of PCNA-immunoreactive cells of all regions of the brain in both pigeon breeds. PCNA-immunoreactive cells were observed at high density in the internal granular layer (IGrL), mitral cell layer (MCL) and external plexiform layer (EPL) layer, while they were found at a lower density in the glomerular layer (GL) and olfactory nerve (ON) layer (Fig. 3.1A, 3.3A, 3.3C).

#### **3.3.2.2. Subventricular zones of the lateral, third, fourth and tectal ventricles**

A high density of PCNA-immunoreactive cells were observed in the walls of the lateral, third, fourth and tectal ventricles, forming an uninterrupted layer, one to four cells thick, of cells with dark staining nuclei in both pigeon breeds. Proliferative hot spots were observed in both dorsal and ventral poles of the lateral ventricle, mostly in rostral levels of the brain (Fig. 3.4A, 3.4C). A higher density of PCNA-immunoreactive cells was observed in the racing homer pigeon associated with the walls of the lateral ventricle abutting the medial striatum (MSt), nidopallium (N), nidopallium caudale (NC) and hyperpallium apicale (HA) when compared to the utility carneau pigeon (Fig. 3.1C – G).

#### **3.3.2.3. Hyperpallium, mesopallium, nidopallium and entopallium**

In the pallial regions (HA, HI, HD, M, and N) PCNA-immunoreactive cells were observed in high density along the cortical margins and regions adjacent to the lateral wall of the lateral ventricle, but this density was obviously reduced in the core regions of HD, M and N in both pigeon breeds (Fig. 3.1C– G). At caudal levels the nidopallium caudale (NC) exhibited an increased density of PCNA-immunoreactive cells at the regions adjacent to the walls of the lateral ventricle and also towards the lateral cortex (Fig. 3.1H – J). The core of the NC had scattered PCNA-immunoreactive cells. In the entopallium (E), there was a higher

density of PCNA-immunoreactive cells rostrally, but the density gradually decreased towards the caudal levels of the telencephalon in all pigeons (Fig. 3.1D, 3.1E). In all these telencephalic regions, the density of PCNA-immunoreactive cells appeared greater in the racing homer pigeons than in the utility carneau pigeons.

#### **3.3.2.4. Hippocampus, piriform cortex and dorsolateral cortical area**

The hippocampus in the pigeons studied herein was defined according to the descriptions provided by Atoji and Wild (2004). PCNA-immunoreactive cells were sparsely distributed throughout the medial layer (Ml) of the ventral hippocampus, while a moderate density of PCNA-immunoreactive cells was observed in the triangular area (Tr), the lateral layer (Ll) (Fig. 3.5A, 3.5D), and the dorsomedial region (DM) in both pigeons. The dorsolateral region (DL) of the hippocampus exhibited the highest density of PCNA immunoreactive cells in both pigeon breeds. Throughout DL, the PCNA-immunoreactive cells were homogeneously distributed, and these cells were observed to continue into the dorsolateral cortical area (CDL) (Fig. 3.1H, 3.1I). The CDL exhibited a high density of PCNA-immunoreactive cells, especially in areas adjacent to the dorsolateral wall of the lateral ventricles in the caudal levels of this region. Ventrolaterally from CDL, towards the temporo-parieto-occipital cortex (TPO), the density of the PCNA-immunoreactive cells gradually decreased to a moderate density. The piriform cortex (CPI) exhibited a high density of PCNA-immunoreactive cells throughout the rostro-caudal axis of the telencephalon in both pigeon breeds (Fig. 3.1H, 3.1I).

#### **3.3.2.5. Striatum, septum and arcopallium**

In the striatum of both pigeon breeds, a moderate density of PCNA-immunoreactive cells was observed in the medial striatum (MSt) adjacent to the ventrolateral wall of the lateral ventricle, while the lateral striatum (LSt) exhibited a low density of PCNA-immunoreactive

cells (Fig. 3.1C – G). Generally in the striatum, there was a rostro-caudal decline in the density of PCNA-immunoreactive cells. In the racing homer pigeon, the medial septum (SM) housed a moderate density of PCNA-immunoreactive cells, the density of which lessened towards the most medial margin of this nucleus. The SM of the utility carneau pigeon had very densely packed PCNA-immunoreactive cells with large nuclei, which was prominent at the level of the anterior commissure (Fig. 3.6A, 3.6C, 3.6D). In the lateral septum (SL) of both pigeon breeds, a higher density of PCNA-immunoreactive cells was observed when compared to the SM, and within this nucleus there was a rostrocaudal decline in PCNA-immunoreactive cellular density. The arcopallium (A) in both pigeon breeds exhibited a moderate density of PCNA-immunoreactive cells along the cortical margins and towards the ventral parts of the NC, while the central areas had a very low to absent density of PCNA-immunoreactive cells (Fig. 3.1G, 3.1H).

#### **3.3.2.6. Diencephalon, optic chiasm and anterior commissure**

The diencephalon exhibited a high density of PCNA-immunoreactive cells in the various nuclei compared to the surrounding tissues. This was observed in the paraventricular nuclei which include nucleus preopticus medialis (POM), nucleus periventricularis magnocellularis (PVM) and nucleus medialis hypothalamic posteriolis (PMH) medially, the dorsal margin in the nucleus dorsomedialis anterior thalami (DMA), nucleus dorsolateralis anterior thalami, pars lateralis (DLL) nuclei and the lateral margin in the nucleus rotundus (Rt) and nucleus geniculatus lateralis, pars ventralis (GLv) (Fig. 3.1G, 3.1H). Further laterally the optic tract (Tro) housed a moderate density of PCNA-immunoreactive cells. In the core regions of the hypothalamus PCNA-immunoreactive cells were either absent or in a low density. The optic chiasm of both pigeon breeds exhibited a cluster of PCNA-immunoreactive cells which increased slightly in density adjacent to the ventral pole of the subventricular zone



of the third ventricle (Fig. 3.7A, 3.7C). There were few scattered PCNA-immunoreactive cells in the anterior commissure.

### **3.3.2.7. Pretectum, tectum, posterior commissure and cerebellum**

The pretectum in both pigeon breeds exhibited a high density of PCNA-immunoreactive cells in the dorsal margins and ventrally in the nucleus subpretectalis (SP). Laterally, the layers of the optic tectum (TeO) had a low density of PCNA-immunoreactive cells, and in the core regions only a few PCNA-immunoreactive cells could be observed (Fig. 3.1H – J). Within the optic tectum a high density of PCNA-immunoreactive cells was observed in the paraventricular nuclei closer to the fourth ventricle medially, the layers of the optic tectum (TeO) laterally in areas adjacent to the tectal ventricle and the nucleus of the basal optic tract (nBOR) ventrally in both pigeon breeds. In the utility carneau pigeons, the nucleus principalis precommissuralis (PPC) near the subventricular zone of the fourth ventricle had a high density of PCNA-immunoreactive cells that exhibited very large cell bodies, but these were not observed in the racing homer pigeons. PCNA-immunoreactive cells were scattered in the central nuclei of the optic tectum, such as the nucleus spiriformis lateralis (SpL), but were absent in the nucleus subpretectalis (SP). In the posterior commissure of both pigeons there were a few PCNA-immunoreactive cells that were intercalated with the commissural fibres. In the cerebellum of both pigeon breeds, PCNA-immunoreactive cells were observed in high density in the Purkinje cell layer and the occasional PCNA-immunoreactive cells were observed in the molecular and granule cell layers (Fig. 3.1J, 3.8A, 3.8C).

### **3.3.3. Distribution of DCX-immunoreactive cells**

DCX-immunoreactive cells were identified throughout the brains of the two adult pigeon breeds and were generally small fusiform shaped, bipolar and round multipolar cells

with slight variations in size in the different brain regions. Any size and/or shape variations are noted in relevant regions.

#### **3.3.3.1. Olfactory bulb**

The olfactory bulb was the region that contained the highest density of DCX-immunoreactive structures in both pigeon breeds. DCX-immunoreactive cells and fibres were seen in high density in the inner layers of the olfactory bulb, but at a lesser density in the outer two layers (Fig. 3.3B, 3.3D). DCX-immunoreactive cells appeared to be in the process of migrating from the olfactory bulbs into the pallial regions of hyperpallium apicalle (HA) and hyperpallium densocellulare (HD).

#### **3.3.3.2. Subventricular zone**

The subventricular zone of the lateral and third ventricles evinced 3 to 4 layers of DCX-immunoreactive cells in both breeds, which intermittently formed cell clusters of intensely stained cells at high density. This arrangement was more apparent on the lateral wall of the lateral ventricle than on the medial wall. The DCX-immunoreactive fibres emanating from the subventricular zone were oriented either parallel or orthogonal to the ventricular wall (Fig. 3.4B, 3.4D). The fourth and tectal ventricles of the racing homer pigeon exhibited a continuous layer of DCX-immunoreactive cells without cell clustering, whereas in the utility pigeon the tectal ventricles had more DCX-immunoreactive fibres than cells.

#### **3.3.3.3. Hyperpallium, mesopallium, nidopallium and entopallium**

There was moderate density of DCX-immunoreactive cells in the divisions of the hyperpallium (HA, HI and HD), with these cells and fibres being of higher density at the periphery and very low density in central areas (Fig. 3.2A – F). At more rostral levels, the hyperpallium densocellulare (HD) appeared to receive migrating neuroblasts from the

olfactory bulb. A similar group of apparently migrating neuroblasts was observed at caudal levels, towards the anterior commissure, where they appeared to migrate from the walls of the lateral ventricle into the hyperpallium apicalle (HA). The mesopallium (M) and nidopallium (N) exhibited a moderate density of DCX-immunoreactive cells and fibres, with higher densities of DCX-immunoreactive structures observed in the peripheral areas adjacent to the lateral wall of the lateral ventricle medially, the dorsoventral wall of the lateral ventricle dorsolaterally and piriform cortex (CPi) ventrolaterally (Fig. 3.2D – G). At caudal levels, posterior to the anterior commissure, the nidopallium caudale (NC) exhibited a similar density of DCX-immunoreactive cells and fibres as seen in the N and M. Higher densities of DCX-immunoreactive cells and fibres were observed near the walls of the lateral ventricle both medially and dorsolaterally in the NC, the CPi ventrolaterally and the arcopallium (A) ventrocaudally (Fig. 3.2H – J). The core areas of M, N and NC had a low to absent density of DCX-immunoreactive structures, but the DCX-immunoreactive cells in these areas were characterised by large round or polygonal somata and thick branching fibres. The entopallium (E) contained a moderated density DCX-immuoreactive cells and fibres in the racing homer pigeon but very low density of DCX-immunoreactive cells and fibres in the utility carneau pigeon (Fig. 3.2D, 3.2E). Generally, there was a rostro-caudal increase in the density and morphological complexity of the DCX-immunoreactive cells in the NC.

#### **3.3.3.4. Hippocampus, piriform cortex and dorsolateral cortical area**

The medial (Ml) region of the ventral hippocampus had sparsely distributed DCX-immunoreactive cells and fibres, while the triangular (Tr) and the lateral (Ll) regions exhibited a moderate density of DCX-immunoreactive cells and fibres in both pigeon breeds (Fig. 3.5B, 3.5C, 3.5E, 3.5F). In the dorsomedial (DM) region of the hippocampus, DCX-immunoreactive cells were observed at a higher density in the central areas and towards the dorsomedial wall of the lateral ventricle. There was low density of DCX-immunoreactive

cells and fibres in the dorsolateral (DL) region of the hippocampus, close to the border with DM medially. The density of DCX-immunoreactive cells in the DL region gradually increased towards the dorsolateral cortical (CDL) region (Fig. 3.2H, 3.2I).

In the CDL, there was a moderate density of DCX-immunoreactive cells and fibres. At caudal levels, the CDL region had a higher density of DCX-immunoreactive cells and fibres in areas adjacent to the dorsolateral margin of the lateral ventricle. The piriform cortex (CPi) had a higher density of intensely stained DCX-immunoreactive cells and fibres in both pigeon breeds (Fig. 3.2B – I).

### **3.3.3.5. Striatum, septum and arcopallium**

In the striatum, the medial striatum (MSt) exhibited a moderate density of DCX-immunoreactive structures with a higher density of stained structures in areas abutting the ventral parts of the lateral wall of the lateral ventricle, while the density of immunoreactive structures was less in the core regions (Fig. 3.2C – F). Ventral to the striatum, there was a high density of DCX-immunoreactive cells in the olfactory tubercle (OTu) and nucleus of the fasciculus diagonalis Brocae (NDB). The lateral striatum (LSt) had moderate to low density of DCX structures in both pigeon breeds (Fig. 3.2E – G), but a high density of DCX-immunoreactive structures was observed in the Globus pallidus (GP) and interpeduncular nucleus (INP) at levels caudal to the anterior commissure.

In the racing homer pigeon the septal nuclear complex exhibited a low density of DCX-immunoreactive structures in the medial septum (SM), whereas the lateral septum (SL) had a moderate density of DCX-immunoreactive structures, although this density was reduced at caudal levels. In the utility carneau pigeon the medial septum exhibited a moderate density of DCX-immunoreactive cells and fibres (Fig. 3.6B). There was a moderate density of DCX-immunoreactive cells and fibres in the arcopallium (A), especially along the ventral margins

in both pigeon breeds, but the core areas had less dense staining in the utility carneau pigeon (Fig. 3.2G).

#### **3.3.3.6. Diencephalon, optic chiasm and anterior commissure**

The diencephalon exhibited a moderate density of DCX-immunoreactive cells and fibres in the paraventricular regions, near the third ventricle, in the nucleus preopticus medialis (POM), and the nucleus medialis hypothalamic posterioris (PMH) (Fig. 3.2G, 3.2H). Dorsally, a moderate density of DCX-immunoreactive structures were observed in the stria medullaris (SMe) and some dorsal thalamic nuclei, including the nucleus dorsolateralis anterior, pars medialis (DLM) and nucleus dorsolateralis, pars lateralis (DLL), and laterally in the thalamus, a low density of DCX-immunoreactive structures were evident in the nucleus geniculatus lateralis, pars ventralis (GLv), nucleus lateralis anterior thalami (LA) and nucleus rotundus (Rt). The subthalamic nuclei (STN) nuclei were devoid of DCX-immunoreactive. The optic chiasm of both pigeon breeds exhibited a cluster of DCX-immunoreactive cells and fibres that spread lateral to the optic tract (TrO) (Fig. 3.7B, 3.7D). In the utility carneau pigeon, the anterior commissure exhibited a few scattered DCX-immunoreactive cells and fibres, but this was not evident in the racing homer pigeon.

#### **3.3.3.7. Pretectum, tectum, posterior commissure and cerebellum**

In the mesencephalic pretectum, a moderate density of DCX-immunoreactive structures were observed in the tegmentum in the decussatio supraoptica (DS). In the tectal region of the mesencephalon, a moderate density of DCX-immunoreactive structures were observed medially in the nucleus ruber (Ru), griseum centralis (GCt), oculomotor nucleus (OMN) and the Edinger-Westphal nucleus (EW), dorsally in the nucleus habenularis medialis (HM) and nucleus spiriformis lateralis (SpL), and ventrally in the nucleus tuberis (TU), pedunculo pontine tegmental nucleus (PPN) and the nucleus of the basal optic root

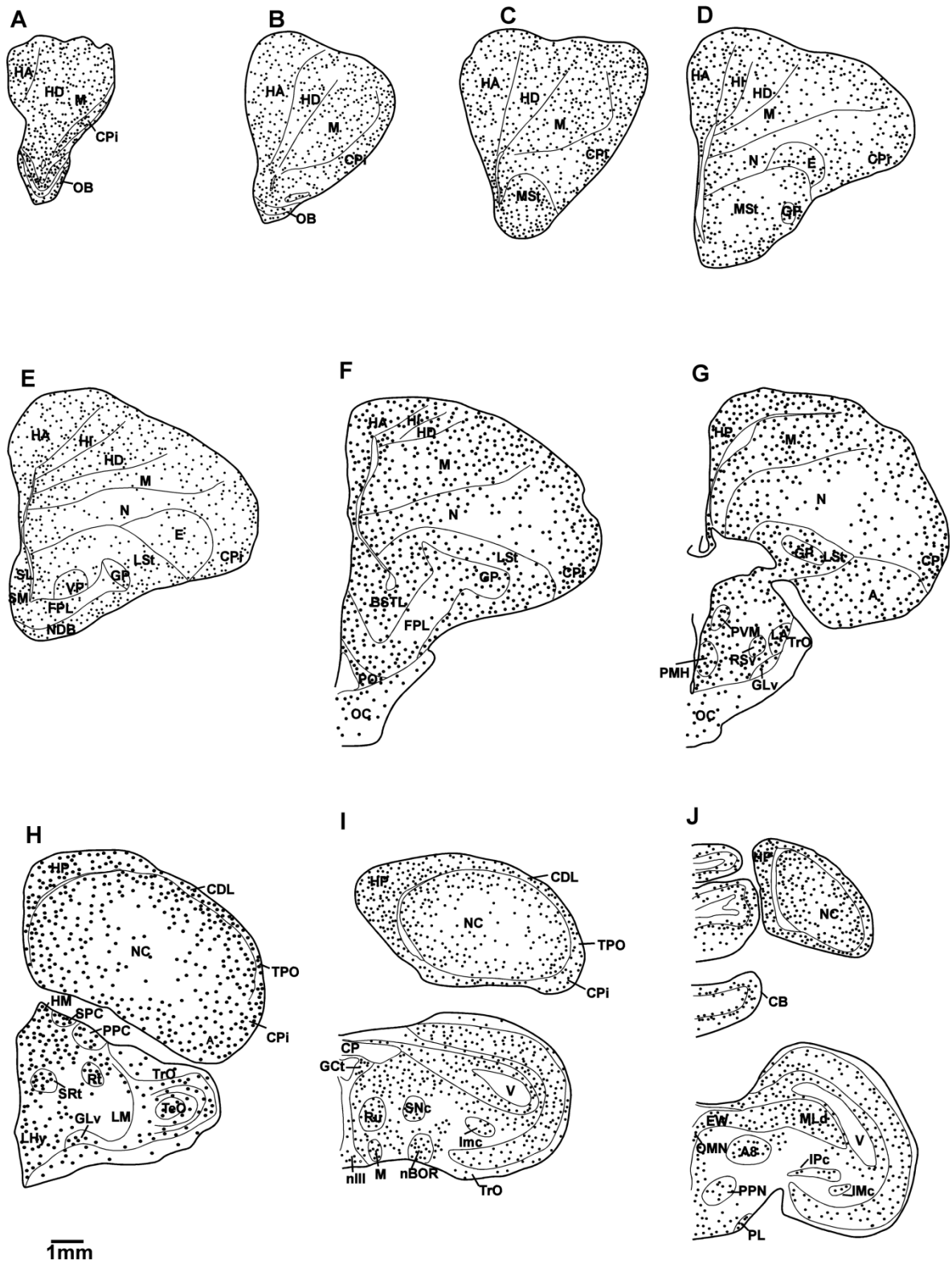
(nBOR) (Fig. 3.2H, 3.2I). The EW and Ru nuclei contained large round DCX-immunoreactive cells. DCX-immunoreactive structures were scattered in the Rt nucleus and in the layers of the optic tectum (TeO). The posterior commissure exhibited a few DCX-immunoreactive structures especially on margins adjacent to the walls of the cerebral aqueduct. In the cerebellum, the granule cell layer of the utility carneau pigeon had a moderate density of DCX-immunoreactive structures, while in both pigeon breeds the Purkinje cell layer had a high density of DCX-immunoreactive structures. The dendrites of the DCX-immunoreactive cells projected into the molecular layer of the cerebellum (Fig. 3.2J, 3.8B, 3.8D).

**Table 3.1.**Summary of qualitative distribution and density of PCNA and DCX immunoreactive structures in the brains of the racing homer and utility carneau pigeons.  
+ low, ++ moderate, +++ high density of DCX immunoreactive structures.

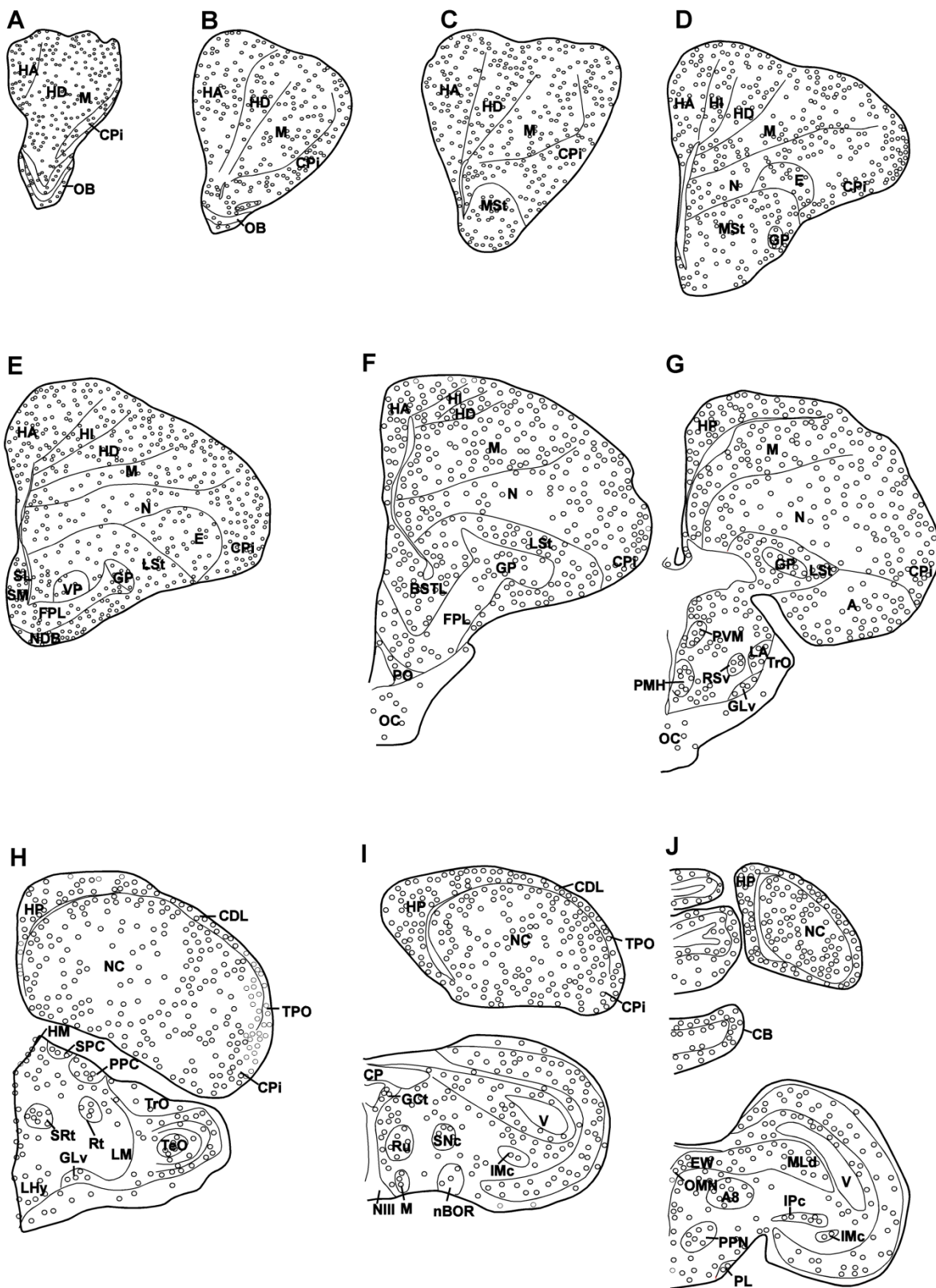
| BRAIN REGION   |             | Racing homer |     | Utility carneau |     |
|----------------|-------------|--------------|-----|-----------------|-----|
|                |             | PCNA         | DCX | PCNA            | DCX |
| SVZ            |             | +++          | ++  | +++             | ++  |
| Olfactory bulb |             | +++          | +++ | +++             | +++ |
| Telencephalon  | HP          | ++           | ++  | ++              | ++  |
|                | HA          | +++          | ++  | +++             | +++ |
|                | HI          | ++           | ++  | ++              | ++  |
|                | HD          | +            | ++  | +               | ++  |
|                | Mesopallium | ++           | ++  | ++              | ++  |
|                | Nidopalium  | ++           | ++  | ++              | ++  |
|                | Entopallim  | ++           | ++  | +++             | +   |
|                | Arcopallium | +++          | ++  | +++             | ++  |
|                | striatum    | ++           | +++ | ++              | +++ |
|                | pallidum    | ++           | ++  | ++              | ++  |
| Diencephalon   |             | ++           | ++  | ++              | ++  |
| Mesencephalon  | pretectum   | ++           | +   | ++              | ++  |
|                | tectum      | ++           | ++  | ++              | +   |
| Metencephalon  | cerebellum  | +            | ++  | +               | +   |



**Figure 3.1.** The reconstruction of coronal sections through one half of the brain of the racing homer pigeon showing areas of the detected PCNA immunoreactive cells (black dots represent proliferating cells). Each drawing of the coronal section is approximately 1500  $\mu\text{m}$  apart, with **A** being the most rostral section and **J** being the most caudal. See list of abbreviations for the abbreviations used on the diagrams.

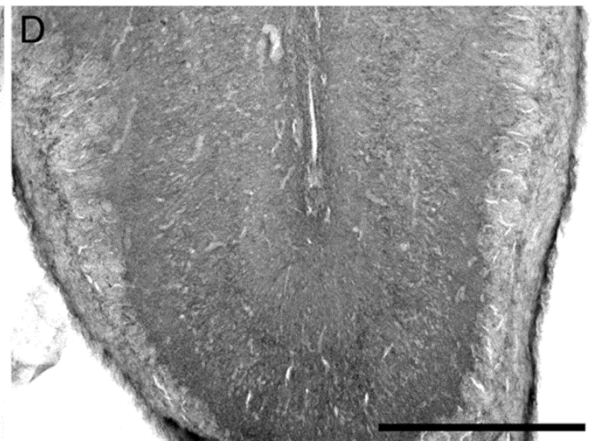
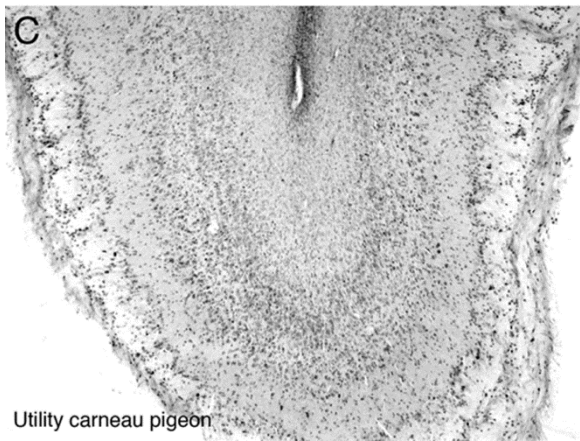
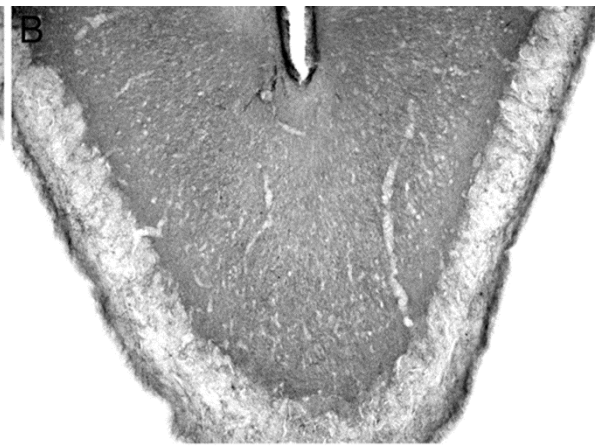
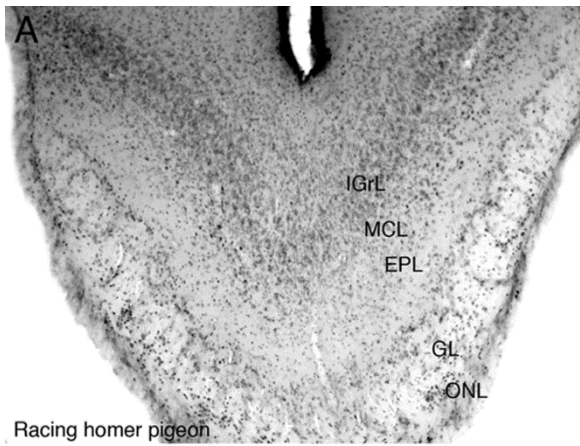


**Figure 3.2.** The reconstruction of coronal sections through one half of the brain of the racing homer pigeon showing areas of the observed DCX immunoreactive immature and migrating neurons (open black circles represent DCX immunoreactive cells). Each drawing of the coronal section is approximately 1500  $\mu\text{m}$  apart, with **A** being the most rostral section and **J** being the most caudal. See list of abbreviations for the abbreviations used on the diagrams.

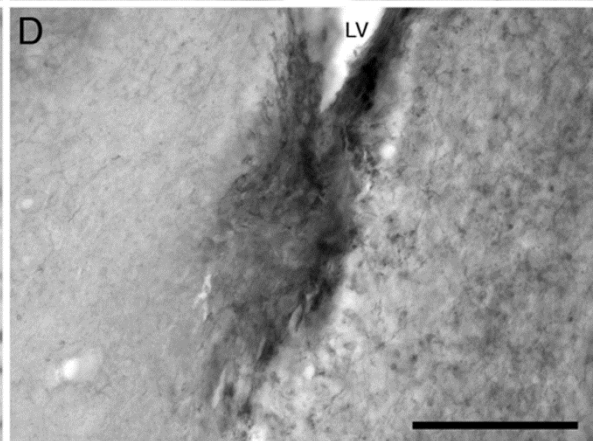
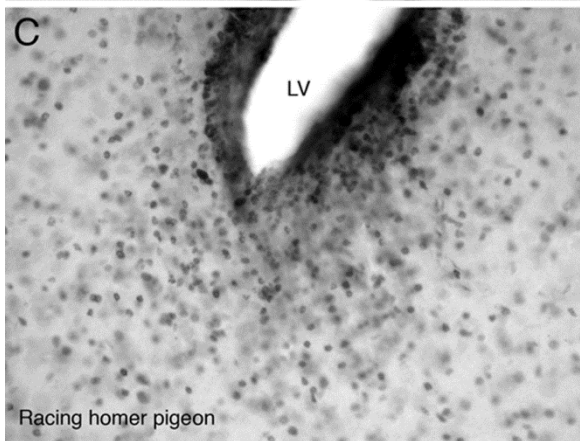
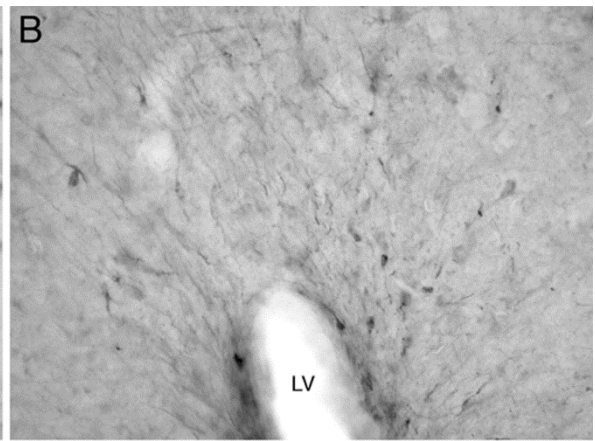
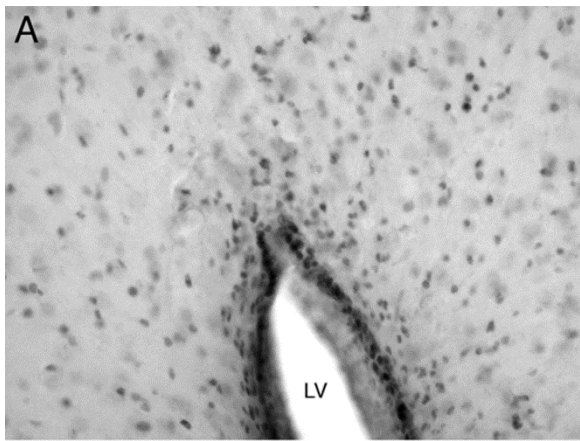


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**Figure 3.3.** Photomicrographs showing the olfactory bulbs (**OB**) of the Racing homer pigeon (**A, B**) and the utility carneau pigeon (**C, D**). The distribution of PCNA immunoreactivity is shown in the cellular layers of the olfactory bulb of the racing homer pigeon (**A**) and utility carneau pigeon (**C**). The distribution of DCX immunoreactivity is shown in the olfactory bulbs of the racing homer pigeon (**B**) and utility carneau pigeon (**D**). Scale bar in **A – D** = 500µm.

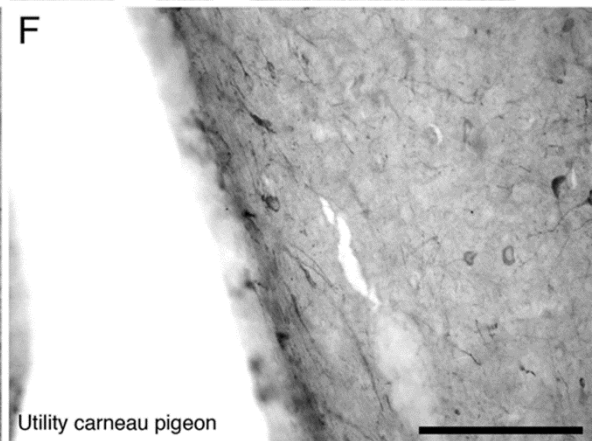
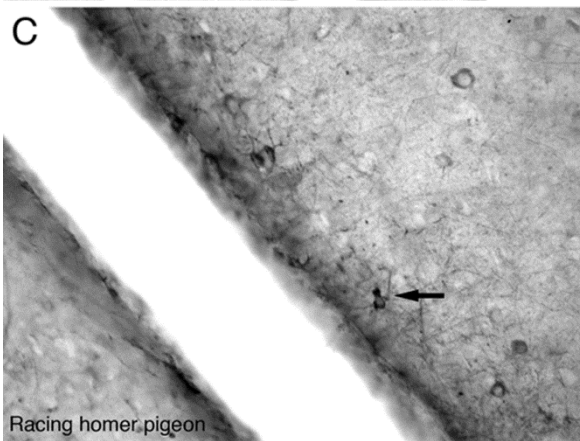
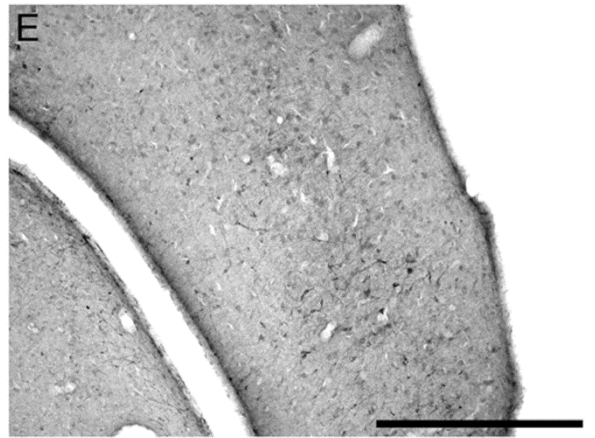
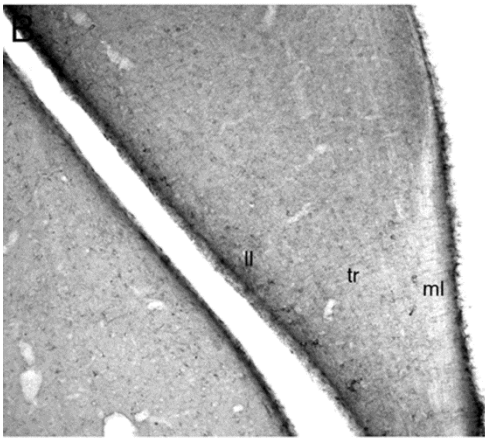
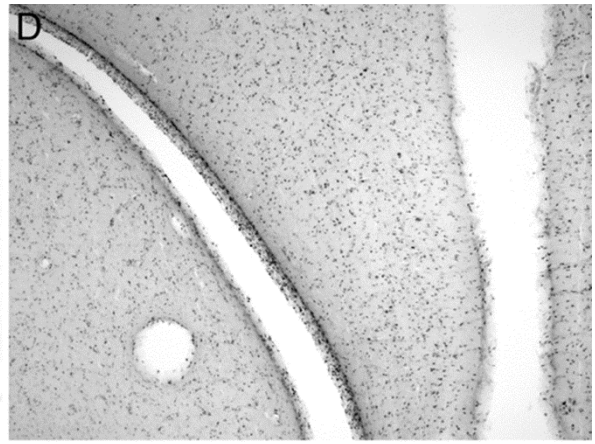
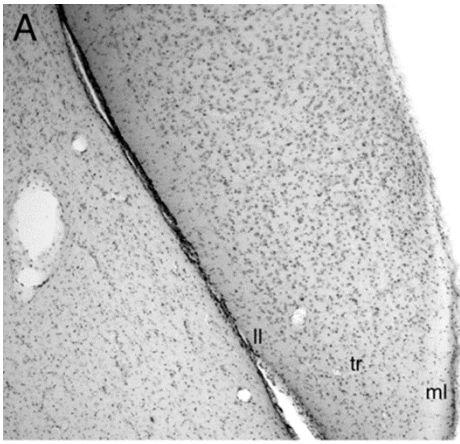


**Figure 3.4.** Photomicrographs showing the distribution of PCNA (**A**, **C**) and DCX (**B**, **D**) immunoreactivity in the dorsal and ventral reaches of the subventricular zone of the lateral ventricle (**LV**) in the racing homer pigeon. Note the intense PCNA staining in the subventricular zone of the lateral ventricle and also migrating DCX immunoreactive cells from the subventricular zone of the lateral ventricle into the parenchyma of the telencephalon. Scale bar in **D** = 100µm also applies to all.

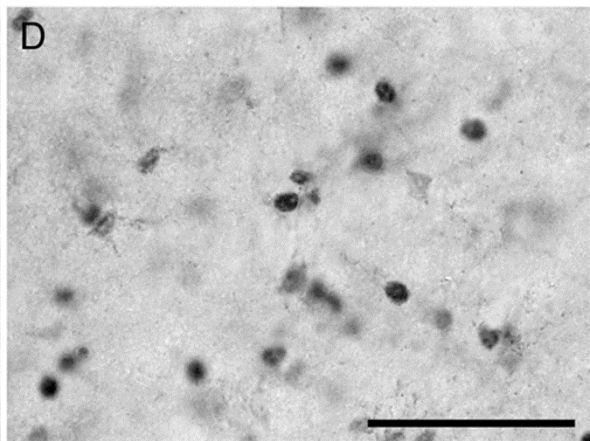
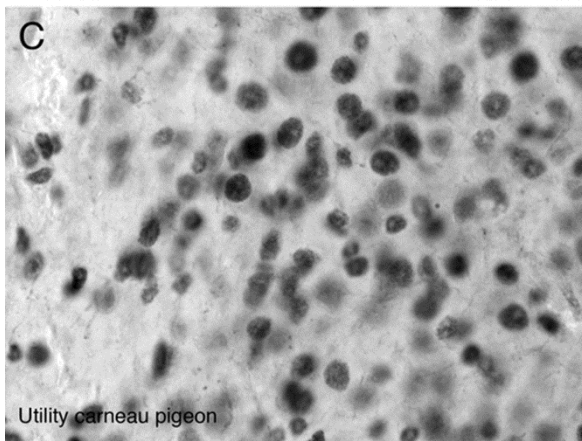
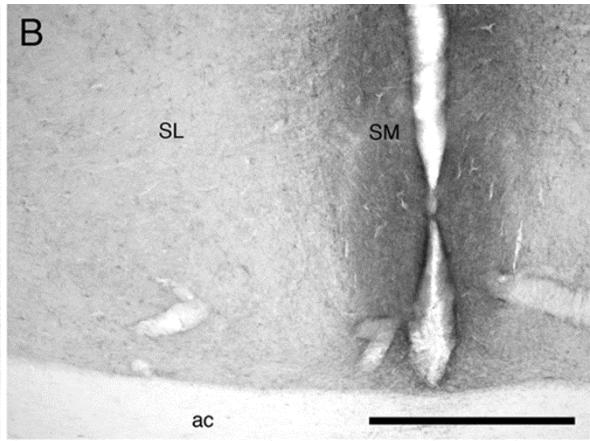
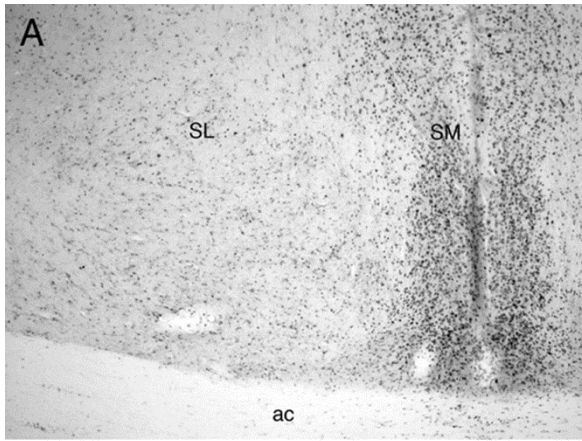




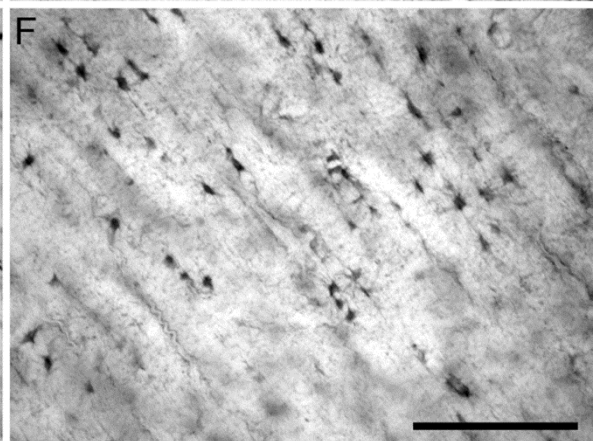
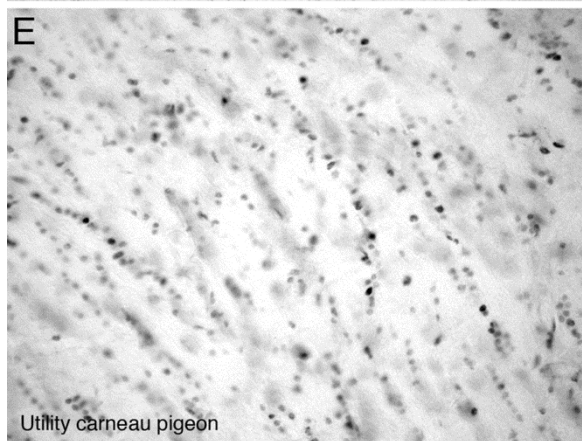
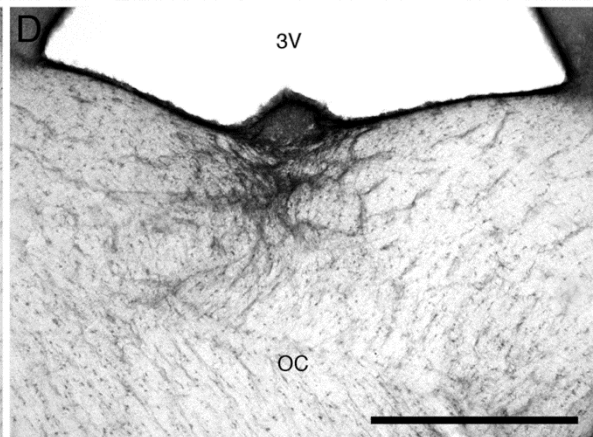
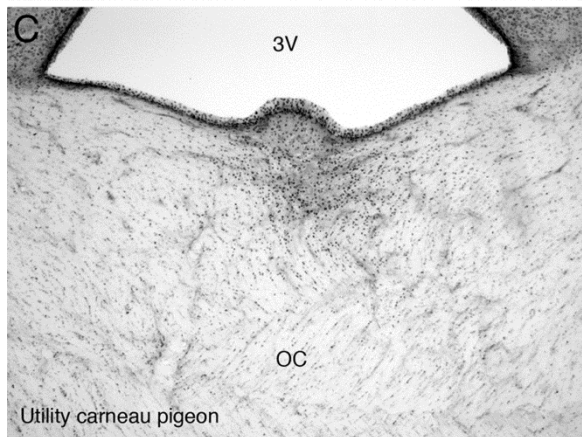
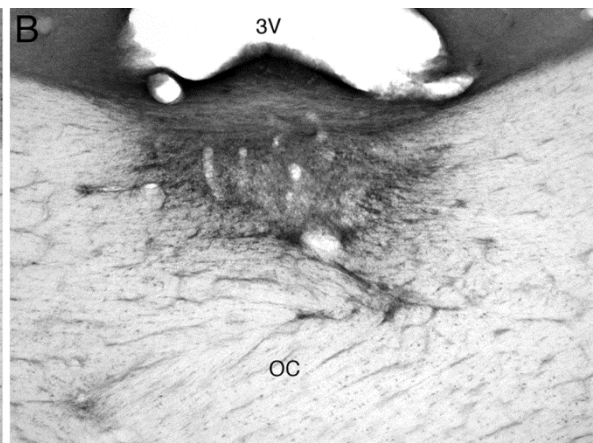
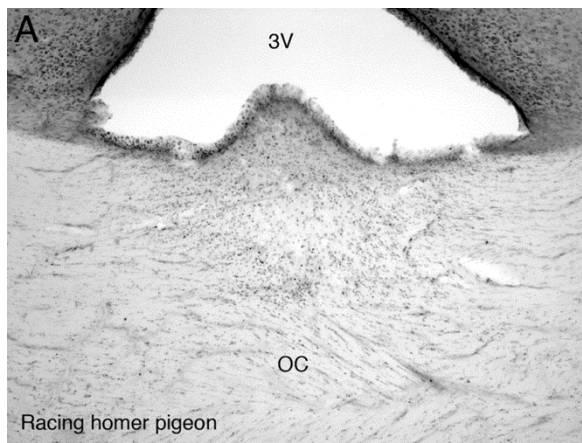
**Figure 3.5.** Photomicrographs showing the distribution of PCNA and DCX in the hippocampus (**Hp**) of the racing homer and utility carneau pigeons. The distribution of PCNA immunoreactivity in the ventral subdivisions of the hippocampus showing mild PCNA immunoreactivity in the medial layer (**ml**), moderate density in the lateral layer (**ll**) and dense reactivity in the triangular area (**tr**) in the racing homer (**A**) and the utility carneau (**D**) pigeons. The distribution of DCX immunoreactivity in the ventral division of the hippocampus showing a moderate concentration of DCX immunoreactive cells and fibres in the lateral layer (**ll**) and triangular area (**tr**) and mild distribution in the medial layer of the ventral hippocampus (**ml**) of the racing homer (**B**) and utility carneau (**E**) pigeons. A higher magnification showing DCX immunoreactive fibres and round unipolar cells in the lateral layer and triangular area of the ventral hippocampus in the racing homer (**C**) and utility carneau (**F**); arrow shows a DCX immunoreactive cell. Scale bar in **E** = 500µm and applies to **A**, **B** and **D**. scale bar in **F** = 100µm and applies to **C**.



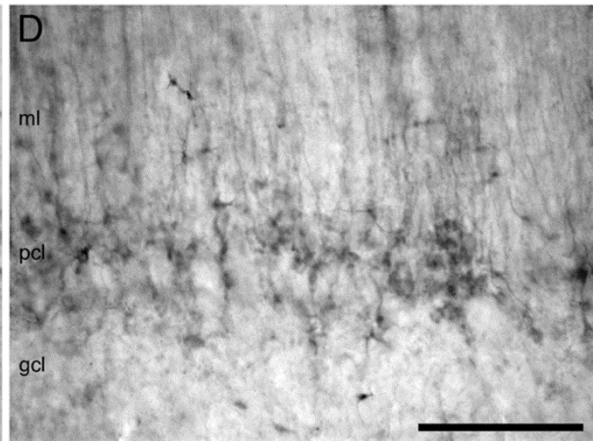
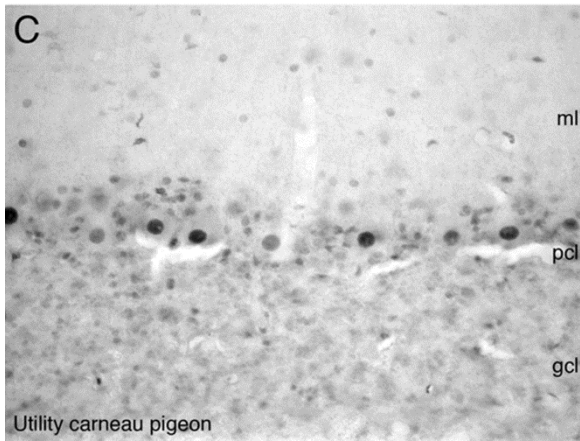
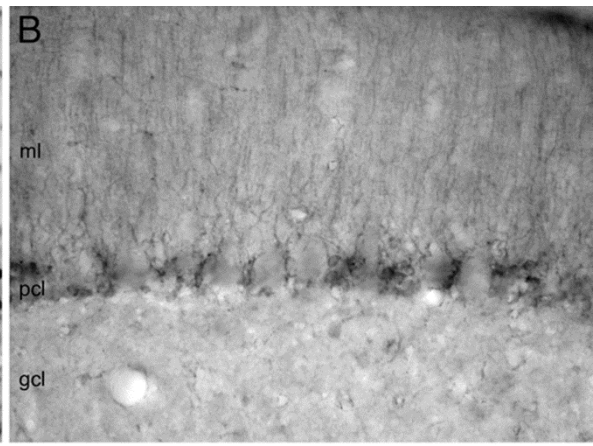
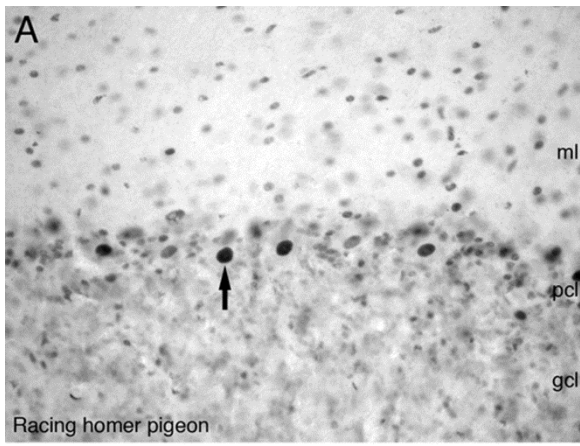
**Figure 3.6.** Photomicrographs showing the septum of the utility carneau pigeon. **(A)** Distribution of PCNA immunoreactive cells in the septum. **(B)** Distribution of DCX immunoreactive cells and fibres in the septum. **(C)** A higher magnification of the medial septum (**SM**) showing dense PCNA immunoreactive cells. **(D)** A higher magnification of the lateral septum (**SL**) showing sparse PCNA immunoreactive cells. Scale bar in **B** = 500µm and applies to **A**. scale bar in **D** = 50µm and applies to **C**. see list of abbreviations section for abbreviations.



**Figure 3.7.** Photomicrographs of PCNA (**A, C, E**) and DCX (**B, D, E**) immunostained sections through the dorsal aspect of the optic chiasm (**OC**), at its border with the floor of the third ventricle (**3V**) in racing homer (**A, B**) and utility carneau (**C, D, E, F**) pigeons. These images depict a potential neurogenic zone, or bulge, at the dorsal midline of the optic chiasm that appears to produce newly generated cells (shown by PCNA immunostaining) and appear to migrate (as shown by DCX immunostaining) into the optic chiasm, optic nerve (**E, F**) and optic tract of both pigeon types. Scale bar in **D** = 500  $\mu\text{m}$  and applies to **A – D**, scale bar in **F** = 100  $\mu\text{m}$  and applies to **E** and **F**.



**Figure 3.8.** Photomicrographs of the cerebellar cortex (**Cb**) of the racing homer (**A, B**) and utility carneau (**C, D**) pigeons. The distribution of PCNA immunoreactive cells in the granule cell layer (**gcl**), Purkinje cells layer (**pcl**) and the molecular cell layer (**mcl**) of the cerebella cortex in the racing homer (**A**) and utility carneau (**C**) pigeons; arrow shows a PCNA immunoreactive cell. The distribution of DCX immunoreactive cells and fibres in the layers of the cerebella cortex in the racing homer (**B**) and utility carneau pigeon (**D**). Note the large and prominent PCNA immunoreactivity cells in the Purkinje cell layer of both pigeons. Scale bar in **D** = 100µm and applies to **A – C**.





### **3.4. Discussion**

#### **3.4.1. General considerations regarding PCNA- and DCX- immunoreactivity in pigeons**

In the current study we observed widespread potential adult neurogenesis in the brains of the two breeds of the adult pigeons, the racing homer and the utility carneau. Putative adult neurogenesis was demonstrated through the expression of the PCNA and DCX molecules, revealed using immunohistochemical techniques, throughout the brains of both pigeon breeds. The two antibodies were localized in similar brain regions of the pigeon breeds with putative adult neurogenesis including the olfactory bulb, telencephalic subdivisions (HA, HI, HD, M, N, NC, E, MSt, LSt, SL, SM), the subventricular zone (SVZ), the diencephalon, mesencephalon and the cerebellum.

PCNA is a marker for proliferating cells that is essential for DNA replication and repair. It is synthesized during the early G1 and S phases of the cell cycle, abundant during the S phase and declines during G2/M phase (Kurki et al., 1988; Hall et al., 1990). In the two pigeon breeds studied, the density of PCNA-immunoreactive cells was higher in the olfactory bulbs, SVZ, NC and the piriform cortex, but was generally less dense in the potential adult neurogenic sites such as the diencephalon, mesencephalon and the cerebellum. This pattern of cellular staining is similar to that seen in other non-mammalian vertebrate including teleost fish (Zupanc et al., 2005), amphibians (Cerri et al., 2009), and chickens (Hannan et al., 1999). In addition, the distribution of proliferating cells observed in the current study compares with reports from studies on canaries and ring doves which showed wide spread distribution of bromodiuridine (BrdU) and [<sup>3</sup>H]thymidine positive cells in all brain regions (Ling et al., 1997; Vellema et al., 2010). However, BrdU-positive cells in the subtelencephalic regions in canaries did not co-express neuron specific marker Hu protein suggesting that the proliferating cells in this region are of glial cell lineage (Vellema et al., 2010). In the SVZ of the lateral ventricles, a high density of PCNA-immunoreactive cells was observed in the ventral and dorsal borders of the ventricles. In these regions, the putative proliferating cells

formed intermittent clusters that were consistent with the proliferative ‘hot spots’ identified by Alvarez-Buylla et al. (1990b).

Proliferating cells were also observed throughout the telencephalon and four possible reasons can be inferred for this: (1) these putative proliferating cells may have migrated from the SVZ throughout the parenchyma (Almli and Wilczynski, 2007); (2) resident progenitors may be proliferating (Alvarez-Buylla et al., 1990a; Alvarez-Buylla et al., 1990b; Doetsch et al., 1999a; Zupanc et al., 2005); (3) putative proliferating cells may be resident glial cells (Cerri et al., 2009); and (4) putative proliferative cells may be both neuronal and glial cells undergoing DNA replication and repair (Hall et al., 1990).

DCX is a microtubule associated protein that plays a key role in the migration of neurons during development and in post mitotic neurons undergoing migration, remodelling of their dendritic processes and synaptogenesis in adulthood (Gleeson et al., 1999; Capes-Davis et al., 2005; Couillard-Despres et al., 2005). In this study, we observed that DCX expression was widespread and heterogeneous in the brains of the two breeds of domestic pigeons (*Columba livia domesticus*). The density of DCX-immunoreactive cells and fibres was most intense in the olfactory bulbs, associated olfactory cortices, telencephalic pallial structures including the hippocampus, the striatum and the NC, but less dense in subtelencephalic structures such as the diencephalon, mesencephalon and cerebellum. This widespread expression of DCX was consistent with observations made in other non-song birds such as the developing domestic chick brain (Capes-Davis et al., 2005), in song birds such as adult canaries (Boseret et al., 2007; Vellema et al., 2014) and in sub-adult zebra finches (Kim et al., 2006). DCX expression in the prosencephalon has been reported in higher density in numerous studies (Kim et al., 2006; Boseret et al., 2007; e.g. Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013; Vellema et al., 2014). The majority of pioneering studies on adult neurogenesis in birds concurred that the process was only limited to the prosencephalon (Alvarez-Buylla and Nottebohm, 1988; Kirn et al., 1994). Few studies

established the presence of DCX expression in the subtelencephalic regions in pathologically normal adult species (Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014). However, increased levels of adult neurogenesis have been reported in the subtelencephalic regions during injury and pathological conditions (Cao et al., 2002; Chen et al., 2006). We also noted that the areas with the densest expression of DCX-immunoreactive structures were adjacent to the walls of the ventricles in all brain regions, suggesting that new neurons were generated in the SVZ and migrated to their target regions. Migration of newly born neurons follows well defined routes in mammalian species, where they migrate from the subgranular zone of the dentate gyrus to the granule cell layer, and the SVZ of the lateral ventricle to the olfactory bulbs through the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Chawana et al., 2013; Patzke et al., 2013). In birds studied to date, including the current study, the migratory routes appear diffuse, with many regions of the brain appearing to incorporate new neurons. Despite this, as in mammals, a rostral migratory-like stream has been identified in rock pigeons, seen to stretch from the caudal nidopallium to the rostral mesopallium, hyperpallium apicale and densocellulare (Melleu et al., 2013). New neurons migrate from the SVZ into the brain parenchyma through radial glia scaffolding (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988). This phenomenon led to the identification of DCX-immunoreactive neuroblasts with the morphology typical of migrating cells (small round and fusiform bipolar cells) (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988; Melleu et al., 2013) in the parenchyma away from the SVZ. However, DCX immunoreactivity was also observed in post-migratory cells of the Purkinje cell layer of the cerebellum including in the current study (e.g. Capes-Davis et al., 2005) and in areas of the telencephalic structures where large round and polygonal multipolar cells with elaborate dendritic processes were also seen to express DCX. In the rock pigeons, these mature-like DCX-immunoreactive cells did not co-express neuron specific marker NeuN (Melleu et al., 2013), suggesting that these cells were immature neurons establishing synaptic

contacts in local circuits (Gleeson et al., 1999). Despite this, DCX-immunoreactive cells outside the SVZ in the mouse brain were found to express NeuN (Yang et al., 2004), indicating that DCX is also expressed in mature neurons that undergo dendritic arborization and axonal growth (Nacher et al., 2001; Brown et al., 2003).

DCX expression has been found to persist for more than 20 days in cells in the adult canary brain (Balthazart et al., 2008), explaining its presence in morphologically distinct neurons in the cores of the hippocampus and other pallial structures as observed in the current study, and in rock pigeons (Melleu et al., 2013). This suggests that DCX-immunoreactive cells in deeper areas of the telencephalic parenchyma represent earlier generations of new-born neurons that completed migration prior to the sacrifice of the animal. In addition, the concept also explains the abundance of DCX-immunoreactive cells observed in the telencephalon of avian species including the current study (Kim et al., 2006; Melleu et al., 2013; Vellema et al., 2014).

### **3.4.2. Olfactory neurogenesis in pigeons**

The olfactory bulbs are known to continuously incorporate new neurons in adulthood in various mammalian species (Kempermann, 2012). We found that the olfactory bulbs and associated brain areas such as the piriform cortex, olfactory tubercles and dorsolateral cortical areas exhibited high densities of PCNA- and DCX-immunoreactive structures. Similar findings were reported in other bird species such as domestic chicks and rock pigeons (Mezey et al., 2012; Melleu et al., 2013). In contrast to olfactory neurogenesis in mammals, where new neurons migrate long distances from the SVZ, the presence of PCNA- and DCX-immunoreactive cells in the walls of the olfactory ventricle and adjacent olfactory bulb layers suggests that new neurons are generated and incorporated in the olfactory bulbs locally rather than by migration. Melleu et al. (2013) also found conspicuous BrdU labelled cells in the walls of the olfactory ventricles. Retrograde tracing studies in pigeons have shown that

olfactory bulb neuronal fibres project to the piriform cortex (CPi), medial septum (SM) (Reiner and Karten, 1985) and the hyperpallium densocellulare (HD) (Patzke et al., 2011). Similar to reports by Melleu et al. (2013) in rock pigeons, we also observed a stream of DCX-immunoreactive cells that appeared to be migrating from the olfactory bulbs to the hyperpallium densocellulare (HD). Processing of the olfactory sense varies greatly in different orders of birds studied to date (Balthazart and Taziaux, 2009). In homing pigeons, olfactory processing is used during navigation from unfamiliar places to home lofts (Patzke et al., 2010) by following familiar airborne odours (Papi et al., 1972). In other species of the order columbiformes, olfactory cues were associated with parental behaviour in ring doves (Bonadonna et al., 2003), social and reproductive behaviours in rock pigeons (Patzke et al., 2010), feeding behaviours in domestic chicks (Jones and Roper, 1997), and food localization in vultures (Graves, 1992) and sea birds (Grubb, 1972). Thus, the process of adult neurogenesis and neural plasticity in birds might aid in replenishing neurons and renewing circuits to facilitate adaptation into natural environments using the olfactory sense.

### **3.4.3. Hippocampal neurogenesis**

The density of PCNA- and DCX-immunoreactive structures was lower in the hippocampus of domestic pigeons especially in the medial layer (ML) of the ventral hippocampus when compared to the surrounding brain parenchyma. The triangular region (Tr) and the lateral layers (Ll) exhibited a moderate density of PCNA- and DCX-immunoreactive structures. The dorsolateral (DL) regions of the hippocampus had a higher density of PCNA- and DCX-immunoreactive structures than the dorsomedial (DM) region. Similar differential densities were also reported in rock pigeons (Melleu et al., 2013), but domestic pigeons have been found to have a significantly larger hippocampus than the ancestral wild rock pigeons (Rehkämper et al., 2008), indicating that the similarity in the

extent of adult hippocampal neurogenesis between rock and domestic (homing) pigeons may be best ascertained by quantitative methods.

In birds, adult hippocampal neurogenesis has been associated with the integration of new experiences, such as new songs, and the clearance of old memories (Nottebohm, 1981; Wilbrecht and Kirn, 2004; Kempermann, 2008), thus, the process may serve to prevent interference between old and new memories, especially in food caching birds that need to recall both old and new food caches accurately (Barnea et al., 2006; Wiskott et al., 2006; Pravosudov and Smulders, 2010). Kempermann (2008) also suggested that AHN may contribute to a neurogenic reserve that can be incorporated only when there is a need for new learning. Generally, AHN decreases with advancing age in both mammals and non-mammalian vertebrates (Barnea and Nottebohm, 1994; Kempermann et al., 1997; Eriksson et al., 1998; Kim et al., 2006); however, in some reptiles and fish, AHN appears to contribute, in part, to the continuous growth of the nervous system (Zupanc and Horschke, 1995; Ngwenya et al., 2013). Barnea and Nottebohm (1994) found that AHN varies seasonally, with more neuronal accumulation occurring in the autumn in black capped chickadees. The hippocampal formation has also been associated with spatial memory dependent tasks related to learning and acquisition of a spatial map and its operation in homing pigeons (Bingman et al., 2005), migration and food caching (Bingman and Cheng, 2005; Hoshooley and Sherry, 2007).

#### **3.4.4. Neurogenesis in other brain areas**

Our observation of wide spread DCX immunoreactivity throughout the brains of domestic pigeons is similar to that reported for adult chickens, canaries and zebra finches (Capes-Davis et al., 2005; Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008). In the pallial structures PCNA and DCX-immunoreactive structures were apparent in the

margins of the HA, M, N, and NC rather than their core areas. In addition, we observed PCNA and DCX immunoreactivity in the septum, striatum and the entopallium in both pigeons. Expression of the DCX antibody in the entopallium was comparatively denser in racing homer pigeon than the utility carneau pigeon. Mezey et al. (2012) reported similar findings but mild DCX expression in the entopallium of chickens. The striatum complex and the entopallium participate in the processing of visual information which is vital during navigating long distances particularly by homing pigeons. Birds are heavily visual and auditory reliant during feeding, reproduction, socialisation and many other daily functions (Grubb, 1972; Papi et al., 1972; Graves, 1992; Bonadonna et al., 2003; Patzke et al., 2011), but the cores of the telencephalon that process visual and auditory information exhibited very low to absent densities of PCNA- and DCX-immunoreactive structures in the domestic pigeon breeds studied. In contrast, the diencephalic and mesencephalic regions associated with these senses exhibited moderate densities of PCNA- and DCX-immunoreactive structures for example the dorsal nuclei of the thalamus which connects with the medial striatum (MSt) and also the tectum which connects with the lateral striatum (LSt) (Kuenzel et al., 2011; Shanahan et al., 2013). We also observed PCNA and DCX immunoreactivity in the optic chiasma, anterior and posterior commissures and suggested that glial cells and neuronal axons may be proliferating and remodelling respectively. In conclusion, adult neurogenesis appears to be a conserved process in the pigeon brain and may help in continuous reinforcement and remodelling of neuronal circuits and behaviour.

## **CHAPTER FOUR: Putative adult neurogenesis in old world parrots: the Congo African grey parrot (*Psittacus erithacus*) and Timneh grey parrot (*Psittacus timneh*)**

### **4.1. Introduction**

Adult neurogenesis encompasses the birth and maturation of new neurons that become incorporated into existing circuitry or replace old and damaged neurons under normal physiological and or pathological conditions (Lindsey and Tropepe, 2006). The process of adult neurogenesis was confirmed in a variety of species from different taxa ranging from insects to humans (see reviews, Cayre et al., 2002; García-Verdugo et al., 2002; Lindsey and Tropepe, 2006; Chapouton et al., 2007). Although this process has been confirmed in wide spectrum of species, the animals studied to date represent only a small fraction of the extant species, and studying further species will allow a broader understanding of the potential diversity of the process and function of adult neurogenesis (Barnea and Pravosudov, 2011). Across many species similarities in the process of adult neurogenesis have been observed, which include the origin of neurons, the phenotype of stem cells and their proliferative mechanisms, the migration and differentiation of neurons (for a review, see Doetsch and Scharff, 2001); however, they are also important differences among species in the spatial distribution of adult born neurons, their mode of migration and phenotypic diversity (Doetsch and Scharff, 2001).

Numerous studies have revealed widespread adult neurogenesis in non-mammalian species such as fish, amphibians, reptiles and birds when compared to mammalian species (García-Verdugo et al., 2002). Generally, the rate of adult neurogenesis decreases with age across vertebrate taxa (Kuhn et al., 1996; Knoth et al., 2010; Amrein et al., 2011; LaDage et al., 2011), and the process is affected by factors such as genetics, endogenous and exogenous factors, and seasonal variation (for a review, see Barnea and Pravosudov, 2011).



African grey parrots are stocky, short tailed birds with an average body mass of 400 grams. The Congo African grey parrot (*Psittacus erithacus*) is larger and lighter grey in colour compared to the smaller and darker grey Timneh grey parrot (*Psittacus timneh*). The African grey parrots live a long life span ranging between 20-50 years in the wild, but this can increase to nearly 100 years in captivity (Carey and Judge, 2001; Schmid et al., 2006). African grey parrots lead a complex social life, coupled with long term monogamous relationships (Seibert, 2006; Emery et al., 2007). In addition to displaying complex social interactions, African grey parrots are thought to exhibit potentially advanced cognitive capabilities (Pepperberg et al., 1999; Iwaniuk et al., 2004; Pepperberg, 2006). These parrots display vocal learning and mimicry abilities that rival those of young humans (Iwaniuk et al., 2004; Pepperberg, 2006), show cooperative problem solving and the ability to discriminate discrete and continuous variables (Al Aïn et al., 2009; Péron et al., 2011). Here we investigated the generation and maturation of new neurons throughout the brains of the two subspecies of African grey parrots, the Congo African grey parrot (*Psittacus erithacus*) and the Timneh grey parrot (*Psittacus timneh*) using proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) immunohistochemistry.

## **4.2. Experimental procedures**

### **4.2.1. Animals and tissue processing**

Two brains of adult (sexually mature) male Congo African grey (*Psittacus erithacus*) and two brains of adult male Timneh grey parrots (*Psittacus timneh*) were used in the current study. The birds were purchased from a local breeder in South Africa, and were sacrificed and perfused in November 2013. The birds were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (clearance no: 2013/05/02B), which parallel those of the NIH for the care and use of animals in scientific experimentation. Five minutes prior to being euthanized, both birds were given an intramuscular dose of

heparin, 2500 units (0.5 ml) to prevent blood clotting. Animals were then injected with an intraperitoneal dose of Euthapent (1 ml/kg) and body mass recorded. The average body mass of the two Congo African grey parrots was 453.75 g, and the two Timneh grey parrots was 286.36 g. All animals were transcardially perfusion-fixed, initially with a rinse of 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). The brains were carefully removed from the skull, and post-fixed overnight in 4% paraformaldehyde in 0.1M PB. The average brain mass of the Congo African grey parrots was 10.30 g and that of the Timneh grey parrot was 7.85 g. Before sectioning, the tissue was allowed to equilibrate in a 30% sucrose in 0.1M PB solution at 4°C for 4 days. The brains were then frozen in dry ice and sectioned in the coronal plane, 50 µm thick sections, on a sliding microtome. A one in ten series of sections were taken and three series stained for Nissl substance, PCNA and DCX. The remaining series of sections were placed in an antifreeze solution and are stored at -20°C for future use. The series of sections used for Nissl staining were mounted on 0.5% gelatine coated slides, dried overnight, cleared in a 1:1 mixture of 100% ethanol and 100% chloroform and stained with a 1% cresyl violet solution.

#### **4.2.2. Immunohistochemistry for PCNA and DCX**

The series of sections used for free floating PCNA and DCX immunohistochemistry were initially treated for 30 min at room temperature under gentle shaking with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1M PB: 1.6% of 30% H<sub>2</sub>O<sub>2</sub>), followed by three 10 min rinses in 0.1 M PB. To block non-specific binding sites the sections were then preincubated for 2 h, at room temperature under gentle shaking, in a blocking buffer solution consisting of 3% normal horse serum (NHS) for PCNA sections or 3% normal rabbit serum (NRS) for DCX sections, 2% bovine serum albumin, and 0.25% Triton X-100 in 0.1 M PB. Following preincubation the primary antibodies were added to the blocking buffer solution and the sections were incubated for 48 h at 4°C under gentle shaking (PCNA - 1: 500

dilution of mouse anti-PCNA, NCL-L-PCNA Leica Biosystems, Newcastle, United Kingdom; DCX - 1:300 dilution of goat anti-DCX antibody, C-18, Santa Cruz Biotechnology, Dallas, Texas, U.S.A) under gentle agitation. The primary antibody incubation was followed by three 10-min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (PCNA sections - 1:1000 dilution of biotinylated anti-mouse IgG [BA-2001, Vector Labs] in 3% normal horse serum and 2% bovine serum albumin in 0.1 M PB; DCX sections - 1:1000 dilution of anti-goat IgG [BA-5000, Vector Labs] in 3% normal rabbit serum and 2% bovine serum albumin in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin biotin solution (1:125 in 0.1 M PB; Vector Labs, Burlingame, California, USA), followed by three 10-min rinses in 0.1 M PB. Sections were then transferred to a solution consisting of 0.05% diaminobenzidine tetrahydrochloride in 0.1M PB for 5 min at room temperature, after which 3.3  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub>/ml of solution was added. With the aid of a low power stereomicroscope the progression of the staining was visually followed and allowed to continue until a level was reached where the background staining could assist in architectonic matching to the Nissl stained sections without obscuring the immunopositive structures. The tissue was then rinsed twice more in 0.1M PB before being mounted on glass slides coated with 0.5% gelatine and allowed to dry overnight. Once dry, the slides were placed in a solution of 70% ethanol for 2 h and then dehydrated, cleared in xylene and coverslipped with Depex. To test for non-specific staining of the immunohistochemical protocol, the primary and secondary antibodies were omitted from random sections and no staining was evident. The observed immunostaining patterns support the specificity of the antibodies and are compatible with observations made in pigeons, parakeets and quails (Charvet and Striedter, 2008; Charvet and Striedter, 2009; Melleu et al., 2013; Melleu et al., 2015).

### 4.2.3. Analysis

The Nissl stained sections were examined with a low power stereomicroscope and the architectonic borders traced using a camera lucida. The PCNA and DCX immunostained sections were then matched to the drawings from the Nissl stained sections and the location of immunopositive soma marked on the drawings. Selected drawings were then scanned and redrawn using the Canvas 8 Software (Deneba Software, Miami, Florida, USA). High power microscopic observation allowed for the determination of the relative densities of stained structures throughout the various regions of the brain. The relative densities of immunostained structures were visually compared and recorded on a scale ranging from low (+) to moderate (++) to high (+++). A second observer was used to eliminate observer bias. The brain regions were identified and named in accordance with the stereotaxic atlas of the brain of the budgerigar (*Melopsittacus undulatus*)(Brauth et al., 2011) using the nomenclature recommended by the Avian Brain Nomenclature Forum (Reiner et al., 2004). Digital photomicrographs were captured using a digital camera (Axio Cam HRc, Zeiss South Africa) mounted on the light microscope (Axioskop 2 plus, Ziess, South Africa) and operating on the ZEN 2010 computer software (Zeiss, South Africa). No pixilation adjustments or manipulation of the captured images were undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

### Abbreviations

A – arcopallium

AL – ansa lenticularis

An – nucleus angularis

APH – area parahippocampalis

Bas – nucleus basorostralis

CA – commissura anterioris

Cb – cerebellum

CbL – nucleus cerebellaris lateralis

CbM – nucleus cerebellaris medialis

OC – optic chiasm

CP – commissura posterior

CS – nucleus centralis superior (Bechterew)

DBC – brachium conjunctivum decendens

DCX – doublecortin

DLL – nucleus dorsolateralis anterior thalami pars lateralis

DLP – nucleus dorsolateralis posterior thalami

DMA – nucleus dorsomedialis anterior thalami

DMM – magnocellular nucleus of the dorsomedial thalamus

DMP – nucleus dorsomedialis posterior thalami

E – entopallium

EM – nucleus ectomammillaris

EPL – external plexiform layer of the olfactory bulb

FPL – fasciculus prosencephali lateralis

GCt – substantia grisea centralis

GL– glomerular layer of olfactory bulb

GP – Globus pallidus

HA – hyperpallium apicale

HD – hyperpallium densocellulare

HI – hyperpallium intercalatus

Hp – hippocampus

IGrL – internal granular layer of the olfactory bulb

Imc – nucleus isthmi, pars magnocellularis

IP – nucleus interpeduncularis

Ipc – nucleus isthmi pars parvocellularis

L1 – lamina 1 of Field L

L2a – lamina 2a of Field L

La – nucleus laminaris

ll – lateral layer of the ventral hippocampus

LLV – ventral nucleus of the lateral lemniscus

LoC – locus coeruleus

LSt – lateral striatum

LV – lateral ventricle

M – mesopallium

MCL – mitral cell layer of the olfactory bulb

ml – medial layer of ventral hippocampus

MPV – nucleus mesencephalicus profundus

MSt– medial striatum

N – nidopallium

NC – nidopallium caudale

NI – nidopallium intermoderate

nIII – nucleus oculomotorius

NIVL – nidopallium intermoderate pars ventrolateralis

NLc – central nucleus of the lateral nidopallium

nV – nucleus motorius nervi trigemini

nX– vagus nucleus

nXIIts – nucleus nervi hypoglossis pars tracheosyringalis

OI – nucleus olivaris inferioris

OM – tractus occipitomesencephalicus

ON– olfactory nerve

OV– nucleus ovoidalis

PCNA – proliferating nuclear cell antigen

PL– nucleus pontis lateralis

PM – nucleus pontis medialis

POA – nucleus preopticus anterior

POM – nucleus preopticus medialis

PrV – nucleus sensorium principalis nervi trigemini

RP – nucleus reticularis pontis

Rt – nucleus rotundus

SCv – nucleus subcoeruleus ventralis

SGC – stratum griseum centrale

SGF – stratum griseum et fibrosum

SL– lateral septum

SLu – nucleus semilunaris

SM – medial septum

SNc – substantia nigra pars compacta

PC – nucleus superficialis parvocellularis

SpM – nucleus spiriformis medialis

SpL – nucleus spiriformis lateralis

TeO – Optic tectum

tn – nucleus taenia of the amygdala

tr – triangular area of ventral hippocampus

TrO – Optic tract

TTD – nucleus et tractus descendens nervi trigemini

Tu – nucleus tuberis

VMH – ventromedial hypothalamus nucleus

VSt – ventral striatum



## **4.3. Results**

### **4.3.1. General observations**

In the present study we examined putative adult neurogenesis throughout the brains of the Congo African grey parrot (*Psittacus erithacus*) and the Timneh grey parrot (*Psittacus timneh*) using immunohistochemical techniques for the endogenous markers PCNA and DCX. The distribution of PCNA and DCX immunoreactivity was almost identical in both subspecies, but a few minor differences were observed (Table 4.1). Due to this extensive similarity we depict only the mapping of the distribution of the PCNA and DCX immunoreactive cells in the Congo African grey parrot (Fig. 4.1, 4.2). In both parrots, PCNA and DCX immunoreactivity was observed in the layers of the olfactory bulbs (OB), the subventricular zone of the lateral, third and fourth ventricles and the cerebral aqueduct, subdivisions of the pallium (Hippocampus [Hp], hyperpallium apicalle [HA], hyperpallium intercalatum [HI], hyperpallium densocellulare [HD], mesopallium [M], nidopallium [N], entopallium [E], and arcopallium [A], subpallium (medial and lateral striatum [MSt and LSt], medial and lateral septum [SM and SL], diencephalon, mesencephalon and rhombencephalon. Generally, the telencephalic regions had a higher density of PCNA and DCX immunoreactive cells than other brain regions in both subspecies of parrots examined. In the majority of the telencephalic regions, the density of PCNA and DCX immunoreactive cells increased from rostral to caudal in both parrot subspecies. DCX immunoreactivity was more intense in fibres than in cell bodies and the majority of DCX immunopositive cells included small rounded cells, fusiform unipolar and bipolar cells.

### **4.3.2. Distribution of PCNA immunoreactivity**

#### **4.3.2.1. Olfactory bulb**

PCNA immunoreactive cells were observed at high density in the internal granular layer (IGrL), mitral cell layer (MCL) and external plexiform layer (EPL) layer, while they

were found in low density in the glomerular layer (GL) and olfactory nerve (ON) layer (Fig. 4.1A, 4.1B, 4.3A, 4.3C).

#### **4.3.2.2. Subventricular zone**

In both parrot species the subventricular zone of the lateral ventricle exhibited a high density of PCNA immunoreactivity with occasional cells clustering (Fig. 4.1B – K). There was evidence of proliferative hot spots as described by (Alvarez-Buylla et al., 1990b) in the dorsal and ventral poles of the lateral ventricle in the Congo African grey parrots at rostral levels, but only a dorsal accumulation of PCNA immunoreactive cells was observed in the Timneh grey parrots (Fig. 4.4A, 4.4C). The third and fourth ventricles and the cerebral aqueduct of both parrot species exhibited a moderate density of PCNA immunoreactivity without cell clustering. There was a low density of PCNA immunoreactivity in the subventricular zone of the tectal portion of the cerebral aqueduct in both parrot species.

#### **4.3.2.3. Pallial regions**

In both parrot subspecies examined the HA, HD, HP and APH exhibited a moderate density of PCNA immunoreactive cells (Fig. 4.1A – O, 4.5A). In the ventral hippocampus the medial layer (ml) showed a low density of PCNA immunoreactive cells compared to the triangular (tr) and lateral layers (ll) which presented a moderate density (Fig. 4.5C). The hyperpallium intercalatum (HI) showed a low density of PCNA immunoreactive cells in the medial and core regions but an increase in the density of PCNA immunoreactive cells was observed in its lateral regions.

The mesopallium (M) exhibited a homogenous moderate density of PCNA immunoreactive cells at rostral levels, but at caudal levels the density of PCNA immunoreactive cells were reduced to low density in core regions, but moderate density in the medial and lateral regions (Fig. 4.1A – O). The entopallium exhibited a moderate density of

PCNA immunoreactive cells and their distribution decreased markedly in caudal sections in both species of African grey parrots (Fig. 4.1K – M). There was a high density of PCNA immunoreactive cells in the nidopallium frontale (NF) in the medial and lateral regions in both parrot subspecies (Fig. 4.1A – E). The core region of nidopallium frontale (NF) of the Congo African grey parrot exhibited a large cluster of PCNA immunoreactive cells. At caudal levels, the nidopallium intermoderate (NI) and nidopallium caudale (NC) exhibited a moderate density of PCNA immunoreactive cells in the lateral regions, while the medial and core regions showed a low density of PCNA immunoreactive cells in both parrot species (Fig. 4.1I – O) .

#### **4.3.2.4. Subpallial regions**

In the striatum of both parrot species, a moderate density of PCNA immunoreactive cells was observed in the medial and lateral striatum (MSt and LSt) (Fig. 4.1D – L). PCNA immunoreactive cells were distributed in higher density in ventral regions of the medial striatum and also in regions adjacent to the ventrolateral wall of the lateral ventricle. No detectable PCNA immunoreactive cells were observed in the regions corresponding to the globus pallidus (GP). Generally in the striatum, there was a rostro-caudal decline in the density of PCNA immunoreactive cells. In the septal complex of both parrots, the moderate septum (SM) exhibited a low density of PCNA immunoreactive cells while the lateral septum (SL) showed a moderate density of PCNA immunoreactive cells (Fig. 4.1J, 4.1K). The arcopallium exhibited a moderate density of PCNA immunoreactive cells which were distributed in higher density in the ventral regions encompassing the nucleus taeniae (Tn) than the dorsal regions, which showed a low density to almost no PCNA immunoreactive cells (Fig. 4.1I – L).

#### **4.3.2.5. Diencephalon**

The diencephalon exhibited a high density of PCNA immunoreactive cells in the paraventricular nuclei, including nucleus preopticus anterior and medialis (POA and POM), the dorsal margin in the nucleus dorsomedialis anterior thalami (DMA), nucleus dorsolateralis anterior thalami, pars lateralis (DLL) and nucleus dorsolateralis posterior thalami (DLP) and the lateral margin in the nucleus rotundus (Rt), nucleus superficialis parvocellularis (SPC) and nucleus spiriformis lateralis (SpL) (Fig. 4.1I – L). In the core regions of the diencephalon, the African grey parrot exhibited a moderate density of PCNA immunoreactive cells in nucleus ovoidalis (OV). The ventromedial hypothalamus nucleus (VMH) in the ventral hypothalamus of both parrots exhibited the highest density of PCNA immunoreactive cells. The optic chiasm (OC) showed PCNA immunoreactive cells ventral to the inferior pole of the third ventricle in both species (Fig. 4.6A, 4.6C).

#### **4.3.2.6. Mesencephalon, rhombencephalon and cerebellum**

Most of the regions of the midbrain exhibited a moderate density of PCNA immunoreactive cells, including the nucleus oculomotorius (nIII), substantia griesea centralis (GCt), nucleus interpeduncularis (IP) and the isthmic region in the nucleus semilunaris (SLu) nucleus isthimi pars parvocellularis (Ipc) and nucleus isthmi, pars magnocellularis (Imc). In the optic tectum in both parrots a higher density of PCNA immunoreactive cells was observed in the stratum griseum et fibrosum (SGF) and stratum griseum centrale (SGC) (Fig. 4.1K, 4.1L).

In the cerebellum, a moderate density of PCNA immunoreactive cells was observed in the Purkinje cell layer and the medial and lateral cerebellar nuclei (CbM and CbL) (Fig. 4.7A and C). Occasional PCNA immunoreactive cells were observed in the molecular and granule cell layers. In the pons and the medulla oblongata, a moderate density of PCNA immunoreactive cells was observed in some regions not limited to the Locus coeruleus (Loc),

medial and lateral pontine nuclei (PM and PL), nucleus sensorium principalis nervi trigemini (PrV), nucleus et tractus descendens nervi trigemini (TTd), and nucleus raphe (R) (Fig. 4.1M – P) .

### **4.3.3. Distribution of DCX immunoreactivity**

#### **4.3.2.1. Olfactory bulb**

The olfactory bulb was the region that contained the highest density of DCX immunoreactive structures in both parrots. DCX immunoreactive cells and fibres were seen in high density in the inner layers of the olfactory bulb, but in lower density in the outer layers (Fig. 4.2A, 4.2B, 4.3B, 4.3D).

#### **4.3.2.2. Subventricular zone**

In both parrot subspecies, the SVZ of the lateral ventricle exhibited a high density of DCX immunoreactive cells and fibres (Fig. 4.2B – O). The majority of the DCX immunoreactive cells were orientated parallel to the walls of the lateral ventricles, depicting tangential migration, while a few DCX immunoreactive cells were oriented perpendicular to the walls of the lateral ventricle, suggesting radial migration. At some rostral levels in the Congo African grey parrot there were occasional accumulations of DCX immunoreactive cell clusters in the dorsal and ventral poles of the lateral ventricle, similar to the dorsal and ventral ‘hot spots’ suggested by Alvarez-Buylla et al. (1990b) (Fig 4.4B, 4.4D). Such accumulations of DCX immunoreactive cells were only observed in the dorsal pole of the lateral ventricle of the Timneh grey parrots. The SVZ of the third and fourth ventricles and that of the cerebral aqueduct showed a low density of DCX immunoreactive fibres with no cells clearly discernible.

#### **4.3.2.3. Pallial regions**

The pallial portion of the telencephalon exhibited the highest density of DCX immunoreactive cells and fibres, although the density of DCX immunopositive structures varied across different pallial regions in both parrots. Moderate densities of DCX immunoreactive cells and fibres were present in the hyperpallium apicale (HA), hyperpallium densocellulare (HD), medial and lateral nidopallium frontale (NF), nidopallium (N) and nidopallium intermoderate (NI) (Fig. 4.2A – O). The medial and lateral regions of the hyperpallium intercataum (HI) and the mesopallium (M) showed a low density of DCX immunoreactive cells and fibres (Fig. 4.2D – N). There was almost no DCX immunoreactivity detected in the core of HI and M. In the entopallium a low density DCX immunoreactive cells and fibres was observed (Fig. 4.2K – M). At rostral levels of these telencephalic pallial regions, DCX immunoreactive cells and fibres were present in a slightly higher density laterally than medially. Generally there was a rostro-caudal decrease in the amount of DCX immunoreactive material in these pallial regions. The nidopallium caudale (NC) and the nidopallium caudolaterale (NCL) exhibited a much higher density of DCX immunoreactive cells and fibres than any other pallial region, particularly in the ventral and lateral aspects (Fig. 4.2K – O). The hippocampus (HP) and area parahippocampalis (APH) contained a moderate density of DCX immunoreactive cells and fibres (Fig. 4.2M – O, 4.5 B). In the ventral hippocampus, a moderate density DCX immunoreactive material was observed in the triangular (tr) and lateral layers (ll) while the medial layer (ml) showed a low density (Fig. 4.5D).

#### **4.3.2.4. Subpallial regions**

The medial and lateral striatum (Mst and Lst) exhibited a moderate density of DCX immunoreactive cells and fibres at rostral levels, but the density of these cells and fibres decreased noticeably at levels caudal to the anterior commissure (CA) (Fig. 4.2 D – M). In the

medial striatum, the DCX immunoreactive cells and fibres were observed in higher density in the medial and ventral aspect than in the dorsal and lateral aspects. The arcopallium exhibited a very low density of DCX immunoreactive cells and fibres in both parrot species, especially in the ventral regions containing the nucleus taenia of the amygdala (Fig. 4.2I – L). A low density of DCX immunoreactive cells and fibres was observed in the medial and lateral septum (SM and SL); however, at caudal levels in both parrots the lateral septum showed a moderate density of DCX immunoreactive cells and fibres (Fig. 4.2J, 4.2K). No detectible DCX immunoreactive structures were observed in the regions corresponding to the globus pallidus (GP).

#### **4.3.2.5. Diencephalon**

The diencephalon exhibited a very low density of DCX immunoreactive cells and fibres. A weak DCX immunoreactivity was observed in the paraventricular nuclei, including nucleus preopticus anterior and medialis (POA and POM) of the medial hypothalamus and in the ventromedial hypothalamus nucleus (VMH) of the ventral hypothalamus and in the dorsolateral group of nuclei of the diencephalon, which include the nucleus dorsomedialis anterior thalami (DMA), nucleus dorsolateralis anterior thalami, pars lateralis (DLL), nucleus dorsolateralis posterior thalami (DLP), nucleus rotundus (Rt), nucleus superficialis parvocellularis (SPC) and nucleus spiriformis lateralis (SpL) (Fig. 4.2I – L). The optic chiasm (OC) showed DCX immunoreactive fibres (Fig. 4.6B, 4.6D), while no staining was detected in the anterior commissure (CA).

#### **4.3.2.6. Mesencephalon, rhombencephalon and cerebellum**

A low density of DCX immunoreactive fibres was observed in the layers of the optic tectum (stratum griseum et fibrosum [SGF] and stratum griseum centrale [SGC]) (Fig. 4.2K–M). Some mesencephalic nuclei and tracts also exhibited weak DCX immunoreactive fibres

and vesicles including the substantia grisea centralis (GCt), nucleus nervi trochlear (nIV), nucleus intercollicularis (ICo) and the fasciculus longitudinalis medialis (FLM) (Fig. 4.2L, 4.2M). In the cerebellum, the Purkinje cell layer was weakly stained, with only fibres being observed (Fig. 4.7B, 4.7D). In the pons and medulla oblongata weakly stained fibres and vesicles in very low density were observed in some nuclei including, but not limited to, the ventral nucleus of the lateral lemniscus (LLv), medial and lateral pontine nuclei (PM and PL), nucleus laminaris (La), raphe nucleus (R), nucleus nervi hypoglossis pars tracheosyringalis (nXIIIts), nucleus olivari inferioris and vagal nucleus (nX) (Fig. 4.2N – P).



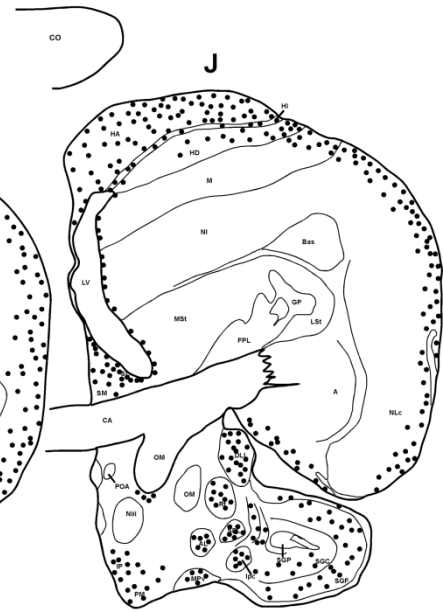
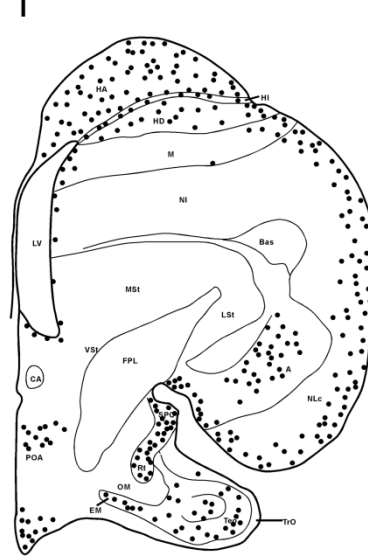
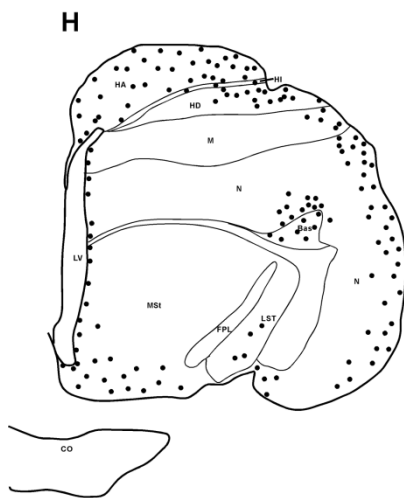
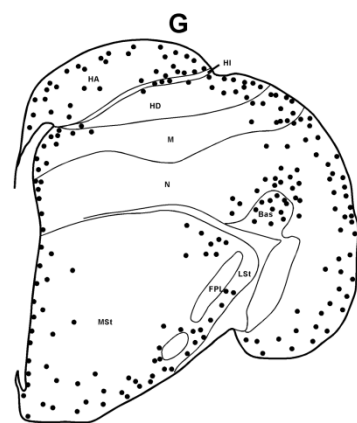
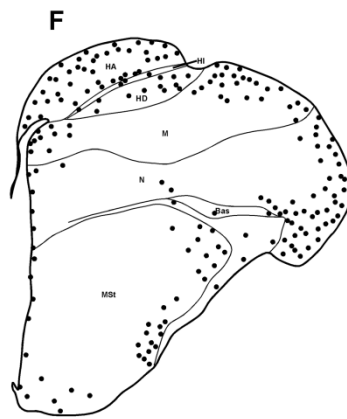
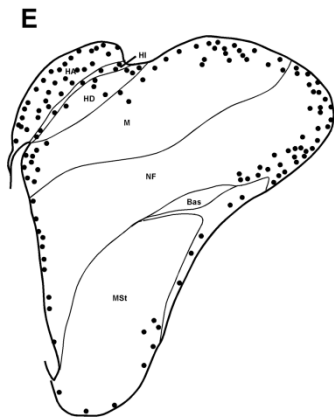
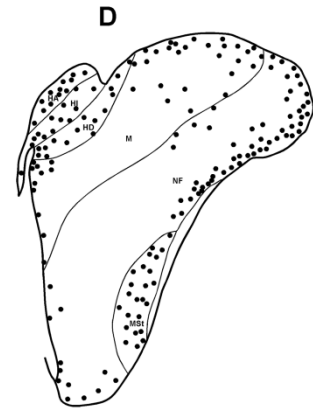
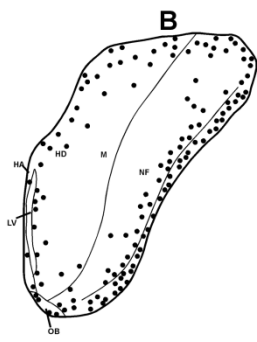
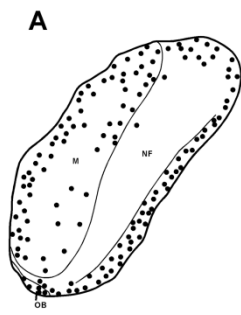
**Table 4.1.** Summary of qualitative distribution and density of PCNA and DCX

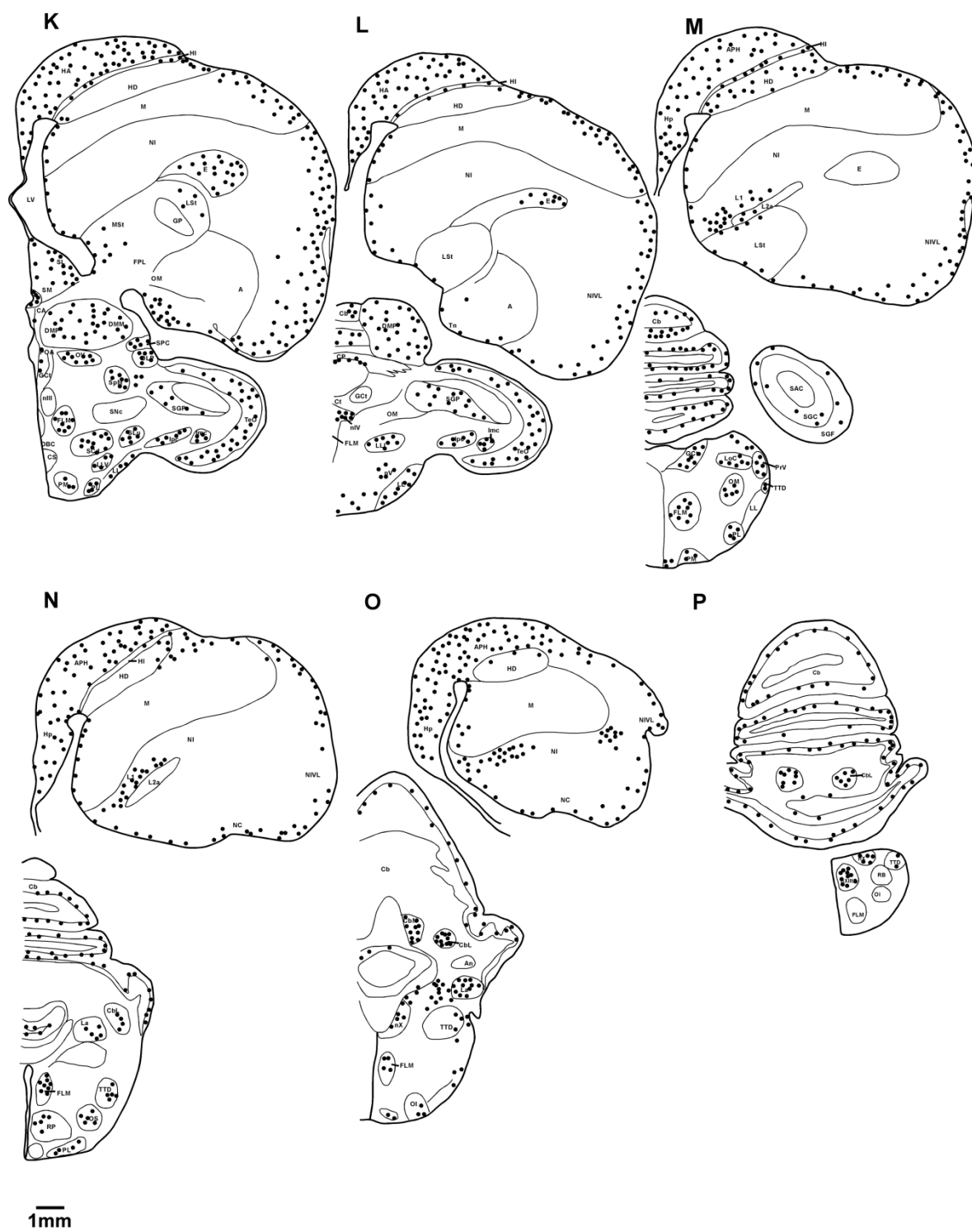
immunoreactive cells in the brain of the Congo African grey parrot and Timneh grey parrot.

- absent, + low, ++ moderate, +++ high density of PCNA and DCX immunoreactive structures

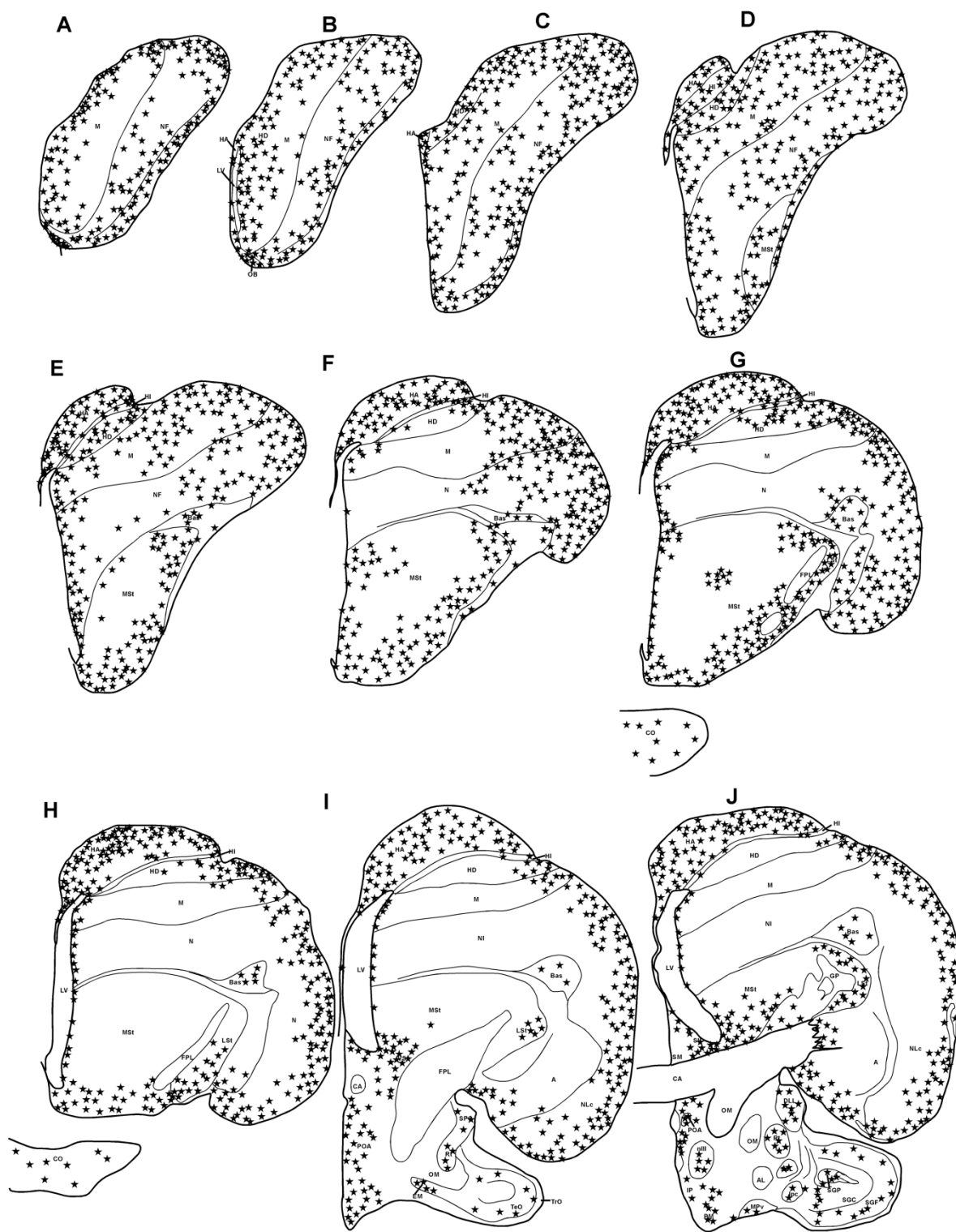
| Brain region    |                   | Congo African grey |     | Timneh grey |     |
|-----------------|-------------------|--------------------|-----|-------------|-----|
|                 |                   | PCNA               | DCX | PCNA        | DCX |
| SVZ             |                   | +++                | +++ | +++         | +++ |
| OB              |                   | +++                | +++ | +++         | +++ |
| Telencephalon   | HP                | ++                 | ++  | ++          | ++  |
|                 | HA                | ++                 | ++  | ++          | ++  |
|                 | HI                | ++                 | ++  | ++          | ++  |
|                 | HD                | ++                 | ++  | ++          | ++  |
|                 | M                 | ++                 | +   | ++          | +   |
|                 | N                 | +++                | ++  | +++         | ++  |
|                 | NC                | ++                 | +++ | ++          | +++ |
|                 | E                 | ++                 | +   | ++          | +   |
|                 | A                 | ++                 | +   | ++          | +   |
|                 | MSt               | ++                 | ++  | ++          | ++  |
|                 | LSt               | ++                 | ++  | ++          | ++  |
|                 | GP                | -                  | -   | -           | -   |
|                 | SM                | +                  | +   | +           | +   |
|                 | SL                | ++                 | +   | ++          | +   |
| Diencephalon    |                   | ++                 | +   | ++          | +   |
| Mesencephalon   | Pretectum         | ++                 | +   | ++          | +   |
|                 | Optic tectum      | +++                | +   | +++         | +   |
| Rhombencephalon | Cerebellum        | +                  | +   | +           | +   |
|                 | Pons              | ++                 | +   | ++          | +   |
|                 | Medulla oblongata | ++                 | +   | ++          | +   |

**Figure 4.1.** Digramatic reconstruction of the drawings of the coronal sections through one half of the brain of the Congo African grey parrot, illustrating the distribution of PCNA-immunoreactive cells. A single black dot indicates a single cell. Drawing **A** represent the most rostral section, **P** the most caudal, and each drawing is approximately 1500  $\mu\text{m}$  apart. See list for abbreviations.





**Figure 4.2.** Digramatic reconstruction of the drawings of the coronal sections through one half of the brain of the Congo African grey parrot, illustrating the distribution of DCX-immunoreactive cells. A single solid black star indicates a single cell body and a single open star represents a weakly stained cell. Drawing A represent the most rostral section, **P** the most caudal, and each drawing is approximately 1500  $\mu\text{m}$  apart. See list for abbreviations.

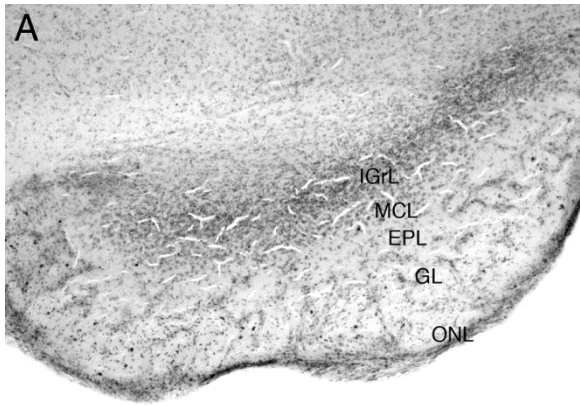




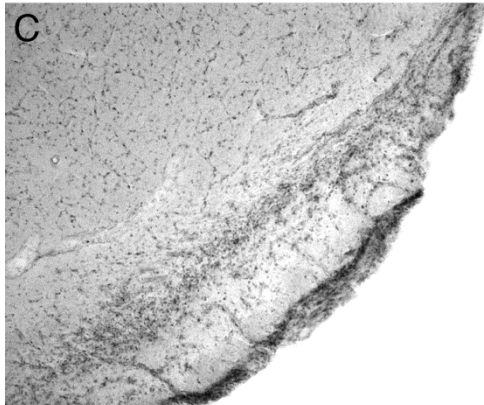
1mm



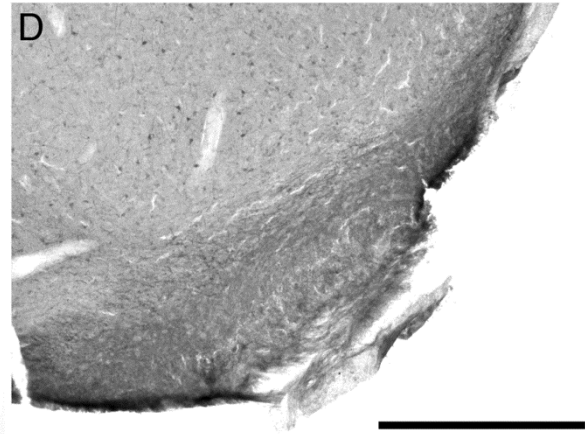
**Figure 4.3.** Photomicrographs of PCNA - (**A, C**) and DCX (**B, D**) - immunostained coronal sections through the olfactory bulb (**OB**) of the two parrot species – **A, B** – Congo African grey parrot (*Psittacus erithacus*), **C, D** – Timneh grey parrot (*Psittacus timneh*). In all images dorsal is to the top and medial to the right. (**A, C**) PCNA immunoreactivity revealed a high number of PCNA- immunoreactive cells in the IGrL, MCL and EPL layers of the olfactory bulb. (**B, D**) DCX immunoreactivity revealed a high density of DCX- immunoreactive cells and fibres in all layers of the olfactory bulb (**IGrL, MCL, EPL** and **GL**) except the olfactory nerve layer. Scale bar in **D** = 500 µm and applies to all. See list for abbreviations.



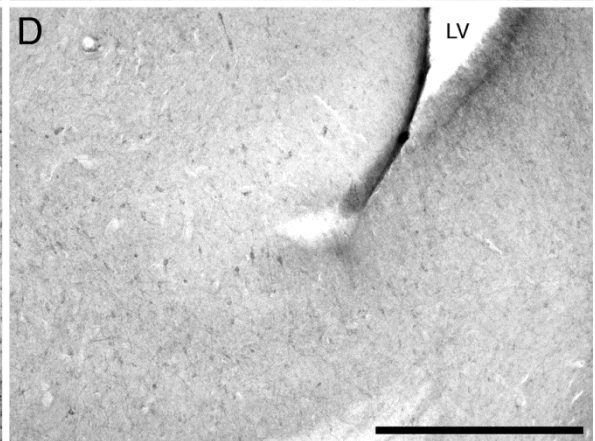
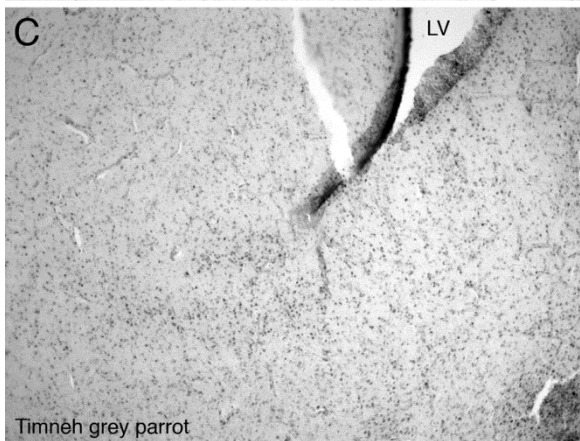
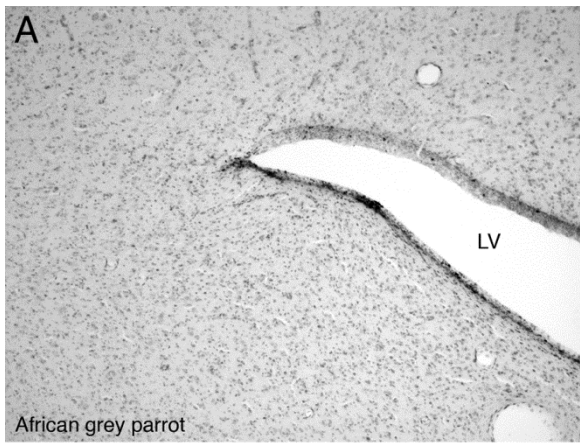
African grey parrot



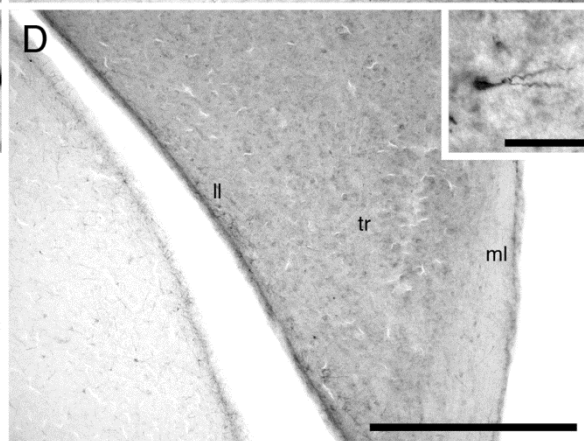
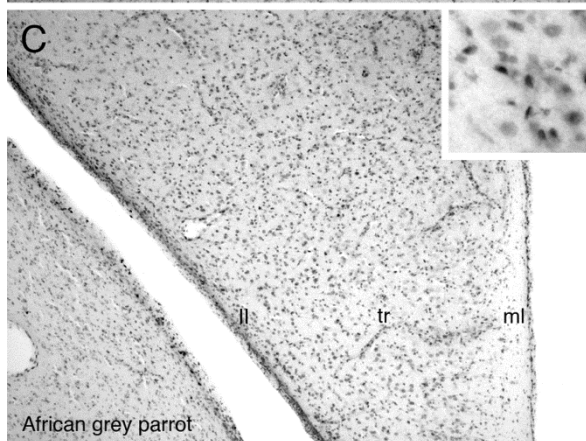
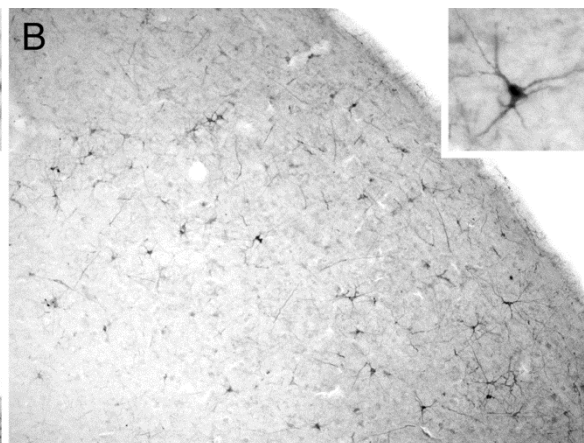
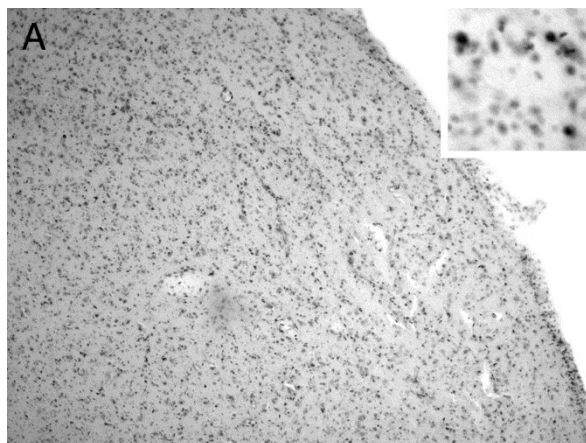
Timneh grey parrot



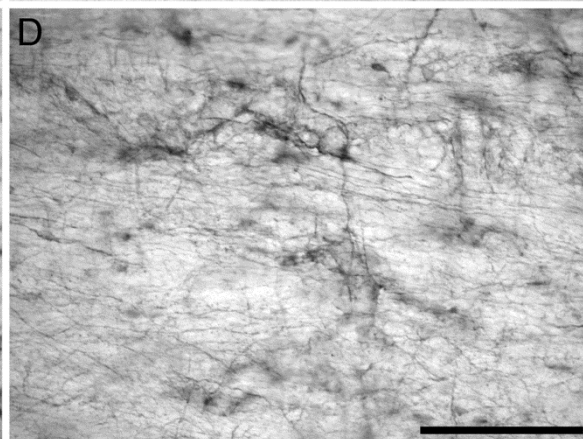
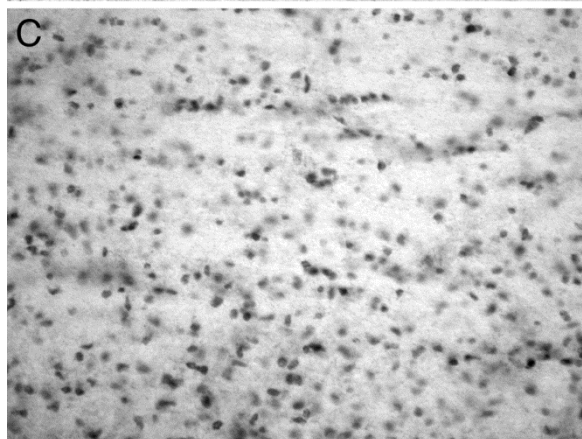
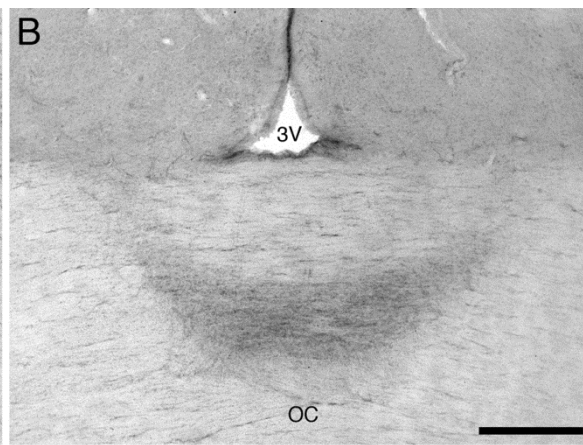
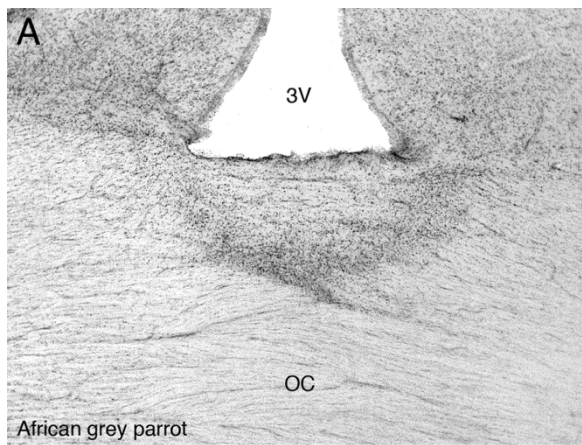
**Figure 4.4.** Photomicrographs of PCNA - (**A**, **C**) and DCX (**B**, **D**) - immunostained coronal sections through the subventricular zones (**SVZ**) of the lateral ventricle in the brains of the two parrot species – **A**, **B** – African grey parrot (*Psittacus erithacus*), **C**, **D** – Timneh grey parrot (*Psittacus timneh*). PCNA immunoreactivity revealed aggregates of proliferating cells (hot spots) in the SVZ of the dorsal pole of the lateral ventricle in the African grey parrot (*Psittacus erithacus*) (**A**), and ventral pole of the lateral ventricle in Timneh grey parrot (*Psittacus timneh*) (**C**). DCX immunoreactivity revealed aggregates of DCX- immunoreactive cells and fibres in the SVZ of the dorsal pole of the lateral ventricle in the African grey parrot (*Psittacus erithacus*) (**B**), and in the ventral pole of the lateral ventricle in the Timneh grey parrot (*Psittacus timneh*) (**D**). In all images dorsal is to the top and medial to the right. Scale bar in **D** = 500 µm and applies to all. **LV** – lateral ventricle.



**Figure 4.5.** Photomicrographs of PCNA - (**A**, **C**) and DCX (**B**, **D**) - immunostained coronal sections through the hippocampus (**Hp**) in the brain of the African grey parrot (*Psittacus erithacus*). Distribution of PCNA immunoreactive cells in the dorsal hippocampus (**A**), and ventral layers of the hippocampus (**C**). Insets in (**A**, **C**) show a higher magnification images of PCNA immunoreactive cells. Distribution of DCX immunoreactive cells in the dorsal hippocampus (**B**), and ventral layers of the hippocampus (**D**). Insets in (**B**, **D**) show a higher magnification of DCX immunoreactive cells. Note multipolar DCX immunoreactive cells in **B** compared to unipolar DCX immunoreactive cells in **D**. In all images dorsal is to the top and medial to the right. Scale bar in **D** = 500  $\mu\text{m}$  and applies to **A** – **D**. The scale bar in the inset in **D** = 50  $\mu\text{m}$  and applies to all insets. See list for abbreviations.

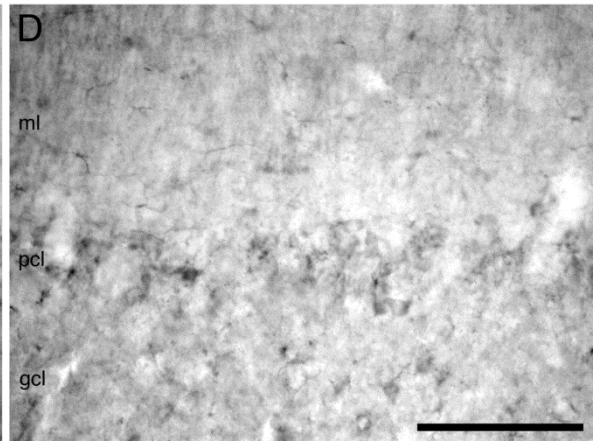
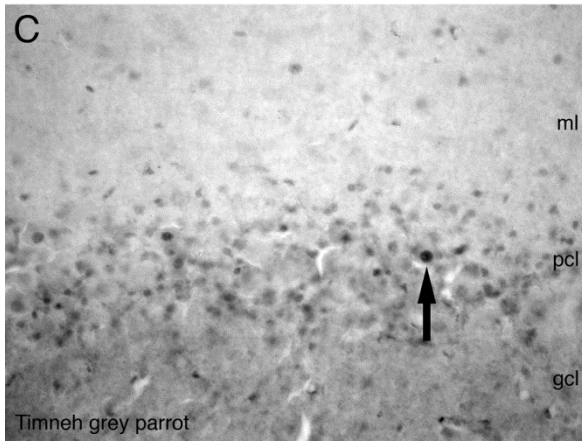
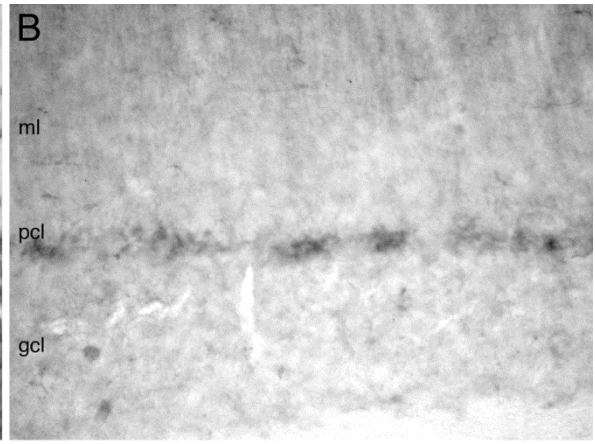
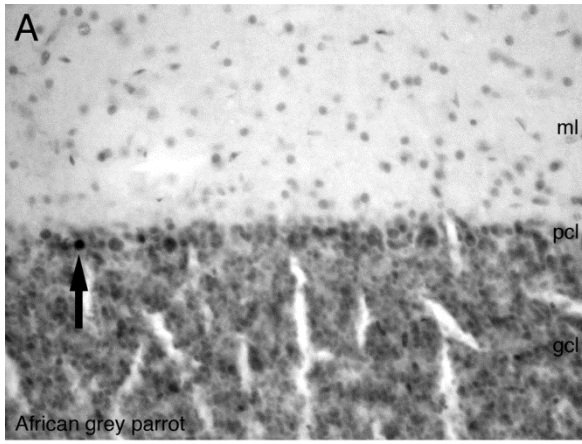


**Figure 4.6.** Photomicrographs of PCNA - (**A**, **C**) and DCX (**B**, **D**) - immunostained coronal sections through the optic chiasm (**OC**) at its border with the floor of the third ventricle (**3V**) in the brain of the Congo African grey parrot (*Psittacus erithacus*). (**A**) Accumulation of PCNA immunoreactive cells in the optic chiasm (**OC**). (**C**) A high magnification image of the PCNA immunoreactive cells in the optic chiasm. (**B**) A concentration of DCX immunoreactive cells and fibres in the optic chiasm. (**D**) A higher magnification image of DCX immunoreactive cells and fibres showing distinct cells and thick fibres. These images depict a potential neurogenic zone at the dorsal midline of the optic chiasm. In all images dorsal is to the top. Scale bar in **B** = 500  $\mu\text{m}$  and applies to **A** and **B**, scale bar in **D** = 100  $\mu\text{m}$  and applies to **C** and **D**.





**Figure 4.7.** Photomicrographs of PCNA - (**A**, **C**) and DCX (**B**, **D**) - immunostained coronal sections through the cerebellar cortex in the brains of the two parrot species – **A**, **B** – African grey parrot (*Psittacus erithacus*), **C**, **D** – Timneh grey parrot (*Psittacus timneh*). A clear similarity in the distribution of PCNA immunoreactive cells in the layers of the cerebellar cortex in the African grey parrot (**A**) and Timneh grey parrot (**C**) is observed. A similar distribution and staining intensity of DCX immunoreactive cells and fibres in the layers of the cerebellar cortex in the African grey parrot (*Psittacus erithacus*) (**B**) and Timneh grey parrot (*Psittacus timneh*) (**D**) is also observed. Note large and deeply stained PCNA immunoreactive cells (*arrows*) in the Purkinje cell layer (pcl) in both parrots. In all images dorsal is to the top and medial to the right. Scale bar in **D** = 100 µm and applies to all. See list for abbreviations.



## **4.4. Discussion**

### **4.4.1. General considerations**

In the current study we examined putative adult neurogenesis in the brains of the two species of African grey parrots the Congo African grey parrot (*Psittacus erithacus*) and Timneh grey parrot (*Psittacus timneh*) using PCNA and DCX immunohistochemistry. The PCNA immunoreactive cells were distributed heterogeneously in all brain regions including the olfactory bulb, telencephalon, diencephalon, mesencephalon and rhombencephalon, while DCX immunoreactive structures were dense in the olfactory bulb, telencephalon and some regions of the diencephalon, but far less dense in some parts of the diencephalon, the mesencephalon and rhombencephalon in both African grey parrot species. To a large extent our results conform and are comparable to the wide spread distribution of adult neurogenesis reported in other birds (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014; Mazenganya et al., 2017). Despite the overall similarity in the distribution of PCNA and DCX in both African grey parrot species, region specific differences, particularly associated with the density of PCNA and DCX immunoreactive structures, were observed in the telencephalon. Such region specific differences might highlight the relationship between adult neurogenesis and brain function, particularly in regions responsible for certain behavioural repertoires in different species.

### **4.4.2. Validity of PCNA and DCX antibodies in determining adult neurogenesis in birds**

The PCNA antibody used in the current study recognises the 36 kDA nuclear protein subunit of the DNA polymerase that is essential for DNA replication and is expressed selectively in proliferating cells (Hall et al., 1990; Balthazart and Ball, 2014b). The PCNA antibody is present during the various stages of the cell cycle except the G<sub>0</sub> stage (Kurki et al., 1988; Hall et al., 1990; Balthazart and Ball, 2014b). In birds, the PCNA antibody has been characterised in developing quail, parakeets, zebra finches (Charvet and Striedter, 2008;

Charvet and Striedter, 2009) and chickens (Hannan et al., 1999; Capes-Davis et al., 2005). The regions where PCNA immunoreactivity was observed in the African grey parrots correspond with those reported in quails after BrdU injection (Charvet and Striedter, 2008). Apart from labelling proliferating cells, the PCNA antibody also labels cells repairing their DNA (Hall et al., 1990; Shivji et al., 1992; Essers et al., 2005) and remains detectable several days after cells exit the cell cycle (Balthazart and Ball, 2014b). Such characteristics can affect the reliability of the PCNA antibody as a marker of adult neurogenesis in birds since they lead to an overestimation of proliferating cells.

DCX is a microtubule associated protein that plays a key role in the migration of neurons during development and in post mitotic neurons undergoing migration, remodelling of their dendritic processes, and synaptogenesis in adulthood (Francis et al., 1999; Gleeson et al., 1999; Nacher et al., 2001; Brown et al., 2003; Yang et al., 2004; Capes-Davis et al., 2005; Couillard-Despres et al., 2005). DCX expression levels have been found to decline with age in mammals (Brown et al., 2003; Couillard-Despres et al., 2005) and in birds (Ling et al., 1997; Hannan et al., 1999; Capes-Davis et al., 2005; Kim et al., 2006). In rats DCX expression is first observed one day after cell division and last for about 2-3 weeks until the neurons begin to express markers of mature neurons such as NeuN (Brown et al., 2003). In birds, DCX expression has been found to last for up to 30 days post mitosis (Balthazart et al., 2008). The DCX antibody was recommended for adult neurogenesis (Rao and Shetty, 2004; Couillard-Despres et al., 2005) and its expression has been consistent in areas known to recruit new neurons in mammals (Brown et al., 2003). In contrast, DCX expression in birds has been found in locations not associated with adult neurogenesis. In adult canaries BrDU immunoreactive cells were found to co-express the DCX antibody in the subtelencephalic regions (Vellema et al., 2014). The DCX antibody used in the current study has been characterised in the following birds: canaries (Boseret et al., 2007; Balthazart et al., 2008; Yamamura et al., 2011; Vellema et al., 2014), chickadees (Fox et al., 2010), zebra finches

(Kim et al., 2006), sparrows (LaDage et al., 2010), starlings (Hall and MacDougall-Shackleton, 2012), pigeons (Melleu et al., 2013; Melleu et al., 2015; Mazenganya et al., 2017) Japanese quail (Balthazart et al., 2010) and chickens (Hannan et al., 1999; Capes-Davis et al., 2005; Mezey et al., 2012).

#### **4.4.3. Adult neurogenesis in African grey parrots compared to other birds.**

Adult neurogenesis has been observed in budgerigars (*Melopsittacus undulatus*), which are small parrots (Nottebohm, 2011), but the current study is the first to report the presence of adult neurogenesis in the brains of the larger African grey parrots. There are remarkable similarities and as well as some variations in the pattern of adult neurogenesis across the avian species studied to date. In the avian brain, new neurons are generated in the subventricular zone of the lateral ventricle, with the largest density of proliferating cells reported in its dorsal and ventral poles. These cells migrate to different target regions of the brain (Alvarez-Buylla et al., 1994). In the present study proliferating cells were observed in high density in the SVZ of the lateral ventricle and throughout its rostrocaudal extent. The Congo African grey parrot exhibited an accumulation of proliferating cells in the dorsal and ventral poles of the lateral ventricle, at the level of the anterior commissure, similar to the proliferative “hot spots” described by Alvarez-Buylla (1990). These aggregates of proliferating cells were only observed in the dorsal pole of the lateral ventricle in the Timneh grey parrot. The proliferative hot spots phenomenon was also reported in other adult birds, including pigeons (Melleu et al., 2013; Mazenganya et al., 2017), canaries (Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1998), chickens (Mezey et al., 2012), and marsh tits (*Poecile palustris*) (Patel et al., 1997); however, they were absent in the adult ring dove (*Streptopelia risoria*) (Ling et al., 1997). The identity of the proliferating cells in these germinal zones remains contentious in both mammals and non-mammals. In the song bird, *Serinus canaria* the proliferative hot spots were associated with large accumulations of radial glial cells

(Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988), suggesting that radial glial cells were the primary cells undergoing mitosis in the germinal zones (Alvarez-Buylla et al., 1990b; Goldman, 1990; Vellema et al., 2010). In contrast, cell proliferation, including in the present study, was observed in the brain parenchyma (Alvarez-Buylla et al., 1988; Vellema et al., 2010; Mazenganya et al., 2017), the subventricular zone of the cerebral aqueduct, third, fourth and tectal ventricles, albeit in low density, and in the midbrain, hindbrain and cerebellum (Vellema et al., 2010). This localised proliferative activity suggests that certain neurons may be produced and integrate locally without the need for cell migration, and or thus proliferating cells can be glia or endothelial cells (Vellema et al., 2010).

The recruitment of new neurons is widespread in the telencephalon of adult birds and affected by various factors including age, environmental complexity, seasonal variation, hormones, stress and social complexity (for review, see Barnea and Pravosudov, 2011). In the African grey parrots, the distribution of DCX immunoreactive cells and fibres exhibited a similar pattern to that observed in the adult canaries (Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014), starlings (Absil et al., 2003) and zebra finches (Kim et al., 2006); however, DCX immunoreactivity was reported to be restricted to the telencephalon in other studies on adult canaries (Alvarez-Buylla and Nottebohm, 1988; Kirn et al., 1994; Vellema et al., 2010), pigeons (Melleu et al., 2013), ring doves (Ling et al., 1997) and chickens (Mezey et al., 2012).

Neuronal recruitment exhibited regional differences in the telencephalic subdivisions of adult canaries, zebra finches, domestic pigeons and rock pigeons (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Melleu et al., 2013; Mazenganya et al., 2017). In the adult canaries and zebra finches, the highest densities of DCX immunoreactive cells were observed in the mesopallium, nidopallium caudale (NC) and the striatum. Intermediate DCX expression was observed in the hyperpallium and rostral nidopallium, while a low

density of DCX immunopositive cells was noted in the hippocampus, arcopallium and subtelencephalic regions (Kim et al., 2006; Boseret et al., 2007; Vellema et al., 2014). We observed similar trends in the adult African grey parrots, but the hippocampus and parahippocampal regions exhibited moderate to high density of DCX immunoreactive cells and fibres. In addition, DCX immunoreactive neurons and fibres showed some area specific distribution in certain regions of the telencephalon and the brain stem nuclei in the two species of African grey parrots. For example, rostral nidopallium exhibited a moderate density DCX immunoreactive cells and fibres, whereas the caudal nidopallium exhibited the densest DCX immunoreactive cells and fibres. In addition, the distribution of DCX immunoreactive cells was not uniform in most telencephalic regions, where DCX immunoreactive cells and fibres were found in higher density in the medial and lateral margins compared to the core regions. The area specific distribution of DCX immunopreactive cells and fibres observed in these parrot species was also reported in adult canaries (Vellema et al., 2010; Vellema et al., 2014) and might highlight brain areas where plastic changes are necessary for the maintenance of behavioural repertoires in different species. In both song and non-song birds, the arcopallium and the entopallium exhibited absent to low densities of DCX immunoreactive cells (Goldman and Nottebohm, 1983; Vellema et al., 2010; Melleu et al., 2013; Mazenganya et al., 2017). Similarly, the two species of African grey parrots showed a low density of DCX immunoreactive cells in the entopallium and the arcopallium. In the arcopallium, the DCX immunoreactive cells and fibres were distributed only in the ventrocaudal margins encompassing the nucleus teanea (Tn).

The morphology of DCX immunoreactive cells varied in the different regions of the brain in the two species of African grey parrots. In regions closer to the lateral ventricle particularly the ventral striatum the DCX immunoreactive cells exhibited elongated somata with unipolar or bipolar morphology. In other regions of the telencephalon elongated DCX immunoreactive cells were mixed with cells with round and multipolar morphology, although

the former were more abundant. Elongated unipolar or bipolar cells represent migrating young neurons, usually emanating from the subventricular zone, whereas the round and multipolar neurons characterise mature neurons (Alvarez-Buylla, 1990). Migration of neurons in the telencephalon of birds follows two defined modes including tangential and radial migration (Alvarez-Buylla et al., 1988; Alvarez-Buylla, 1990). Vellema et al. (2010) further described the random migration of neuroblasts, whereby migrating immature neurons follow undefined routes within the telencephalon. In birds, the most pronounced mode of migration is radial migration, whereby migrating neuroblasts are guided on cellular processes of radial glial cells (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Vellema et al., 2010). Under this mode of migration, young neurons migrate from the subventricular zone (SVZ) of the lateral ventricle preferentially towards the lateral and ventrocaudal aspects of the telencephalon (Alvarez-Buylla, 1990; Vellema et al., 2010). This preferred direction of migration may explain the presence of the high densities of DCX immunoreactive cells and fibres in the lateral and caudal regions of the telencephalon when compared to the medial and rostral regions. According to Melleu et al. (2013), the majority of round and multipolar cells expressing the microtubule marker DCX do not co-express with the adult neuronal marker NeuN, suggesting that they are immature neurons even though they show the morphology of mature neurons.

Recruitment of new neurons in the diencephalon, mesencephalon and rhombencephalon of adult birds has been reported in experimental animals (Cao et al., 2002; Chen et al., 2006), but no studies support adult neuronal recruitment under normal physiological conditions. Despite this, reports in adult canaries (Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014) and zebra finches (Kim et al., 2006) indicate the presence of DCX immunoreactive cells in these regions. In the species of the African grey parrots studied herein, similar, but low density, distributions of DCX immunoreactive cells and fibres were observed. In agreement with Kim et al. (2006) and Boseret et al. (2007) the



low density expression of DCX immunoreactivity in the brain stem may not signify adult recruitment of new neurons, but represent cellular plasticity of adult neurons (Nacher et al., 2001; Brown et al., 2003).

#### **4.4.4. Functional implications of adult neurogenesis related to certain behaviours of the African grey parrots**

The role of adult neurogenesis in brain function and behaviour in various species is still contentious. In mammals adult hippocampal neurogenesis is generally associated with acquisition of new and clearance of old memories (Kempermann et al., 2004; Zhao et al., 2008); however in birds, neuronal recruitment is wide spread in the telencephalon, making it difficult to understand the precise functional implications of this widespread characteristic. In addition, birds live comparably longer than mammals of similar body mass, and adult neurogenesis in avian species may be adaptive, promoting continuous learning by updating and renewing memories (Nottebohm, 1991; Gahr et al., 2002). The effect of learning on adult neurogenesis is bidirectional. Learning has been found to increase the survival, maturation and the response to stimuli of adult born neurons (Kee et al., 2007; Tronel et al., 2010; Lemaire et al., 2012). In contrast, adult neurogenesis facilitates learning in new conditions, which may include new habitats, new members of social groups, or new sources of food. In avian species adult neurogenesis is coupled with cell death (Kirn and Nottebohm, 1993; Rasika et al., 1994; Yamamura et al., 2011) and has been found to be task-dependant in various species (Barker et al., 2011). According to Nottebohm et al. (1986) increased singing in canaries leads to increased neuronal recruitment in the high vocal centre (HVC). In food storing species increased hippocampal neurogenesis was observed during the peak periods of food caching in autumn, whereas in non-storing species no addition of hippocampal neurons was recorded during the same period (Barnea and Nottebohm, 1996; Sherry and Hoshooley, 2009). Moreover in migratory species, which rely on spatial memory, species with larger

home ranges exhibited increased hippocampal neurogenesis compared to related species that inhabit smaller territories (Cristol et al., 2003; Hoshooley and Sherry, 2007; LaDage et al., 2009). The olfactory bulbs recruit new neurons in adults of almost all studied species, except for a few studies of canaries and zebra finches (Barker et al., 2011; Melleu et al., 2013). In the current study we observed a high density of PCNA and DCX immunoreactive structures in the inner layers of the olfactory bulbs in both African grey parrots. The functional implications of olfactory neurogenesis remain elusive, but the olfactory system is involved in reproduction, the monitoring of environmental changes, the maintenance of survival by detecting presence of predators, the identification of clan members and the location of food (Jones and Roper, 1997; Patzke et al., 2010; Barker et al., 2011).

Apart from adult hippocampal and olfactory neurogenesis, neuronal recruitment in the nidopallium caudale (NC) has been shown to increase under various states of environmental enrichment. In a study conducted on zebra finches, adult neurogenesis in the nidopallium caudale was found to increase in adults introduced to communal living compared to individuals living in pairs (Lipkind et al., 2002). The reason associated with this increase is the possibility that individuals introduced to communal living need to identify new members and establish new social relationships (Lipkind et al., 2002; Adar et al., 2008). Similar changes in neuronal recruitment in the nidopallium caudale were identified during the reproductive cycle, when adult individuals of zebra finches were feeding their young (Barkan et al., 2007). In the present study the nidopallium caudale (NC) and the nidopallium caudolaterale (NCL) exhibited the densest DCX immunoreactivity in both species of African grey parrots. The nidopallium caudale is associated with reproductive behaviour (Melleu et al., 2013), whereas the nidopallium caudolaterale, a proposed analogue of the mammalian prefrontal cortex, participates in higher order cognitive functions, such as decision making, speech and planning of behaviour (Güntürkün, 2005).

African grey parrots live in large colonies of approximately 10, 000 individuals (Parr and Juniper, 2010). Although few studies have examined the behaviour of African grey parrots in the wild, results show that their social ecology is comparable to that of primates (Emery et al., 2007). The African grey parrots lead a complex social life coupled with long term monogamous relationships (Seibert, 2006; Emery et al., 2007). Shultz and Dunbar (2010) postulated that species in monogamous relationships develop larger brain to body mass ratios to preserve the stable pair bonded relationships. The African grey parrots may be useful models to assess the impact of changing social environments on adult neurogenesis.

## **CHAPTER FIVE: The distribution of cells immunoreactive to proliferating cell nuclear antigen and doublecortin in the brains of adult pied crows (*Corvus albus*): widespread potential adult neurogenesis.**

### **5.1. Introduction**

Studies of putative adult neurogenesis in non-mammalian vertebrates are not undertaken as regularly as in mammals (Bonfanti et al., 2011), but by examining more species our understanding of the diversity and basic mechanisms underlying adult neurogenesis, its regulating factors, and its attributes in different species, will be enhanced. Adult neurogenesis is a highly conserved neural trait, occurring in both invertebrates and vertebrates (Lindsey and Tropepe, 2006; Amrein et al., 2011; Barker et al., 2011). The process of adult neurogenesis occurs in varied degrees, demonstrating interclass and species-specific similarities and differences (Cayre et al., 2002, 2007; Lindsey and Tropepe, 2006). In birds and mammals adult neurogenesis is coupled with cell death (Barnea and Nottebohm, 1994, 1996; Hastings et al., 2000; Yamamura et al., 2011) and appears to play an important role in the homeostasis of restricted brain regions, particularly those involved in learning and adapting to external environmental variations (Barnea and Pravosudov, 2011; Bonfanti and Peretto, 2011).

In birds the neurogenic sites are restricted to the subventricular zone, predominantly in the dorsal and ventral poles of the walls of the lateral ventricle (Alvarez-Buylla et al., 1990); however, other studies have reported that the resident progenitor cells in the brain parenchyma are able to generate neurons in the adult, especially in larger brained avian species (Vellema et al., 2010; Paredes et al., 2016). Neuronal recruitment in adult birds has been found to be wide-spread in the telencephalon (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2010, 2014; Barnea and Pravosudov, 2011). In addition, neuronal recruitment displayed patterns correlated with species-specific behaviours, such as the learning and production of new songs in song birds, and spatial memory

dependent tasks, including food storing and homing in both song and non-song birds (Sherry and Hoshooley, 2010; Vellema et al., 2010; Barnea and Pravosudov, 2011).

The pied crow (*Corvus albus*) is an indigenous sub-Saharan savannah species, belonging to family Corvidae and order Passeriformes (Winterbottom, 1975; Londei, 2010). The pied crow is a highly social bird, commonly found in pairs or small groups, but large roosts of approximately 300 individuals have been reported (Winterbottom, 1975). Corvids, amongst the birds, have a large relative brain size, produce song, are capable of vocal mimicry, use and manufacture tools, while certain species cache food and appear to show mirror self-recognition (Winterbottom, 1975; Emery and Clayton, 2004; Wang et al., 2009). These ecological and behavioural characteristics indicate that Corvids may exhibit more extensive adult neurogenesis than other avian species (Barnea and Pravosudov, 2011; Kempermann, 2011). Here we investigated, qualitatively, the putative occurrence, extent and distribution of neurogenesis in the brains of adult wild pied crow (*Corvus albus*) using immunohistochemistry to reveal endogenous markers of proliferating cells (PCNA) and immature neurons (DCX).

## **5.2. Experimental procedures**

### **5.2.1. Animals and tissue processing**

The brains of two adult male pied crows (*Corvus albus*) were used in the current study. The pied crows were captured in the north western region of South Africa using baited cages, during late spring (01 and 15 November, 2013). Both birds were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (clearance no: 2013/05/02B), which parallel those of the NIH for the care and use of animals in scientific experimentation. Five minutes prior to being euthanized, both birds were given an intramuscular dose of heparin, 2500 units (0.5 ml), to prevent blood clotting. Animals were

euthanized with an intraperitoneal injection of Euthapent (1ml/kg) and body mass recorded. Both birds were transcardially perfusion-fixed, initially with a rinse of 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). The brains were carefully removed from the skull, and post fixed overnight in 4% paraformaldehyde in 0.1M PB. The brains were then weighed (crow 1: body mass = 425 g, brain mass = 9.7 g; crow 2: body mass = 461.9 g, brain mass = 9.8 g) and placed in a 30% sucrose in 0.1 M PB solution at 4°C for 3 days, and then transferred to an antifreeze solution for storage at -20°C. Before sectioning, the tissue was allowed to re-equilibrate in 30% sucrose in 0.1M PB solution at 4°C for 4 days. The brains were then frozen in dry ice and sectioned in the coronal plane, 50 µm section thickness, on a sliding microtome. A one in ten series of sections were taken and three series stained for Nissl substance, PCNA and DCX. The remaining series of sections were placed back in an antifreeze solution and are stored at -20°C for future use. The first series of sections, used for Nissl staining, were mounted on 0.5% gelatine coated slides, dried overnight, cleared in a 1:1 mixture of 100% ethanol and 100% chloroform and stained with a 1% cresyl violet solution.

### **5.2.2. Immunohistochemistry for PCNA and DCX**

The second and third series of sections were used for free floating PCNA and DCX immunohistochemistry. These sections were initially treated for 30 min at room temperature under gentle shaking with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1M PB: 1.6% of 30% H<sub>2</sub>O<sub>2</sub>), followed by three 10 min rinses in 0.1 M PB. To block non-specific binding sites the sections were then preincubated for 2 h, at room temperature under gentle shaking, in a blocking buffer solution consisting of 3% normal horse serum (NHS) for PCNA sections or 3% normal rabbit serum (NRS) for DCX sections, 2% bovine serum albumin, and 0.25% Triton X-100 in 0.1 M PB. Following preincubation the primary antibodies were added to the blocking buffer solution and the sections were incubated for 48 h at 4°C under gentle

shaking (PCNA - 1: 500 dilution of mouse anti-PCNA, NCL-L-PCNA Leica Biosystems, Newcastle, United Kingdom; DCX - 1:300 dilution of goat anti-DCX antibody, C-18, Santa Cruz Biotechnology, Dallas, Texas, U.S.A) under gentle agitation. The primary antibody incubation was followed by three 10-min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (PCNA sections - 1:1000 dilution of biotinylated anti-mouse IgG [BA-2001, Vector Labs] in 3% normal horse serum and 2% bovine serum albumin in 0.1 M PB; DCX sections - 1:1000 dilution of anti-goat IgG [BA-5000, Vector Labs] in 3% normal rabbit serum and 2% bovine serum albumin in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin biotin solution (1:125 in 0.1 M PB; Vector Labs, Burlingame, California, USA), followed by three 10-min rinses in 0.1 M PB. Sections were then transferred to a solution consisting of 0.05% diaminobenzidine tetrahydrochloride in 0.1M PB for 5 min at room temperature, after which 3.3 µl of 30% H<sub>2</sub>O<sub>2</sub>/ml of solution was added. With the aid of a low power stereomicroscope the progression of the staining was visually followed and allowed to continue until a level was reached where the background staining could assist in architectonic matching to the Nissl stained sections without obscuring the immunopositive structures. The tissue was then rinsed twice more in 0.1M PB before being mounted on glass slides coated with 0.5% gelatine and allowed to dry overnight. Once dry, the slides were placed in a solution of 70% ethanol for 2 h and then dehydrated, cleared in xylene and coverslipped with Depex. To test for non-specific staining of the immunohistochemical protocol, the primary and secondary antibodies were omitted from random sections and no staining was evident. The observed immunostaining patterns support the specificity of the antibodies and are compatible with observations made in pigeons, parakeets and quails (Charvet and Striedter, 2008, 2009; Melleu et al., 2013).

### 5.2.3. Analysis

The Nissl stained sections were examined with a low power stereomicroscope and the architectonic borders traced using a camera lucida. The PCNA and DCX immunostained sections were then matched to the drawings from the Nissl stained sections and the location of immunopositive soma marked on the drawings. Selected drawings were then scanned and redrawn using the Canvas 8 Software (Deneba Software, Miami, Florida, USA). High power microscopic observation allowed for the determination of the relative densities of stained structures throughout the various regions of the brain. The relative densities of immunostained structures were visually compared and recorded on a scale ranging from low (+), moderate (++) to high (+++). A second observer was used to eliminate observer bias.

The brain regions were identified and named in accordance with the stereotaxic atlas of the brain of the Japanese jungle crow (*Corvus macrorhynchos*) (Izawa and Watanabe, 2007) using the nomenclature recommended by the Avian Brain Nomenclature Forum (Reiner et al., 2004). Digital photomicrographs were captured using a digital camera (Axio Cam HRc, Zeiss South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software (Zeiss, South Africa). No pixilation adjustments or manipulation of the captured images were undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

### Abbreviations

3V – third ventricle

A – arcopallium

AL – ansa lenticularis

AP – area pretectalis



APH – area parahippocampalis

B – nucleus basorostralis pallii

BC – brachium conjunctivum

BST – nucleus striae terminalis

CA – commissura anterior

Cb – cerebellum

CbL – nucleus cerebellaris lateralis

CbM – nucleus cerebellaris intermedius

CO – chiasma opticum

CS – nucleus centralis superior

DCX – doublecortin

DLA – nucleus dorsolateralis anterior thalami

DLP – nucleus dorsolateralis posterior

DMA – nucleus doesomedialis anterior thalami

DMP– nucleus doesomedialis posterior

E – entopallium

EM – nucleus ectomamillaris

EPL – external plexiform layer of the olfactory bulb

FA – tractus fronto-arcopallialis

FLM – fasciculus longitudinalis medialis

FPL – fasciculus prosencephali lateralis

FRL – formatio reticularis mesencephalic pars lateralis

GCt – substantia grisea centralis

GL – glomerular layer of olfactory bulb

GLv – nucleus geniculatus lateralis, pars ventralis

GP – globus pallidus

HA – hyperpallium apicale

HD – hyperpallium densocellulare

HI – hyperpallium intercalatus

Hp – hippocampus

HVC – high vocal center

ICo – nucleus intercollicularis

IGrL – internal granular layer of the olfactory bulb

Imc – nucleus isthmi, pars magnocellularis

IO – nucleus isthmo-opticus

Ipc – nucleus isthmi, pars parvocellularis

La – nucleus laminaris

LHy – hypothalamus pars lateralis

ll – lateral layer of the ventral hippocampus

LoC – locus ceruleus

LSt – striatum laterale

LV – lateral ventricle

M – mesopallium

MAN – nucleus magnocellularis nidopallii anterioris

MC – nucleus magnocellularis

MCL – mitral cell layer of the olfactory bulb

ml – medial layer of ventral hippocampus

MLd – nucleus mesencephalicus lateralis, pars dorsalis

MSt – striatum mediale

N – nidopallium

NC – nidopallium caudale

NI – nidopallium intermoderate

nIV – nucleus nervi trochlearis

nIX – nucleus nervi glossopharyngei

nVII – nucleus nervi facialis

nXII – nucleus nervi hypoglossi

OB – olfactory bulb

OI – nucleus olivarius inferior

OM – tractus occipito-mesencephalicus

ON – olfactory nerve

OS – nucleus olivaris superior

Ov – nucleus ovoidalis

PaM – nucleus paramedianus

PCNA – proliferating cell nuclear antigen

PGL – nucleus paragigantocellularis lateralis

PH – plexus of Horsley

PL – nucleus pontis lateralis

PM – nucleus pontis medialis

PMH – nucleus medialis hypothalami posterioris

POA – nucleus preopticus anterior

POM – nucleus preopticus medialis

PrV – nucleus sensorius principalis nervi trigemini

PT– nucleus pretectalis

PV – nucleus posteroventralis thalami

PVM – nucleus periventricularis magnocellularis

R – nuclei rephes

RA – nucleus robustus arcopallialis

RP– nucleus reticularis pontis caudalis

RPgc – nucleus reticularis pontis caudalis, pars gigantocellularis

Rt – nucleus rotundus

Ru – nucleus ruber

SCE – stratum cellulare externum

SCI – stratum cellulare internum

SGF – substantia grisea et fibrosa

SL – nucleus septalis lateralis

SLu – nucleus semilunaris

SM – nucleus septalis medialis

SNc – substantia nigra, pars compacta

SO – statum opticum

SP– nucleus subpretectalis

SPC – nucleus superficialis parvocellularis

SpL – nucleus spiriformis lateralis

SpM – nucleus spiriformis medialis

SpM – nucleus spiriformis medialis;

Ta – nucleus tangentialis (Cajal)

TeO – tectum opticum

TFM – tractus thalamo-frontalis et frontalis-thalamicus medialis thalami

TnA – nucleus taeniae amygdalae

tr – triangular area of ventral hippocampus

TrO – tractus opticus

TSM – tractus septopallio-mesencephalicus

TTD – nucleus tractus descendens trigemini

Tu– nucleus tuberis

V – ventricle

VeL – nucleus vestibularis lateralis

VeM – nucleus vestibularis medialis

X – area X

## 5.3. Results

### 5.3.1. General observations

In the present study we examined putative adult neurogenesis throughout the brain of the pied crow (*Corvus albus*) using immunohistochemical techniques for the endogenous markers PCNA and DCX. Due to the similarity in the pattern of expression of both PCNA and DCX antibodies in the brains of the two crows examined we present only the camera lucida drawings and photographs of the crow 1 brain throughout this chapter. The distribution of PCNA and DCX immunoreactive cells were mapped onto architectonic drawings based on adjacent Nissl stained sections (Fig. 5.1, 5.2). PCNA immunoreactivity was observed in all brain regions including the olfactory bulb, telencephalon, diencephalon, mesencephalon and rhombencephalon, but in varying degrees (Table 5.1). Similar heterogeneity in densities of immunostained structures and distribution was observed for DCX immunoreactivity. Dense DCX immunoreactivity was identified in the layers of the olfactory bulbs (OB), the subventricular zone of the lateral, third and fourth ventricles and the cerebral aqueduct, subdivisions of the pallium (Hippocampus [Hp], hyperpallium apicalle [HA], hyperpallium intercalatum [HI], hyperpallium densocellulare [HD], mesopallium [M], and nidopallium [N]) and subpallium (medial and lateral striatum [MSt and LSt], lateral septum [SL] and the globus pallidus [GP]) except the medial septum (SM). Weaker DCX immunoreactivity was observed in the diencephalon, mesencephalon (except in the optic tectum where a moderate density of DCX immunoreactivity was observed) and the rhombencephalon. Generally, the telencephalic regions had a higher density of PCNA and DCX immunoreactive cells than other brain regions, but the intensity of PCNA immunoreactivity was high in the nuclei of the diencephalon and the hindbrain. DCX immunoreactivity was more intense in fibres than in cell bodies, and the majority of DCX immunoreactive cells were small rounded cells, fusiform unipolar and or bipolar cells.

### **5.3.2. Distribution of PCNA immunoreactive cells**

#### **5.3.2.1. Olfactory bulb**

PCNA immunoreactive cells were observed at high density in the internal granular layer (IGrL), mitral cell layer (MCL) and external plexiform layer (EPL) layer, but with a lower density in the glomerular layer (GL) and olfactory nerve (ON).

#### **5.3.2.2. Subventricular zone**

The subventricular zones of the lateral ventricle, third ventricle, cerebral aqueduct, fourth and tectal ventricles exhibited a high density of PCNA immunoreactive cells (Fig. 5.1C– L). The dorsal and ventral poles of the lateral ventricles showed an accumulation of PCNA immunoreactive cells in high density, especially rostrally within the brain (Fig. 5.3A, 5.3C, 5.4A, 5.4C).

#### **5.3.2.3. Pallial structures**

A heterogeneous distribution of PCNA immunoreactive cells was observed in the different pallial regions of the telencephalon (Fig. 5.1A– O). A relatively higher density and homogeneous distribution of PCNA immunoreactive cells was observed in the frontal regions of the hyperpallium apicale (HA), which decreased in density caudally to very low density in the middle of this region and absent in the caudal portions of this region (Fig. 5.1A– J). The dorsal region of the hippocampus (Hp) and the area parahippocampalis (APH) evinced a high density of PCNA immunoreactive cells (Fig. 5.5A, 5.5C). The ventral region of the hippocampus (Hp) exhibited a moderate density of PCNA immunoreactive cells in the medial layer (ml) and a low density in the triangular (tr) and lateral layers (ll) (Fig. 5.6A, 5.6C). A moderate density of PCNA immunoreactive cells were observed in the medial and lateral regions of the hyperpallium densocellulare (HD) and the mesopallium (M) (Fig. 5.1C– J). A large and dense cluster of PCNA immunoreactive cells was observed at the level of the



anterior commissure (CA) in the mesopallium (M) (Fig. 5.1G – J). The nidopallium frontalis (NF) exhibited a moderate density of PCNA immunoreactive cells, except in the nucleus magnocellularis nidopallii anterioris (MAN, a song nucleus) which showed a high density of PCNA immunoreactive cells (Fig. 5.7A, 5.7C). At mid-telencephalic levels the nidopallium intermoderate (NI) exhibited low density of PCNA immunoreactive cells in the medial regions, but a higher density in the lateral regions (Fig. 5.1F-I). The core region of the nidopallium intermoderate (NI) was devoid of PCNA immunoreactive cells (Fig. 5.1H-J). In the nidopallium caudale (NC), a high density of PCNA immunoreactive cells was observed in the medial region which houses the high vocal centre (HVC, another song nucleus) (Fig. 5.1K, 5.1L, 5.8A), and a moderate density of PCNA immunoreactive cells in the lateral region of the NC (Fig. 5.1K– N). Generally the density of PCNA immunoreactive cells in almost all pallial subdivisions of the telencephalon showed a rostro-caudal decline, except the nidopallium caudale (NC) where the density of PCNA immunoreactivity increased caudally.

#### **5.3.2.4. Subpallial structures**

The medial and lateral striatum (MSt and LSt) contained a moderate density of PCNA immunoreactive cells throughout their rostro-caudal extent (Fig. 5.1E – J). A much higher density of PCNA immunoreactive cells was distributed in the medial regions adjacent to the ventral pole of the lateral ventricle including the area X nucleus (Fig. 5.9A) and the ventrolateral regions of the medial striatum (MSt) (Fig. 5.1E– J). The globus pallidus (GP) exhibited a low density of PCNA immunoreactive cells (Fig. 5.1G – K). Moderate densities of PCNA immunoreactive cells were observed in the medial and lateral septum (SM and SL), entopallium (E), arcopallium (A), robust nucleus of the arcopallium (RA) (Fig. 5.10A), nucleus taeniae amygdalae (TnA) of the amygdala, and the tractus fronto-arcopallialis (FA) (Fig. 5.1H– O).

#### **5.3.2.5. Diencephalon**

PCNA immunoreactive cells in the diencephalon were intensely stained and in high density in the dorsal thalamic nuclei, including nucleus dorsomedialis anterior (DMA), nucleus dorsolateralis anterior thalami (DLA) and nucleus dorsolateralis posterior thalami (DLP), the medial regions of the hypothalamus, including nucleus medialis hypothalami posterioris (PMH), nucleus preopticus medialis (POM), and nucleus periventricularis magnocellularis (PVM) and ventrally in the nucleus tuberis (Tu) (Fig. 5.1I– K) . In the optic chiasm (CO), a large cluster of PCNA immunoreactive cells was observed ventral to the hypothalamus (Fig. 5.11A).

#### **5.3.2.6. Mesencephalon, rhombencephalon and cerebellum**

Most of the nuclei in the midbrain, pons and medulla oblongata exhibited intensely stained PCNA immunoreactive cells. In the midbrain, some of the nuclei that exhibited intense PCNA immunoreactivity include, the nucleus rotundus (Rt), nucleus ruber (Ru), substantia nigra, pars compacta (SNc), substantia nigra centralis (GCT), nucleus ectomammillaris (EM), nucleus isthmi, pars parvocellularis and pars magnocellularis (Ipc and Imc), nucleus mesencephalicus lateralis, pars dorsalis (MLd) and nucleus spiriformis medialis (SpM) (Fig. 5.1I– K). The layers of the optic tectum (TeO) contained a high density of PCNA immunoreactive cells (Fig. 5.1I– M). Various nuclei of the pons and medulla oblongata showed intense PCNA immunoreactivity (Fig. 5.1L– O). In the cerebellum a high density of large PCNA immunoreactive cells was observed in the Purkinje cell layer, but only a moderate density of immunoreactive cells was observed in the granule cell layer (Fig. 5.1K– O, 5.12A).

### **5.3.3. Distribution of DCX immunoreactive cells and fibres**

#### **5.3.3.1. Olfactory bulb**

The olfactory bulbs, although small in crows, exhibited the highest density of DCX immunoreactive cells and fibres, particularly in the inner layers, of any location in the crow brain. The one exception was the external glomerular cell layer (GL), which exhibited a low density of DCX immunoreactive cells and fibres.

#### **5.3.3.2. Subventricular zone**

The subventricular zone of the lateral ventricles contained a high density of DCX immunoreactive cells and fibres (Fig. 5.2C– L). The majority of the DCX immunoreactive cells had their neurites oriented parallel to the wall of the ventricle, indicating tangential migration. Occasionally, a few cells and neurites were observed oriented perpendicular to the wall of the lateral ventricle, particularly in regions adjacent to the medial striatum (MSt) ventrally and the hyperpallium densocellulare (HD) and hyperpallium apicale (HA) dorsally. Additionally, at rostral levels and occasionally at middle levels, accumulations of DCX immunoreactive cells and fibres were observed in the dorsal and ventral poles of the lateral ventricle, comparable to the “hot spots” described by Alvarez-Buylla et al. (1990) (Fig. 5.2C– F, 5.2I, 5.2J, 5.3B, 5.3D, 5.4B, 5.4D). The subventricular zones of the third ventricle, cerebral aqueduct, fourth and tectal ventricles displayed moderate densities of DCX immunoreactive structures, the majority being intensely stained fibres (Fig. 5.2H, 5.2J).

#### **5.3.3.3. Pallial structures**

The pallial subdivisions of the telencephalon contained moderate, but variable, densities of DCX immunoreactive cells and fibres. A moderate density of DCX immunoreactive cells and fibres was observed in the hyperpallium apicale (HA) at rostral levels, but at middle and caudal levels the density of DCX immunoreactive structures

decreased to low and absent respectively (Fig. 5.2A– J). The dorsal hippocampus (HP) and the area parahippocampalis (APH) exhibited low densities of DCX immunoreactive cells and fibres across the rostro-caudal extent (Fig. 5.5B, 5.5D). In the ventral hippocampus, DCX immunoreactive cells were present in a moderate density in the triangular and lateral layers (tr and ll) overlying the lateral wall of the lateral ventricle (Fig. 5.6B, 5.6D). In the mesopallium, a moderate density of DCX immunoreactive cells and fibres was observed at rostral levels, particularly in the ventrolateral regions (Fig. 5.2C– D), but at caudal levels a low density of DCX immunoreactive cells and fibres was observed in the medial and lateral regions, and DCX immunoreactive structures were absent in the core region (Fig. 5.2E – J). The nidopallium frontalis (NF) exhibited a moderate density of DCX immunoreactive cells and fibres, particularly in the medial and lateral regions, but a much higher density of these structures was observed in the lateral part of the nucleus magnocellularis nidopallii anterioris (MAN) (Fig. 5.2D – F, 5.7B, 5.7D). In the nidopallium intermoderate (NI), DCX immunoreactive cells and fibres were distributed in a low density in the medial regions and in a higher density in the lateral regions, but in the core region DCX staining was absent (Fig. 5.2G– J). The nidopallium caudale (NC) showed three prominent clusters of moderate to high density of DCX immunoreactive cells and fibres, including the medial region of the high vocal centre (HVC) (Fig. 5.8B), the core cluster above the arcopallium, and in the lateral region (Fig. 5.2K– N).

#### **5.3.3.4. Subpallial structures**

The medial and lateral striatum (MSt and LSt) exhibited a moderate density of DCX immunoreactive cells and fibres throughout their rostro-caudal extent (Fig. 5.2E – K). In the medial striatum (MSt), a much higher density of DCX immunoreactive cells and fibres was observed in the regions adjacent to the ventral pole of the lateral ventricle and ventral regions (Fig. 5.2E – J). The song nucleus, area X, showed a moderate density of DCX

immunoreactive cells and fibres, similar to the surrounding regions (Fig. 5.9B). The nucleus basorostralis pallii (B), globus pallidus (GP) and the entopallium (E), exhibited very low densities of DCX immunoreactive cells and fibres (Fig. 5.2E – I). No DCX immunoreactivity was detected in the arcopallium (A) and robust nucleus of the arcopallium (RA) (Fig. 5.2L–N, 5.10B), but regions ventral to the arcopallium, including the nucleus taeniae amygdalae (TnA), contained a low density of DCX immunoreactive cells and fibres (Fig. 5.2L). In the septum, the lateral septum (SL) showed a low density of DCX immunoreactive cells and fibres, but no DCX immunoreactivity was observed in the medial septum (SM) (Fig. 5.2H, 5.2I).

#### **5.3.3.5. Diencephalon**

Weak DCX immunostaining was observed throughout the majority of the diencephalon. The nucleus preopticus medialis (POM), nucleus periventricularis magnocellularis (PVM), nucleus medialis hypothalamic posterioris (PMH) and the nucleus ovoidalis (OV) exhibited a low density of DCX immunoreactive cells and fibres, but the nucleus tuberis (Tu) contained a higher density of intensely stained DCX immunoreactive cells and fibres (Fig. 5.2H – J). The dorsal margin of the diencephalon including the nucleus dorsomedialis posterior thalami (DMA), nucleus dorsolateralis anterior thalami (DLA), and the dorsolateral margin containing nucleus spiriformis lateralis (SPL), displayed weak DCX immunostaining (Fig. 5.2J – K). The optic chiasm (CO) contained an accumulation of DCX immunoreactive fibres, with no apparent cells, ventral to the hypothalamus (Fig. 5.11B).

#### **5.3.3.6. Mesencephalon, rhombencephalon and cerebellum**

Most of the nuclei of the midbrain, pons and medulla oblongata displayed weak DCX immunolabelling. Weak DCX staining was observed in the perikarya of the nuclei of the midbrain including the nucleus isthmi, pars magnocellularis (Imc), nucleus isthmi, pars

parvocellularis (Ipc), nucleus mesencephalicus lateralis, pars dorsalis (MLd) and the substantia grisea centralis (GCt); however, DCX immunoreactive fibres and occasionally small round cell-like structures and synaptic vesicles were observed in the nucleus rotundus (Rt), nucleus laminalis (La), nucleus geniculatus lateralis, pars ventralis (GLv) the nucleus ectomamillaris (EM) and in the fasciculus longitudinalis medialis (FLM) (Fig. 5.2J – L). A moderate density of DCX immunoreactive fibres and occasional cells were observed in the layers of the optic tectum (Fig. 5.2I – M). In the pons and medulla oblongata, weak DCX immunoreactivity was observed in fibres within the nucleus pontis lateralis and medialis (PL and PM), nucleus tractus descendens trigemini (TTD), nucleus reticularis pontis caudalis, pars gigantocellularis (RPgc), nucleus reticularis pontis caudalis (RP), nucleus vestibularis lateralis (VeL) and in the nucleus cerebellaris internus (CbI) (Fig. 5.2K– O). The cerebellar cortex did not show any DCX immunoreactive cells, with only very weakly stained fibres being observed in the Purkinje cell layer (Fig. 5.12B).

**Table 5.1.** Summary of qualitative distribution and density of PCNA and DCX

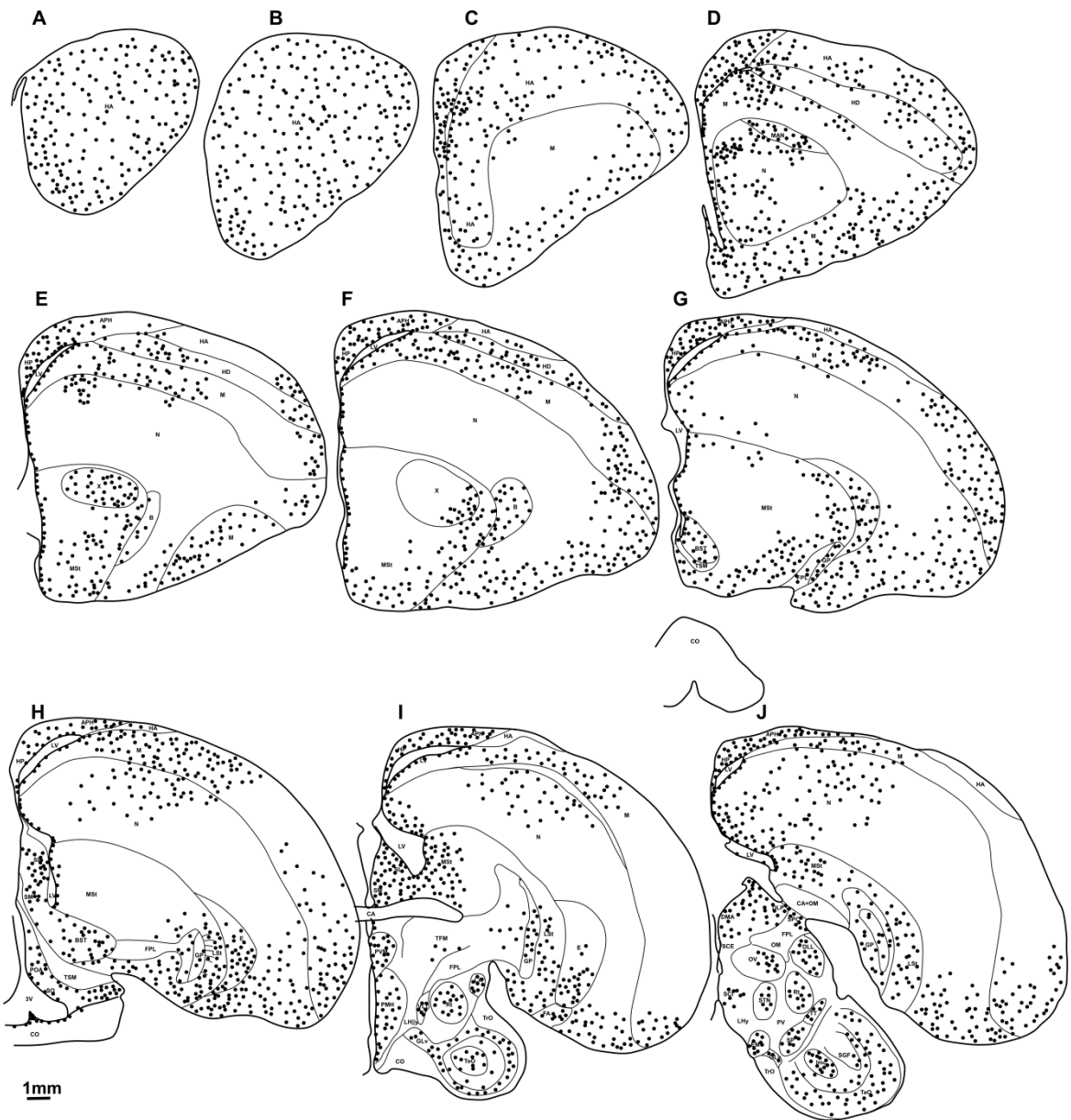
immunoreactive cells in the brains of the pied crow.

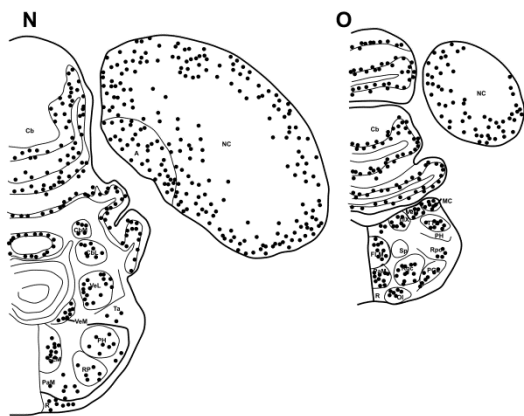
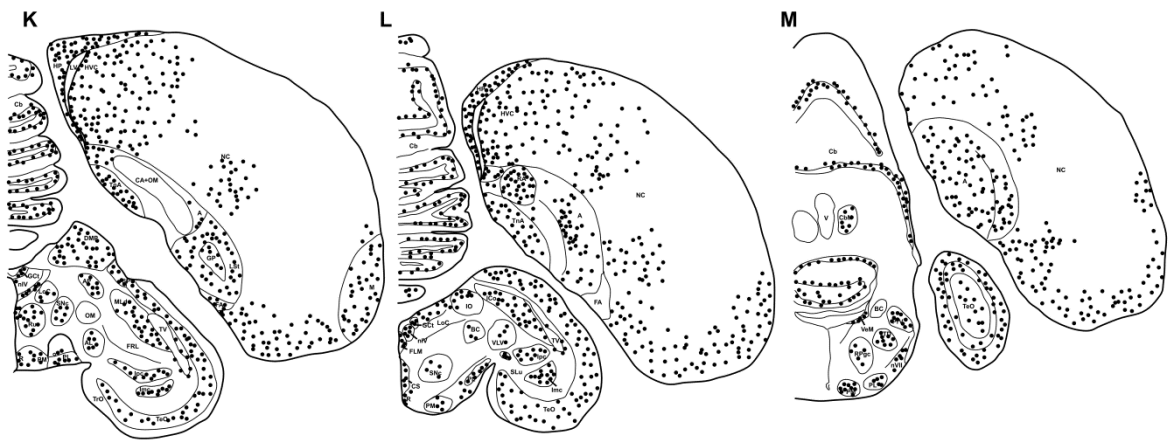
- absent, + low, ++ moderate, +++ high density of PCNA and DCX immunoreactive structures.

| BRAIN REGION    |                   | PCNA | DCX |
|-----------------|-------------------|------|-----|
| SVZ             |                   | +++  | +++ |
| Olfactory bulb  |                   | +++  | +++ |
| Telencephalon   | HP                | ++   | +   |
|                 | HA                | +++  | ++  |
|                 | HI                | ++   | ++  |
|                 | HD                | ++   | ++  |
|                 | Mesopallium       | ++   | ++  |
|                 | Nidopalium        | ++   | ++  |
|                 | Entopallim        | ++   | +   |
|                 | Arcopallium       | ++   | -   |
|                 | Striatum          | ++   | ++  |
|                 | Septum            | ++   | ++  |
| Diencephalon    |                   | ++   | +   |
| Mesencephalon   | Pretectum         | ++   | +   |
|                 | Tectum            | ++   | ++  |
| Rhombencephalon | Cerebellum        | ++   | +   |
|                 | Pons              | ++   | +   |
|                 | Medulla oblongata | ++   | +   |



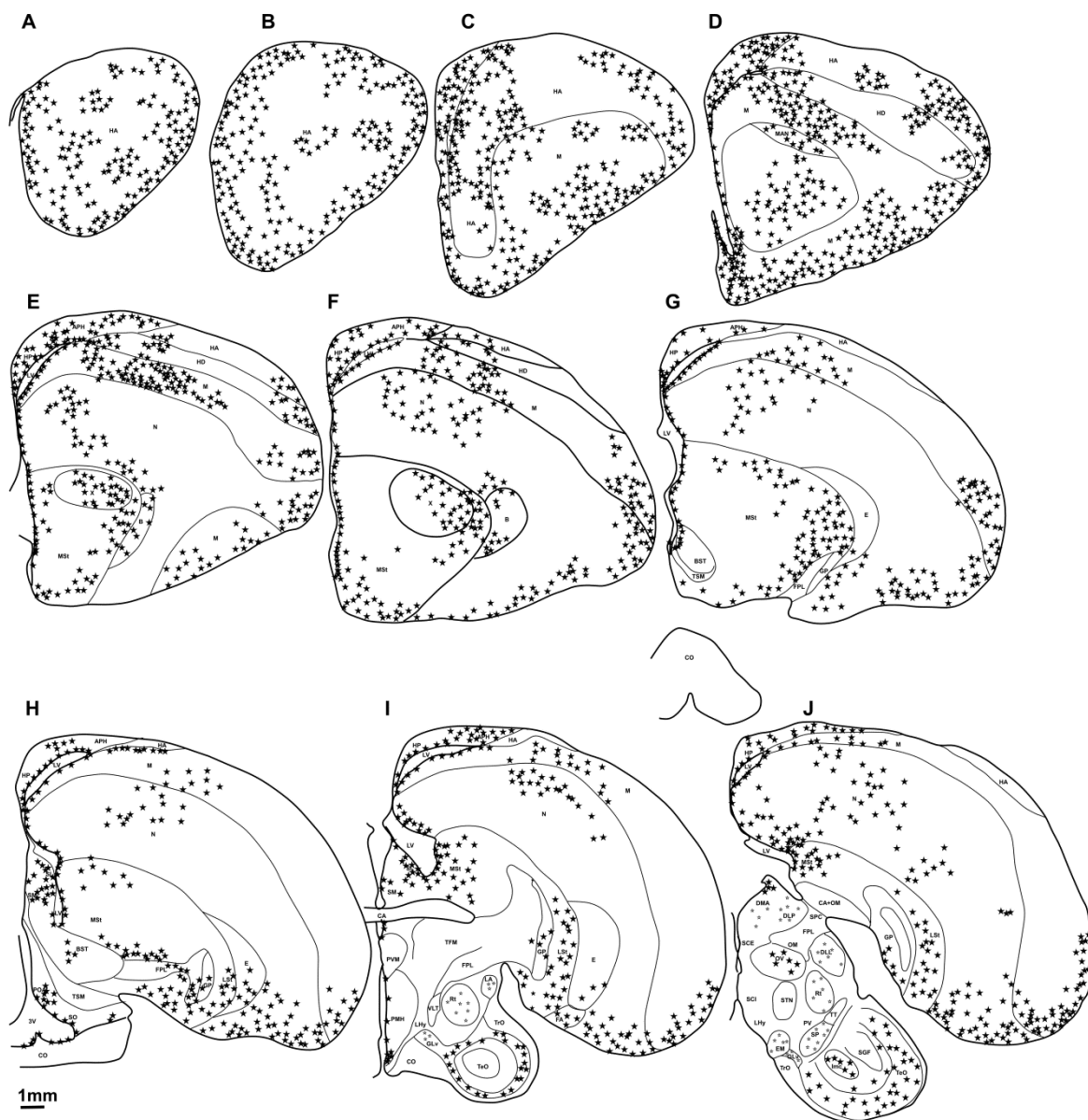
**Figure 5.1:** Diagrammatic reconstructions of coronal sections through one half of the brain of the pied crow (*Corvus albus*), illustrating the distribution of PCNA immunoreactive cells. A single black dot indicates a single immunolaballed cell. Drawing **A** represent the most rostral section, **O** the most caudal. Each drawing is approximately 1500 µm apart. See list for abbreviations.

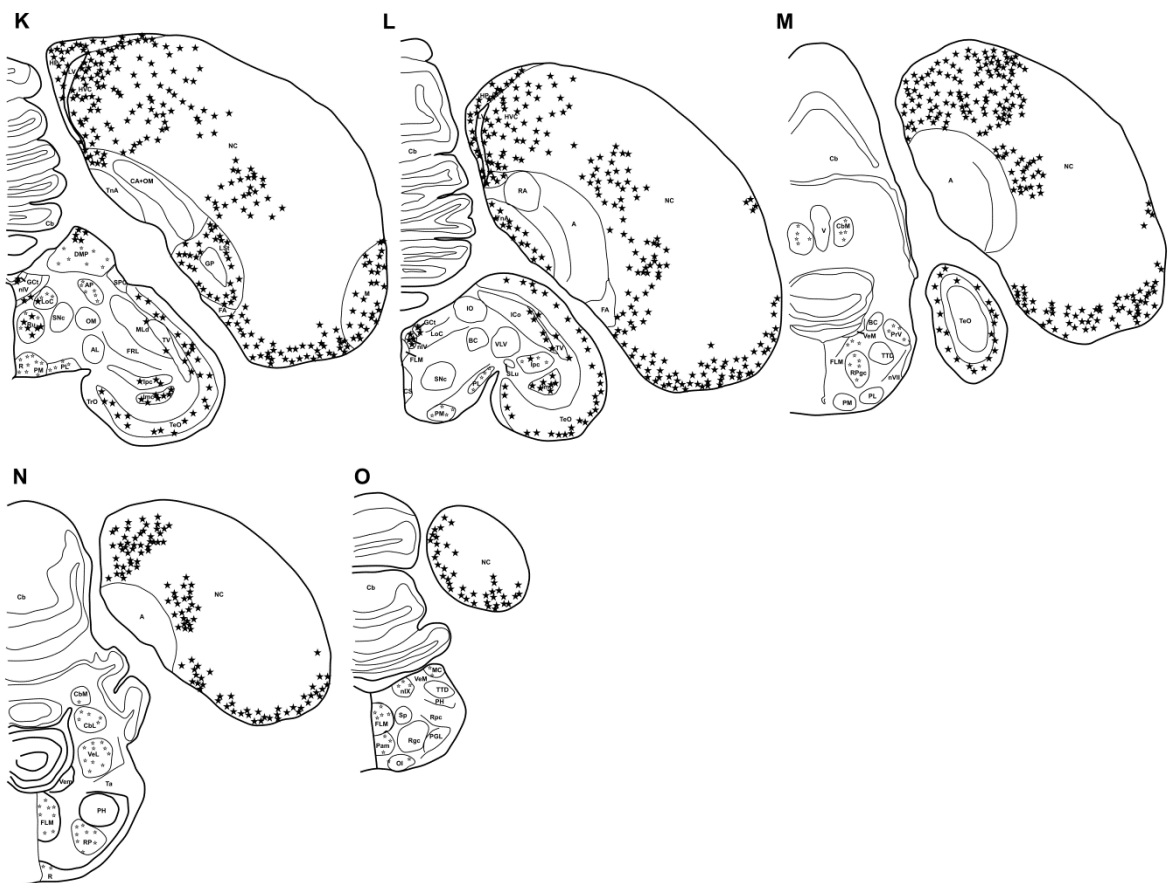




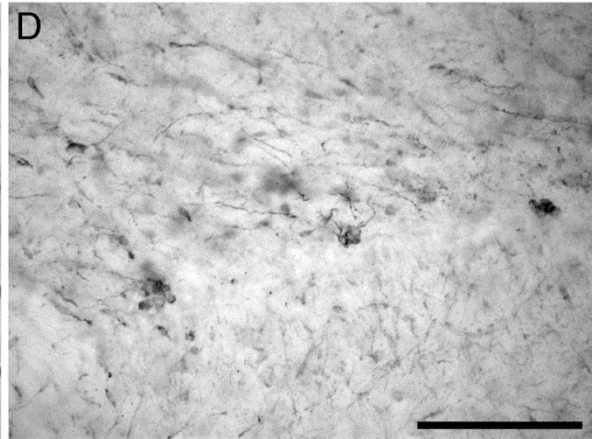
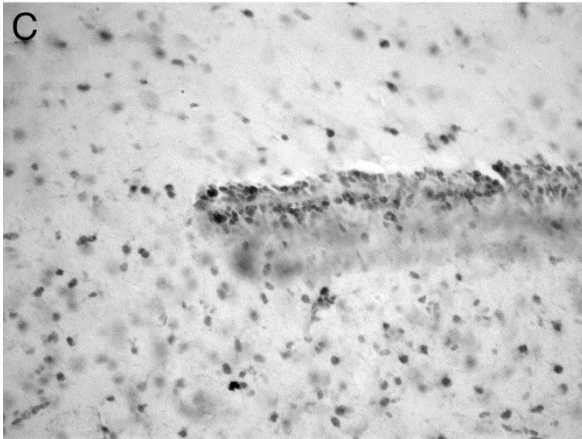
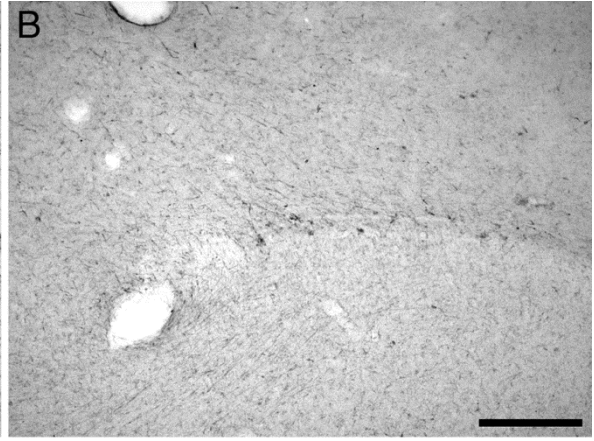
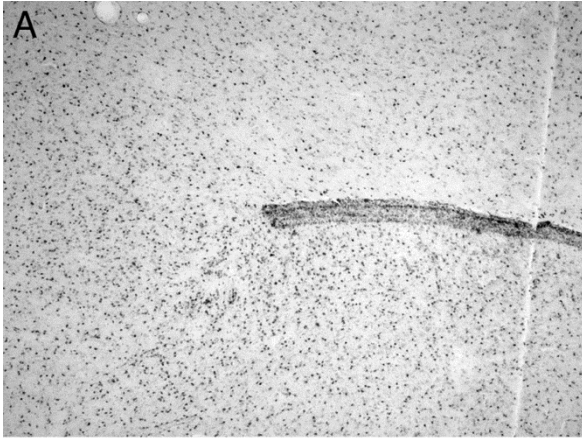
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**Figure 5.2:** Diagrammatic reconstructions of coronal sections through one half of the brain of the pied crow (*Corvus albus*), illustrating the distribution of DCX immunoreactive cells. A single solid black star indicates a single cell body and a single open star represents a weakly stained cell body. Drawing **A** represent the most rostral section, **O** the most caudal, with each drawing matching those in Figure 1. Each drawing is approximately 1500  $\mu\text{m}$  apart. See list for abbreviations.



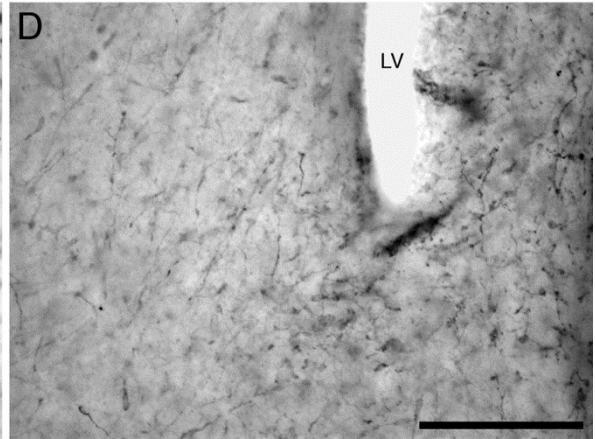
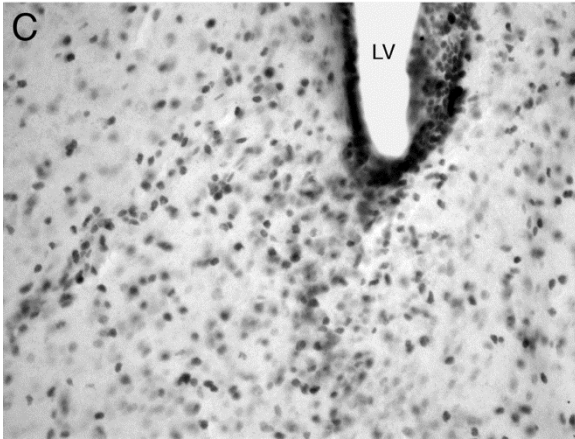
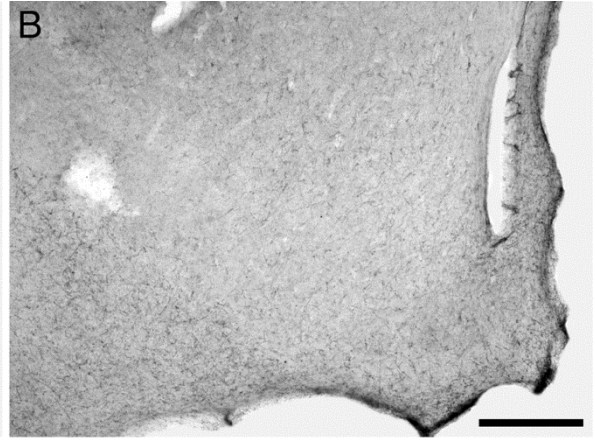
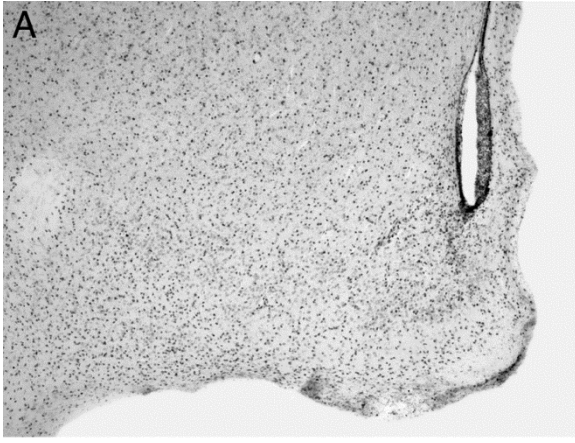


**Figure 5.3:** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the dorsal pole of the subventricular zone (**SVZ**) of the lateral ventricle in the brain of the pied crow (*Corvus albus*). PCNA immunoreactivity revealed aggregates of proliferating cells (hot spots) in the SVZ of the dorsal pole of the lateral ventricle (**A**). DCX immunoreactivity revealed aggregates of DCX immunoreactive cells and fibres in the SVZ of the dorsal pole of the lateral ventricle (**B**). (**C**) A high magnification image of the PCNA immunoreactive cells in the dorsal pole of the lateral ventricle. (**D**) A high magnification image of the DCX immunoreactive cells in the dorsal pole of the lateral ventricle. In all images dorsal is to the top and medial to the right. Scale bar in **B** = 250  $\mu\text{m}$  and applies to **A** and **B**. Scale bar in **D** = 100  $\mu\text{m}$  and applies to **C** and **D**.

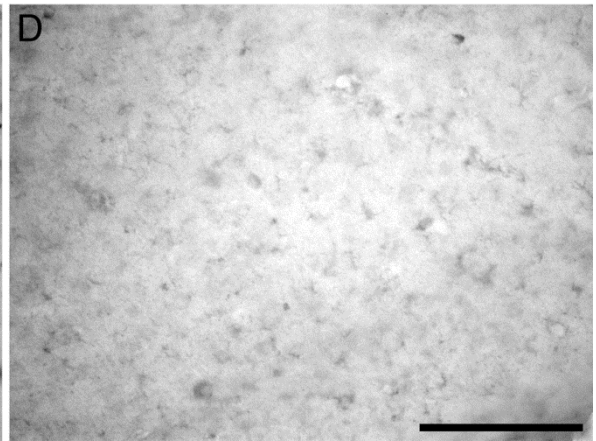
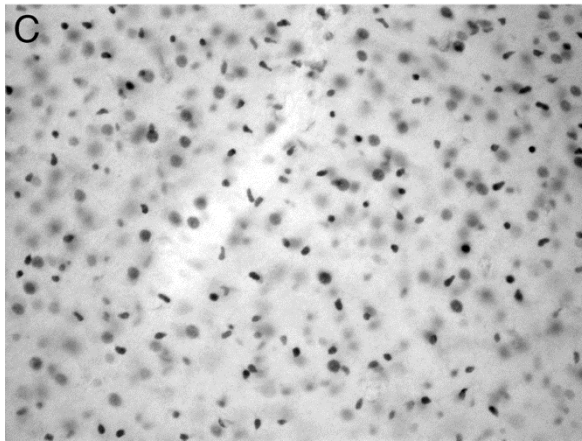
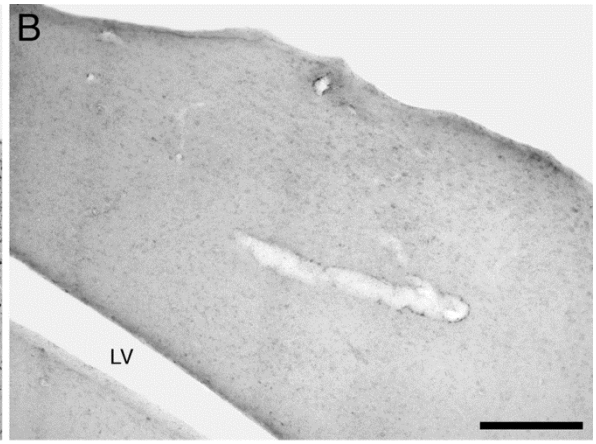
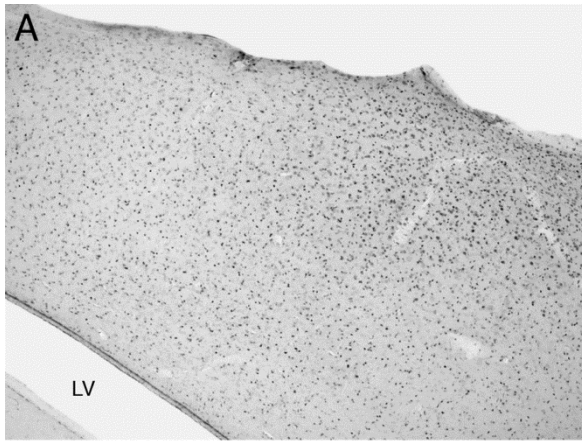




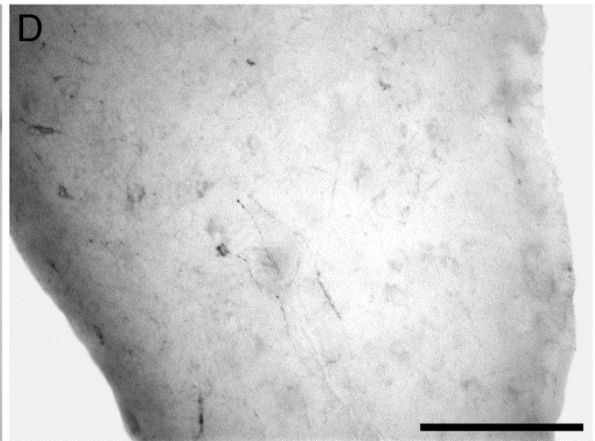
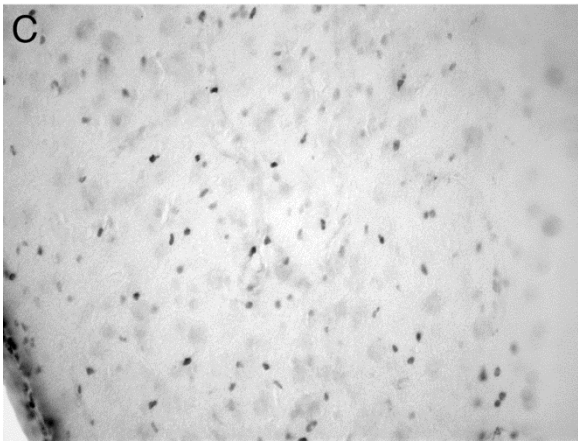
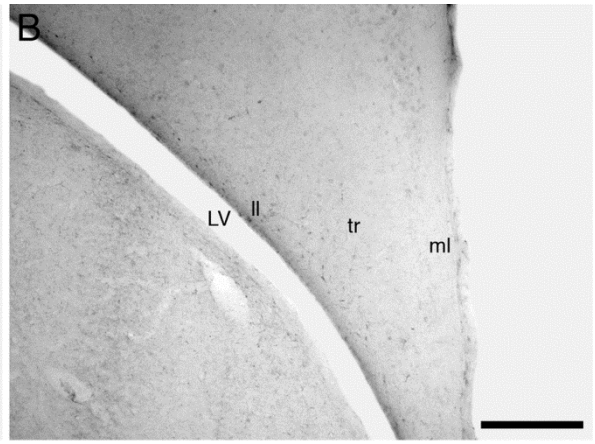
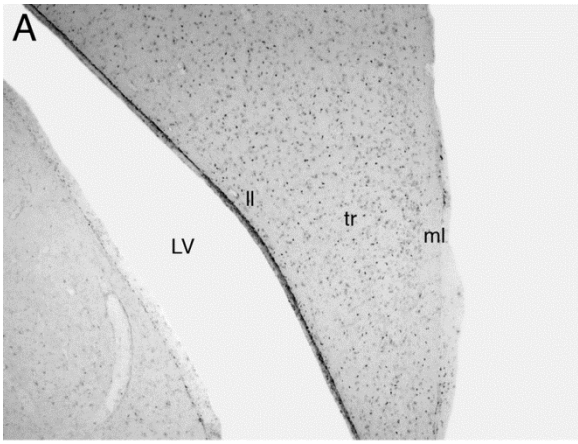
**Figure 5.4:** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the ventral pole of the subventricular zone (**SVZ**) of the lateral ventricle in the brain of the pied crow (*Corvus albus*). PCNA immunoreactivity revealed aggregates of proliferating cells (hot spots) in the SVZ of the ventral pole of the lateral ventricle (**A**). DCX immunoreactivity revealed aggregates of DCX immunoreactive cells and fibres in the SVZ of the ventral pole of the lateral ventricle (**B**). (**C**) A high magnification image of the PCNA immunoreactive cells in the ventral pole of the lateral ventricle. (**D**) A high magnification image of the DCX immunoreactive cells in the ventral pole of the lateral ventricle. In all images dorsal is to the top and medial to the right. Scale bar in **B** = 250  $\mu\text{m}$  and applies to **A** and **B**. Scale bar in **D** = 100  $\mu\text{m}$  and applies to **C** and **D**. **LV** – lateral ventricle.



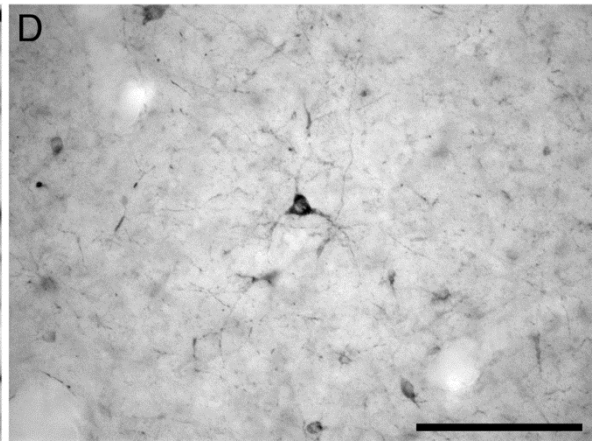
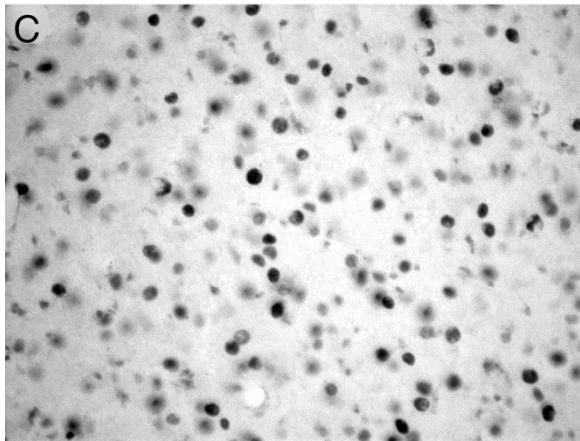
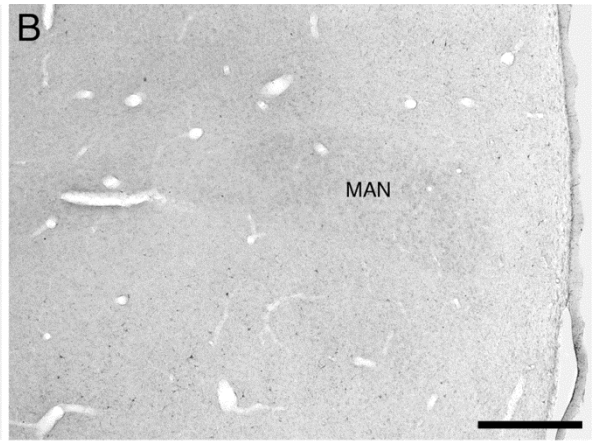
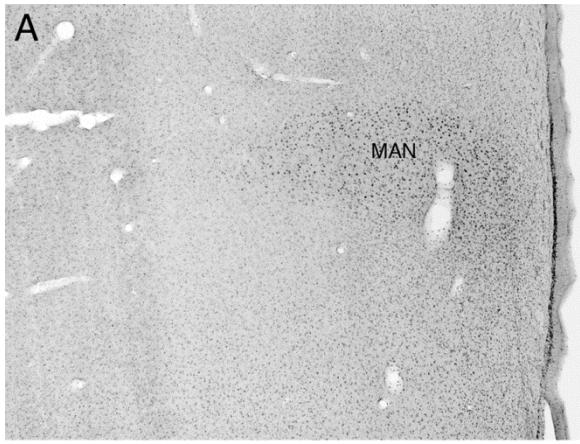
**Figure 5.5:** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the dorsal hippocampus in the brain of the pied crow (*Corvus albus*). (**A**) PCNA immunoreactive cells in the dorsal hippocampus. (**B**) DCX immunoreactive cells in the dorsal hippocampus. (**C**) A high magnification image of PCNA immunoreactive cells in the dorsal hippocampus. (**D**) A high magnification image of DCX immunoreactive cells in the dorsal hippocampus. Note the low density of DCX immunoreactive cells in the dorsal hippocampus (**B, D**). In all images dorsal is to the top and medial to the right. Scale bar in **B** = 250  $\mu\text{m}$  and applies to **A** and **B**. Scale bar in **D** = 100  $\mu\text{m}$  and applies to **C** and **D**. **LV** – lateral ventricle.



**Figure 5.6:** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the ventral hippocampus in the brain of the pied crow (*Corvus albus*). (**A**) PCNA immunoreactive cells in the ventral layers of the hippocampus. (**B**) DCX immunoreactive cells in the ventral layers of the hippocampus. (**C**) A high magnification image of the PCNA immunoreactive cells in the ventral layers of the hippocampus. (**D**) A high magnification image of the DCX immunoreactive cells in the ventral layers of the hippocampus. Note the density gradient of unipolar DCX immunoreactive cells in the ventral layers of hippocampus in (**B, D**). In all images dorsal is to the top and medial to the right. Scale bar in **B** = 250  $\mu\text{m}$  and applies to **A** and **B**. Scale bar in **D** = 100  $\mu\text{m}$  and applies to **C** and **D**. **LV** – lateral ventricle. See list for remaining abbreviations.

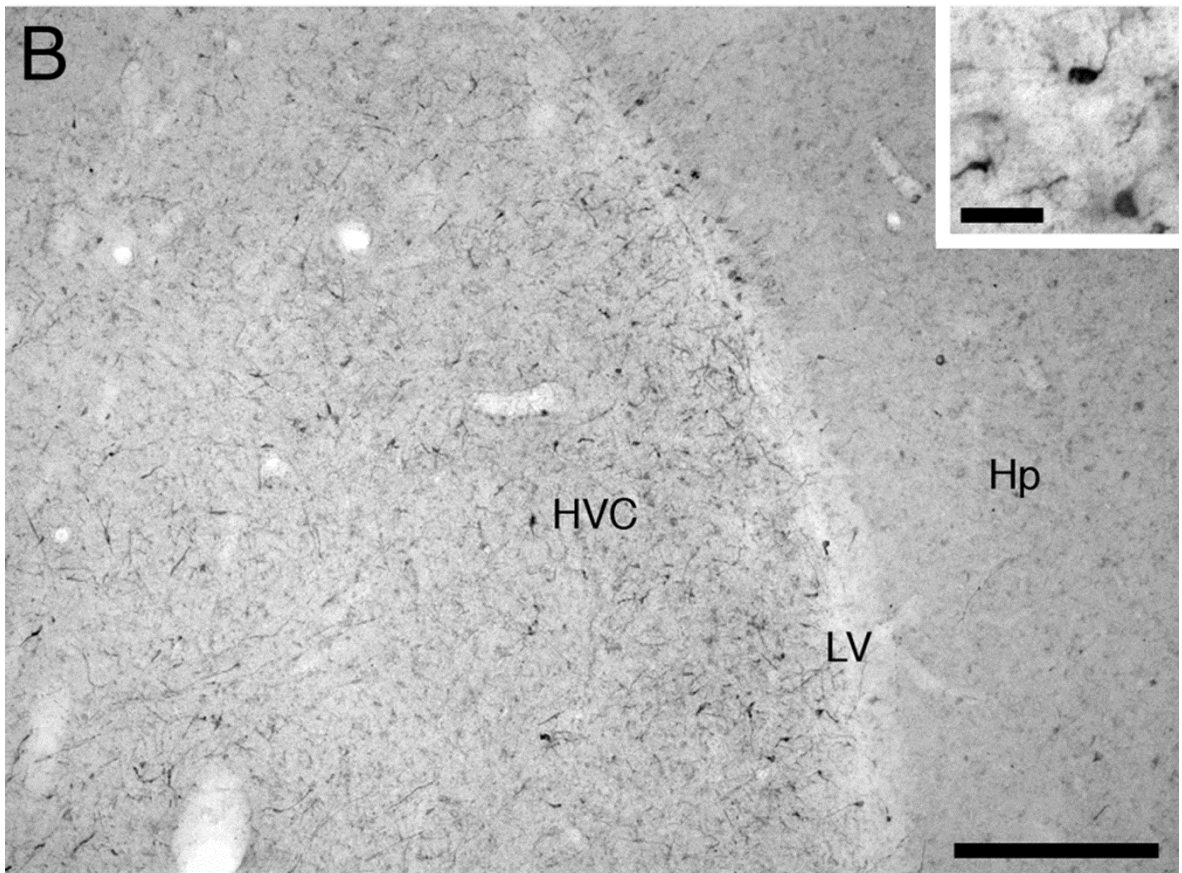
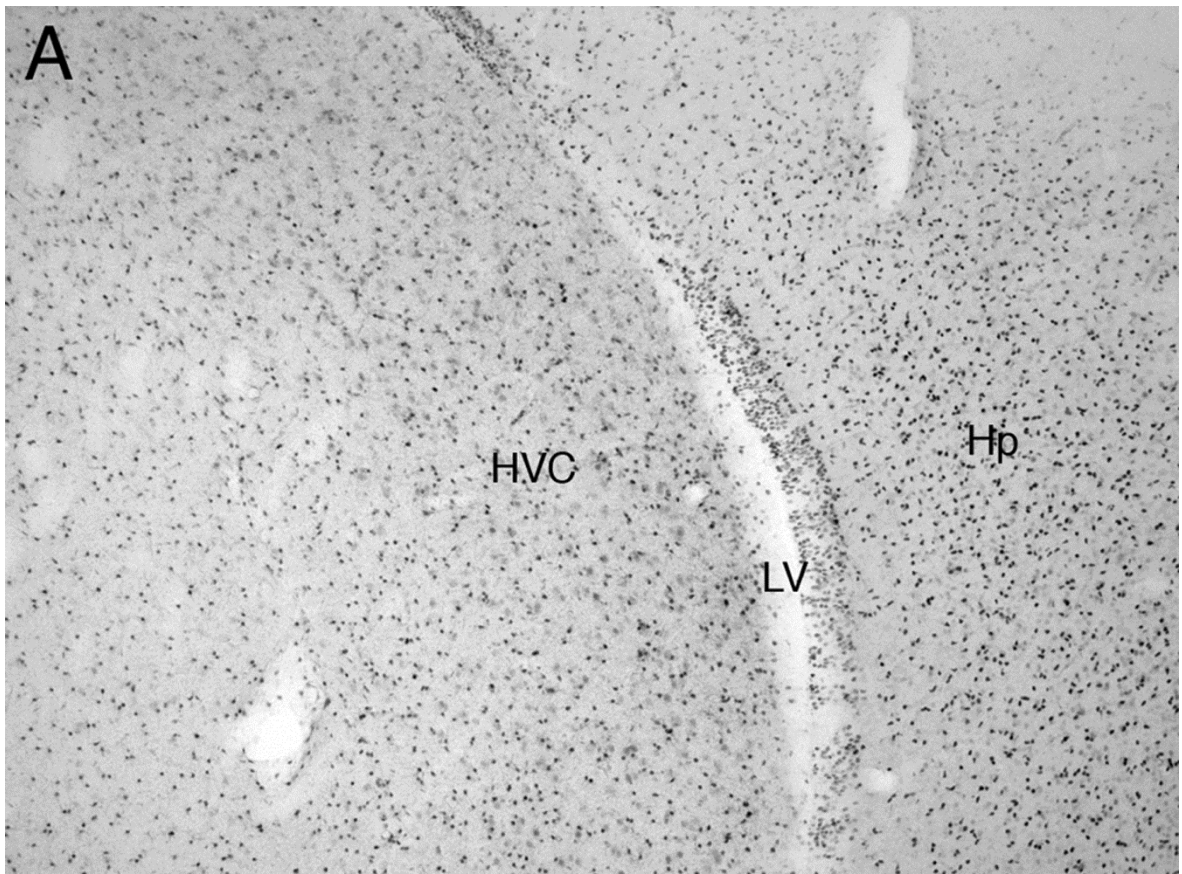


**Figure 5.7:** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the nucleus magnocellularis nidopallii anterioris (**MAN**) in the brain of the pied crow (*Corvus albus*). (**A**) PCNA immunoreactive cells in **MAN**. (**B**) DCX immunoreactive cells in **MAN** showing a very low density in the medial regions of the nucleus. (**C**) A high magnification image of the PCNA immunoreactive cells in **MAN**. (**D**) A high magnification image of the DCX immunoreactive cells in the lateral regions of **MAN**. Note the multipolar and unipolar DCX immunoreactive cells in the lateral region of **MAN** (**D**). In all images dorsal is to the top and medial to the right. Scale bar in **B** = 500  $\mu\text{m}$  and applies to **A** and **B**. Scale bar in **D** = 100  $\mu\text{m}$  and applies to **C** and **D**.

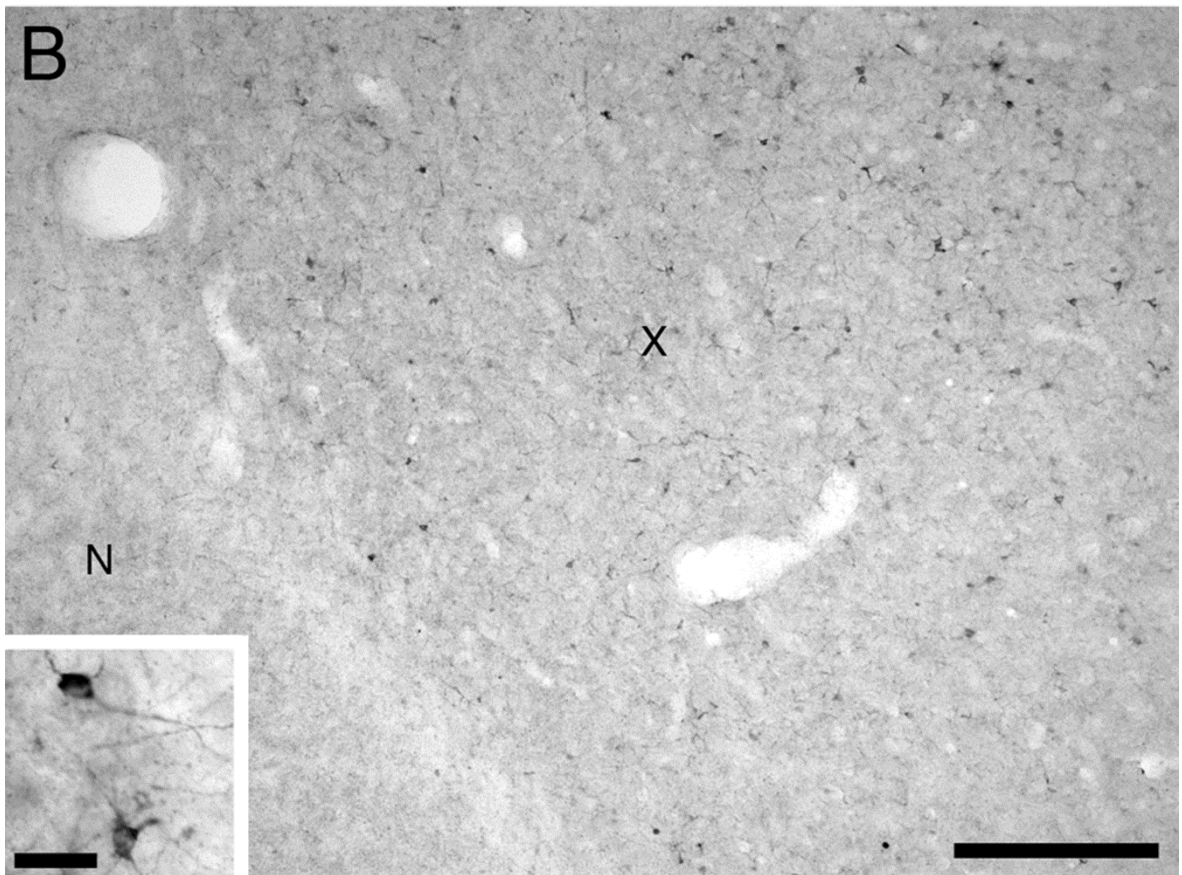
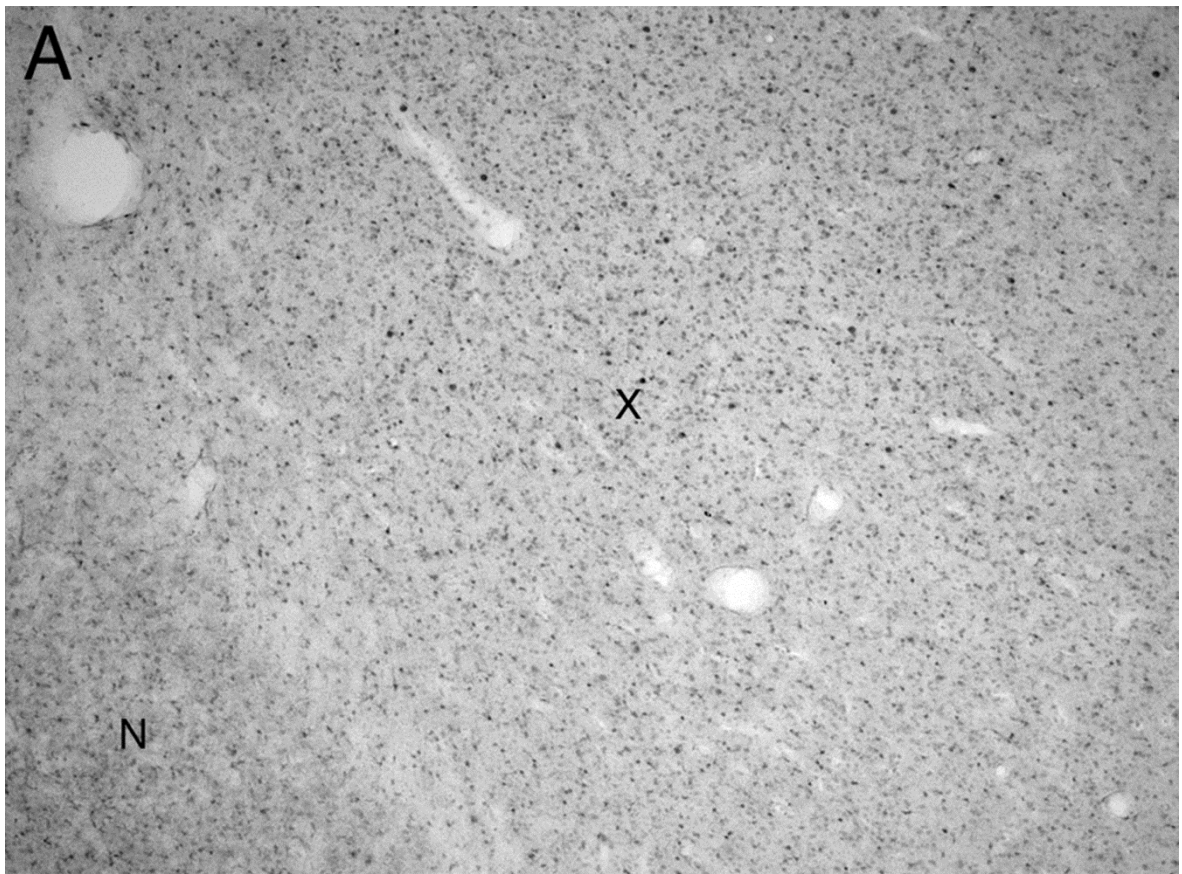




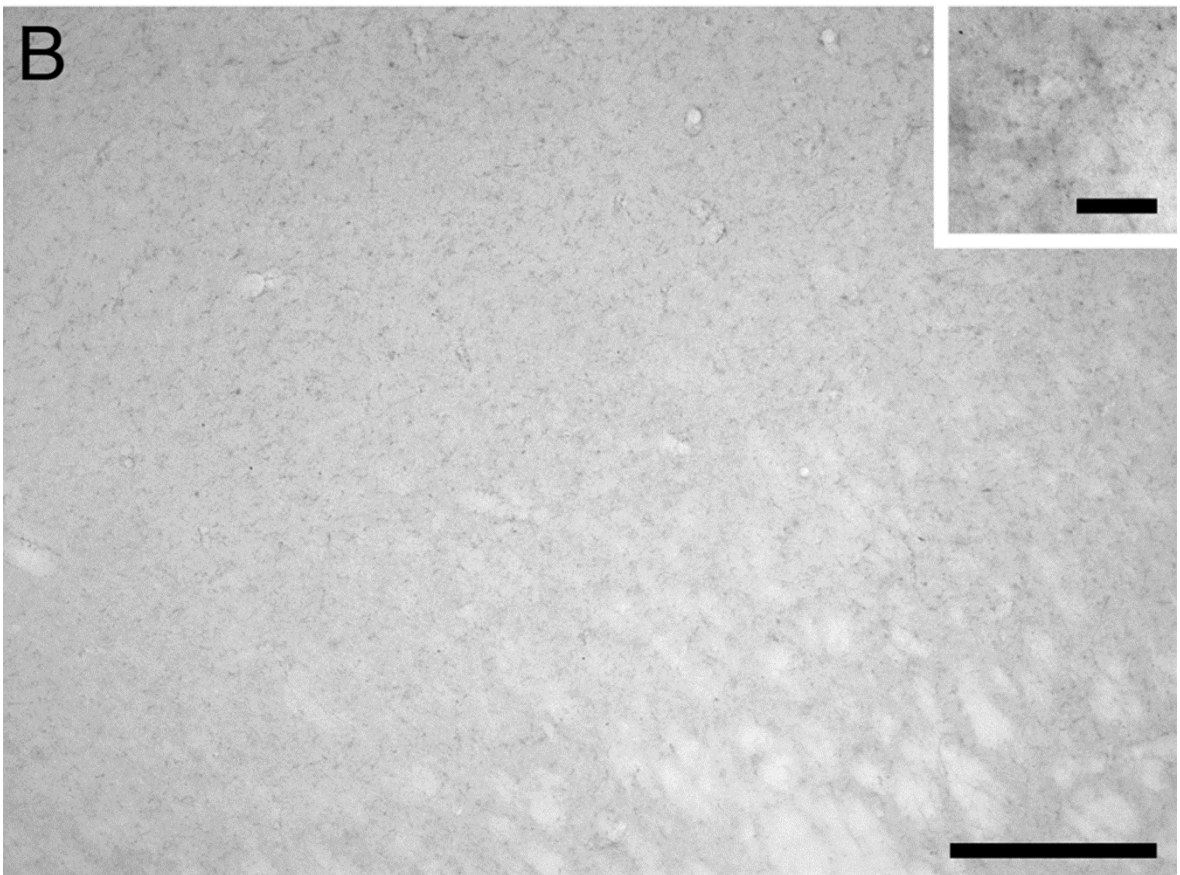
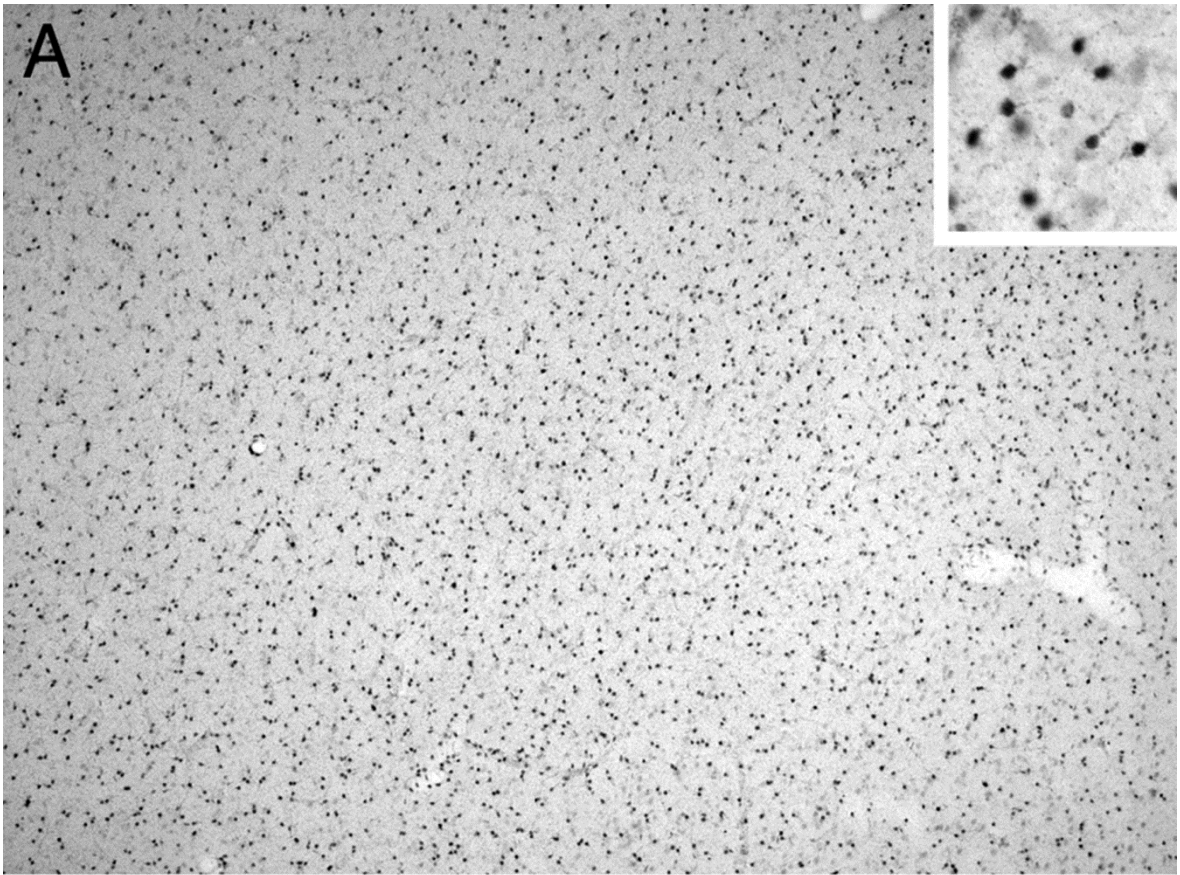
**Figure 5.8:** Photomicrographs of PCNA (**A**) and DCX (**B**) immunostained coronal sections through the high vocal center (**HVC**) in the brain of the pied crow (*Corvus albus*). (**A**) A high density of PCNA immunoreactive cells in the **HVC**. (**B**) A high density of DCX immunoreactive cells in the **HVC**. Inset in (**B**) shows a higher magnification of DCX immunoreactive cells showing their unipolar morphology. In all images dorsal is to the top and medial to the right. Scale bar in **B** = 250  $\mu\text{m}$  and applies to **A** and **B**. Scale bar in inset **B** = 25  $\mu\text{m}$  and applies to inset only. **Hp** – hippocampus, **LV** – lateral ventricle.



**Figure 5.9:** Photomicrographs of PCNA (**A**) and DCX (**B**) immunostained coronal sections through area X (**X**) in the brain of the pied crow (*Corvus albus*). (**A**) A high density of PCNA immunoreactive cells in area X. (**B**) A high density of DCX immunoreactive cells in area X. Inset in (**B**) shows a higher magnification of DCX immunoreactive cells showing multipolar morphologies. In all images dorsal is to the top and medial to the right. Scale bar in **B** = 250  $\mu\text{m}$  and applies to **A** and **B**. Scale bar in inset **B** = 25  $\mu\text{m}$  and applies to inset only. **N** – nidopallium.

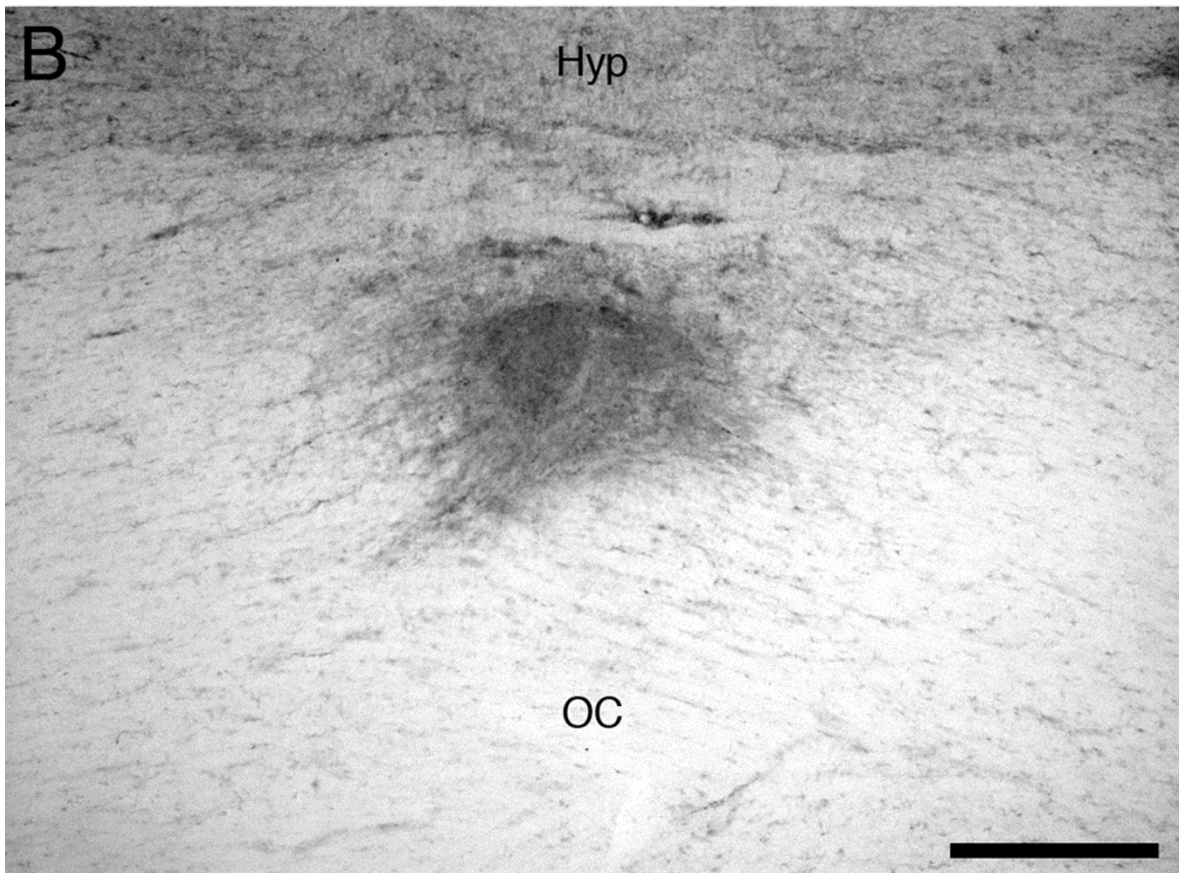
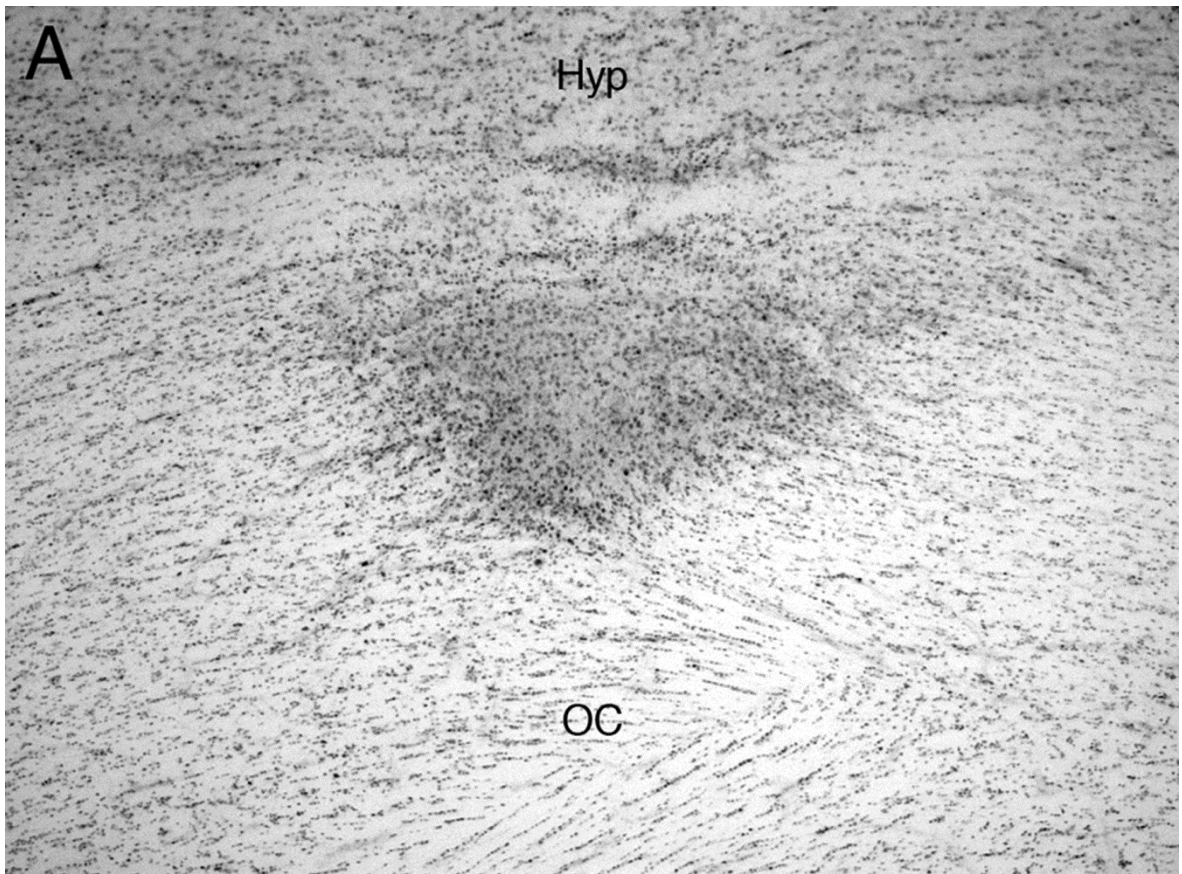


**Figure 5.10:** Photomicrographs of PCNA (**A**) and DCX (**B**) immunostained coronal sections through robust nucleus of the arcopallium (RA) in the brain of the pied crow (*Corvus albus*). (**A**) A high density of PCNA immunoreactive cells in RA. (**B**) A lack of DCX immunoreactivity in RA. Insets in (**A**) show a higher magnification of PCNA immunoreactive cells and (**B**) the absence of DCX immunoreactive fibres. In all images dorsal is to the top and medial to the right. Scale bar in **B** = 250  $\mu\text{m}$  and applies to **A** and **B**. Scale bar in inset **B** = 25  $\mu\text{m}$  and applies to both insets.



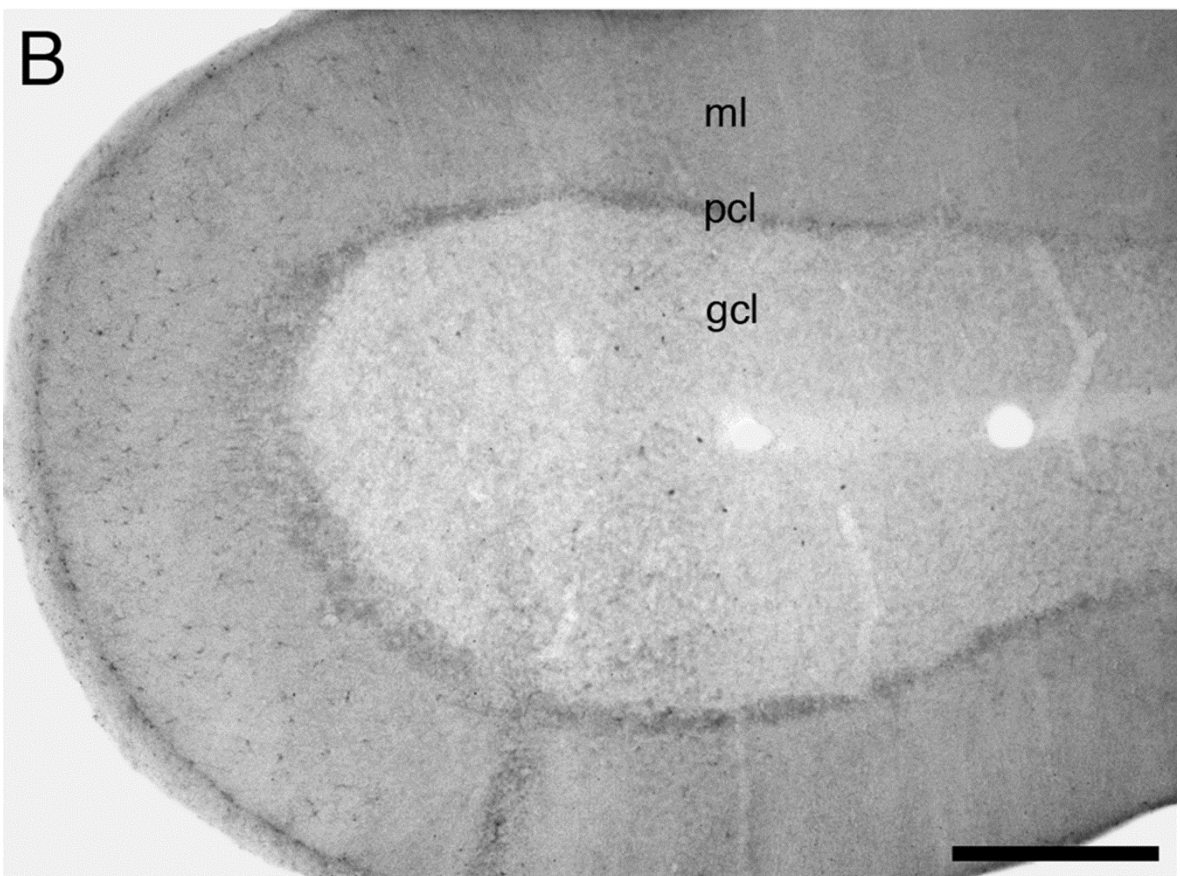
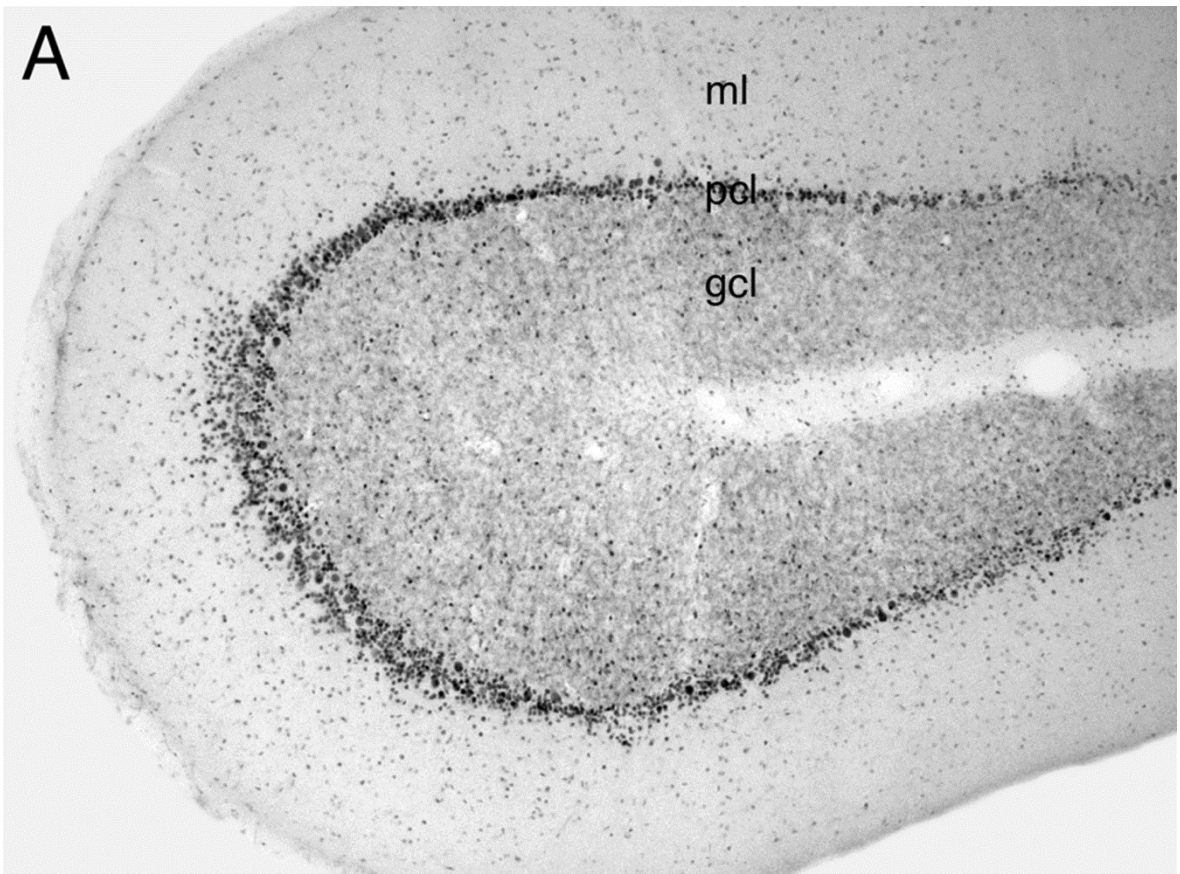
**Figure 5.11:** Photomicrographs of PCNA (**A**) and DCX (**B**) immunostained coronal sections through the optic chiasm (**OC**) ventral to the hypothalamus (**Hyp**) in the brain of the pied crow (*Corvus albus*). (**A**) Accumulation of PCNA immunoreactive cells in the optic chiasm. (**B**) Concentration of DCX immunoreactive fibres in the optic chiasm. These images depict a potential neurogenic zone at the midline of the optic chiasm. In both images the midline is the centre of the image and dorsal to the top of the image. Scale bar in **B** = 250  $\mu\text{m}$  and applies to both **A** and **B**.







**Figure 5.12:** Photomicrographs of PCNA (**A**) and DCX (**B**) immunostained coronal sections through the cerebellar cortex in the brain of the pied crow (*Corvus albus*). (**A**) PCNA immunoreactive cells in the layers of the cerebellar cortex. (**B**) DCX immunoreactive structures in the cerebellar cortex showing very weak to absent DCX immunostaining. Note large and intensely stained PCNA immunoreactive cells in the Purkinje cell layer (**pcl**). In all images dorsal is to the top and medial to the right. Scale bar in **B** = 250  $\mu$ m and applies to both **A** and **B**. **gcl** – granule cell layer, **ml** – molecular cell layer.



## **5.4. Discussion**

### **5.4.1. General observations**

In the current study we examined putative adult neurogenesis in the brain of the pied crow (*Corvus albus*). PCNA and DCX immunoreactivity was observed in all brain regions but to varying degrees. The results of the current study corroborates results from a similar study in domestic pigeons where distribution of the two markers were found to be widespread in the brain but in different proportions across brain regions (Mazenganya et al., 2017). The distribution patterns of these endogenous markers highlights the importance of brain plasticity in certain regions, in accord with the behavioural demands encountered by this species.

### **5.4.2. Proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) immunoreactive specificity**

The present study examined the distribution of PCNA and DCX in the brains of the pied crow (*Corvus albus*) using immunohistochemical techniques. PCNA is an endogenous marker for proliferating cells and its presence has been characterised using immunohistochemical techniques in the brains of developing parakeets and quails, as well as adult domestic pigeons (Charvet and Streidter, 2008; Mazenganya et al., 2017). DCX is a microtubule associated protein endogenously expressed in young and migrating neurons (Brown et al., 2003). In birds, the presence and distribution of DCX immunolabelling has been characterised in both song birds (e.g. Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014) and non-song birds (e.g. Capes-Davis et al., 2005; Mezey et al., 2012; Melleu et al., 2013; Mazenganya et al., 2017).

PCNA immunoreactive cells were observed throughout the brains of the pied crows, although the presence and density of labelled cells varied. The subdivisions of the telencephalon contained the highest density of PCNA immunoreactive cells, particularly in the medial and lateral regions, when compared to the subtelencephalic regions (diencephalon,

mesencephalon and rhombencephalon). The PCNA staining was conspicuous in the various regions and nuclei of the subtelenencephalon. Similar observations were made regarding the distribution of proliferating cells in the domestic pigeon using PCNA immunohistochemistry (Mazenganya et al., 2017), and with other markers of proliferative cells (bromodiuridine, BrdU, and [ $^3\text{H}$ ] thymidine) in canaries (*Serinus canaria*) and ring doves (*Streptopilia risoria*) (Ling et al., 1997; Vellema et al., 2010). Given the similarities in the distribution and densities of proliferating cells across species and with different techniques, it is reasonable to conclude that the findings present herein for the pied crow are a true reflection of these cells in this species.

DCX immunoreactivity was widespread, but heterogeneously expressed in the brain of the pied crows, and showed substantial overlap with the distribution of PCNA immunoreactive cells. DCX immunoreactive structures were observed in high density, with intense labelling, in the olfactory bulb (OB) and the subdivisions of the telencephalon, whereas in the subtelencephalic regions both the density and intensity of DCX labelled structures was lower. These observations are in agreement with previous similar studies in song birds including canaries (Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014), zebra finches (Kim et al., 2006), and starlings (Absil et al., 2003), and in non-song birds such as chickens (Capes-Davis et al., 2005; Mezey et al., 2012) and pigeons (Melleu et al., 2013; Mazenganya et al., 2017). However, these results are at variance with other previous studies that reported DCX immunoreactive structure being found only in the telencephalon of adult birds (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1994; Barnea and Nottebohm, 1994; Absil et al., 2003). This discrepancy may result from different methodological approaches, such as different antibodies being used or the use of older animals. The expression of the DCX antigen is correlated with age, and available evidence indicates that DCX expression decreases with increased age in both mammals and birds (Amrein et al., 2004, 2011; Kim et al., 2006; Knoth et al., 2010). Thus, while there are

some potential methodological caveats needed in the interpretation of the results of DCX expression in the brain of the pied crow, the similarity of distribution of this marker to that seen in many other studies of adult birds and the extensive overlap with PCNA immunoreactive cells, indicate that the reported DCX expression and distribution within the brain of the pied crow is likely to be accurate for this species.

#### **5.4.3. Cell proliferation in the brain of adult pied crows – hot spots**

Proliferative cells have been observed throughout the brains of adult birds, including in the pied crows studied herein. While the olfactory bulbs and the telencephalon of the pied crows contained the highest density of proliferative cells, they were also observed in all subtelencephalic regions, albeit heterogeneously. In the pied crow, high density aggregates of proliferating cells were observed in the subventricular zones of the dorsal and ventral poles of the lateral ventricles at rostral levels of the brain. These aggregates are consistent with proliferative hot spots described by Alvarez-Buylla et al. (1990). Several studies in birds have reported the presence of these proliferative hot spots, but their locations along the rostro-caudal axis of the lateral ventricles has been seen to vary. Hot spots have been reported rostrally (Vellema et al., 2010; Mazenganya et al., 2017), at middle levels of the ventricles (Alvarez-Buylla et al., 1990; Kim et al., 2006), and caudally (Melleu et al., 2013). In some species the identification of hot spots has been uncertain, or they are considered to be absent (e.g. Ling et al., 1997; Boseret et al., 2007; Balthazart et al., 2008). This variation in hot spot presence and location appears to reflect a species-specific or phylogenetic group-specific, trait. For example in Columbiformes, hot spots were identified in the dorsal and ventral poles of the lateral ventricle in rock and domestic pigeons (Melleu et al., 2013; Mazenganya et al., 2017), but were less pronounced or absent in the closely related ring doves (Ling et al., 1997). It is likely that further studies in other avian species will describe further variation in the

presence and location of these proliferative hot spots, or neurogenic zones, within the avian telencephalon.

Cell proliferation not associated with the proliferative hot spots, was observed in the subventricular zones of the third, fourth and tectal ventricles and the telencephalic parenchyma at low densities, but the morphology of these cells was similar to those near the hot spots (Vellema et al., 2010). Ling et al. (1997) observed proliferating cells in the telencephalic parenchyma but did not observe any [ $^3\text{H}$ ] thymidine activity in the subventricular zones third, fourth and tectal ventricles. These findings suggest the presence of additional proliferative or neurogenic sites in addition to the subventricular zones. Paredes et al. (2016) have suggested that animals with large brains have neurogenic or proliferative zones in addition to the subventricular and subgranular zones, indicating that these additional zones may increase the potential for brain plasticity in larger brained species. This concept is somewhat supported by the observations made in the current study of the pied crow, which does have a large (both absolutely and relative to body mass) brain for an avian species.

Apart from the subventricular zones, the identity and fate of proliferating cells in the parenchyma of other brain regions remains elusive. Lineage studies, utilising double immunolabelling may help to reveal the identity and subsequent fate of these cells. In a study conducted in canaries, 93% of BrdU positive cells, including those in the parenchyma, co-expressed Hu protein, which labels mature neurones for 38 days after BrdU injection, suggesting that the majority of dividing cells in the adult telencephalon are neurones (Vellema et al., 2010). However in the subtelencephalic regions, in the same study, BrdU positive cells did not co-express Hu protein, indicating that the cells were of the glial and or endothelial lineage. On the contrary, Ling et al. (1997) found that [ $^3\text{H}$ ] thymidine positive cells in the brain parenchyma of the telencephalon had a very small nucleus compared to those in the subventricular zone (SVZ) of the lateral ventricle, and that the cells did not co-express neuron

specific antibodies such as anti-microtubule associated protein-2 (MAP-2) and anti-neuronal nucleus (NeuN), indicating that they were not neurons.

#### **5.4.4. Neuronal recruitment in the brain of adult pied crows**

Doublecortin (DCX) immunoreactivity in the brains of adult birds is thought to represent the possibility of neuronal recruitment to circuitries in the regions where this is observed (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008). The heterogeneous distribution and density of DCX immunolabelled structures in the brain of the pied crow described herein, is similar to almost all previous studies of DCX expression of the brains of adult birds (e.g. Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Mezey et al., 2012; Vellema et al., 2014; Mazenganya et al., 2017). The olfactory bulbs have been shown to contain a high density of DCX immunoreactive structures in birds (Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013, 2015; Mazenganya et al., 2017), but the origin of these neurons in the olfactory bulbs of adult birds is still uncertain. In adult mammals, olfactory bulb neurons migrate from the subventricular zones through the rostral migratory stream (e.g. Ihunwo et al., 2016). In contrast, few studies in birds indicate the presence of a rostral migratory stream (e.g. Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013), suggesting that in birds, including the pied crow, adult olfactory bulb neurons are generated locally and then assimilated, or recruited, into the local circuitry.

Another region of the pied crow brain that evinced a high density of DCX immunoreactive structures was the subventricular zone (SVZ) of the lateral ventricles throughout their rostro-caudal axis. Aggregates of DCX immunoreactive cells and fibres were observed at rostral levels, in the dorsal and ventral poles of the lateral ventricles adjacent to the hyperpallium and the medial striatum respectively, indicating the presence of neurogenic hot spots as described in other birds and considered a primary neurogenic niche in the avian brain (Alvarez-Buylla et al., 1988, 1990; Alvarez-Buylla, 1990). Neuroblasts from the dorsal

hot spot migrate into the hyperpallium, whereas those from the ventral hot spot migrate into the striatum (Alvarez-Buylla, 1990). The definitive routes of migration of neuroblasts in birds are yet to be understood. A few studies have described the migration of neuroblasts from the ventral hot spot into the olfactory bulb, through the rostral migratory stream, and their contribution into the olfactory circuitry (Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013; Vellema et al., 2014). Migrating neuroblasts from the SVZ into the telencephalic parenchyma utilise both radial and random migration, where in radial migration immature neurons utilise radial scaffolds to travel short distances into the parenchyma, whereas in random migration the migrating neuroblasts use unspecified modes and routes to penetrate the brain tissue (Alvarez-Buylla, 1990; Vellema et al., 2014). In this sense the pied crow is similar to many other species of birds previously studied.

In the pied crows studied herein, the hippocampus and area parahippocampalis (HP and APH) showed a very low density of DCX immunoreactive structures. Previous studies have reported variation in neuronal recruitment levels in the adult avian hippocampal complex, from absent in the canaries and starlings (Alvarez-Buylla et al., 1994; Absil et al., 2003; Hoshooley and Sherry, 2007; Vellema et al., 2010), low rates in pigeons and canaries (Melleu et al., 2013; Vellema et al., 2014), moderate rates in finches and canaries (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008) and high rates in marsh tits (Patel et al., 1997) and food caching birds such as the black capped chickadee (La Dage et al., 2010; Sherry and Hoshooley, 2010). Hippocampal neurogenesis in food caching birds was associated with spatial memory dependant tasks (Sherry and Hoshooley, 2009, 2010; La Dage et al., 2010; Chancellor et al., 2011) and showed seasonal variability (Balthazart et al., 2010; Barnea and Pravosudov, 2011). Information on food caching in pied crows is scarce, but the majority of the members in the corvids family demonstrate food caching and retrieving behaviours (Emery and Clayton, 2004).



DCX immunoreactivity in the telencephalic mesopallium (M) and nidopallium (N) was variable in the pied crow, with a high density of immunoreactive structures in the song control nuclei MAN and HVC, and the caudal nidopallium (NC), while the remaining regions contained a moderate density of DCX immunoreactive structures. In contrast to studies in canaries (Vellema et al., 2010), in the pied crow the MAN and HVC nuclei showed pronounced DCX immunoreactivity compared to the surrounding regions, suggesting that these areas are specifically targeted by migrating neurons. Generally in the pied crow, DCX immunoreactive structures were found in higher density in the lateral regions of the mesopallium (M) and nidopallium (N), were less dense in the medial regions, and were absent to very low densities in the core regions. A similar pattern of DCX immunopositive structure densities was observed in pigeons, chickens, canaries and zebra finches (e.g. Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Mezey et al., 2012; Vellema et al., 2014; Mazenganya et al., 2017). In the majority of studies on adult neurogenesis in birds, the nidopallium caudale (NC) was found to be highly neurogenic (Barnea and Pravosudov, 2011), a region thought to be analogous to the mammalian prefrontal cortex (Güntürkün, 2005).

In the pied crow the subpallial structures, including the medial and lateral striatum (MSt and LSt) and area X, showed a moderate density of DCX immunoreactive structures, while the globus pallidus (GP), nucleus basorostralis pallii (B), entopallium (E), nucleus taeniae of the amygdala (TnA) and the lateral septum (SL) contained a low density of DCX immunoreactive structures, while the medial septum (SM), arcopallium (A) and the robust nucleus of the arcopallium (RA) showed no DCX immunoreactivity. These findings are in agreement with previous studies in both song and non-song birds (Boseret et al., 2007; Balthazart et al., 2008; Mezey et al., 2012; Mazenganya et al., 2017).

In the subtelencephalic regions of the avian brain, numerous studies, including the current study on pied crows, have observed low densities of DCX immunoreactive structures in a few selected regions and nuclei of the diencephalon, mesencephalon and

rhombencephalon (Boseret et al., 2007; Balthazart et al., 2008; Mezey et al., 2012; Melleu et al., 2013; Mazenganya et al., 2017). The characteristic staining of DCX immunoreactive cells was not observed in these regions, including in the pied crow. Instead punctate-like staining on synaptic vesicles and very few cells in the medial hypothalamus, ventral tegmental area of the midbrain and some selected brainstem nuclei showed DCX immunoreactivity. In addition no DCX immunoreactivity was observed in the cerebellar cortex. Studies in canaries (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1994) and a study in chickens (Mezey et al., 2012) did not observe DCX immunoreactive structures in the subtelencephalon, but it was reported to be present in a low density in other studies in canaries (Kim et al., 2006; Boseret et al., 2007; Vellema et al., 2014) and in moderate density in a study in chickens (Capes-Davis et al., 2005). Such variations may indicate the importance of adult neurogenesis in brain plasticity in some species, particularly in relation to environmental adaptation for some species compared to others. For example, altricial birds which are hatched with an immature brain and require lengthy periods in the nursery will show protracted adult neurogenesis in the subtelencephalic structures compared to precocious birds which are hatched with almost a mature brain (Charvet and Striedter, 2008, 2009).

#### **5.4.5. Possible behavioural implications of adult neurogenesis in the pied crow**

The distribution of the endogenous markers PCNA and DCX in the brain of the pied crow showed extensive similarities to other birds, particularly song birds, where significant densities of immunoreactivity structures to both markers was observed in the song control nuclei. The observation of both PCNA and DCX immunoreactivity in the song control nuclei of the pied crow indicates that crows are song birds, although their song is not as elaborate as other Passerineformes (Wang et al., 2009). Adult neurogenesis in these nuclei is associated with the plastic changes necessary for song acquisition and maintenance (Alvarez-Buylla et al., 1994). However, the family of corvids is well-known for food caching and retrieval

behaviours (Emery and Clayton, 2004), a spatial memory dependant task which relies on hippocampal plasticity (Hoshooley and Sherry, 2007; La Dage et al., 2010, 2011). In the current study we observed very low levels of DCX immunoreactivity in the hippocampus of pied crows and this may suggest that behavioural plasticity in these birds does not correspond with neuronal and synaptic plasticity in this region. In addition, recruitment of neurons in the hippocampus has been shown to be influenced by seasonal changes and environmental complexities (Barnea and Nottebohm, 1994; Barnea and Pravosudov, 2011). The low levels of neuronal recruitment in the hippocampus of pied crow in the current study may indicate that the experimental animals were sacrificed during late spring, a time of the year when food caching and retrieval behaviour is likely to not have been necessary. Another neurogenic region associated with behavioural plasticity in birds is the olfactory bulb. The olfactory bulbs in the pied crow are comparably small, but in the current study we observed a high density of both PCNA and DCX immunoreactive structures, indicating high levels of adult neurogenesis and plasticity. The olfactory sense in birds has been associated with migratory, reproduction, parenting and feeding behaviours (Balthazart and Taziaux, 2009), and the time of year when these crows were sacrificed coincides with the period when their chicks were likely to be fledglings. In conclusion, our findings were comparable to the majority of previous studies on adult neurogenesis in birds. The results also confirm that pied crows possess song control systems that are neurogenic, although their song is not elaborate compared to many other song birds. We did not find anything specific that might suggest that the well-documented behavioural complexity of a range of corvid species is strongly reliant upon the process of adult neurogenesis.

## **CHAPTER SIX: Adult neurogenesis in the paleognathous birds: the common ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*)**

### **6.1. Introduction**

Adult neurogenesis encompasses the birth, migration and maturation of new neurons that subsequently integrate into existing circuitry by adding or replacing worn out neurons under normal physiological or pathological conditions (Lindsey and Tropepe, 2006). The process of adult neurogenesis has been reported in varying magnitudes in different species from different orders and classes ranging from invertebrates to vertebrates including humans (Cayre et al., 2002; Lindsey and Tropepe, 2006; Barker et al., 2011; Barnea and Pravosudov, 2011; Ihunwo et al., 2016). Adult neurogenesis is profuse in the brains of invertebrates, comparably less in non-mammalian vertebrates and least in mammals (Cayre et al., 2002; Chapouton et al., 2007; Kaslin et al., 2008). The abundance of adult neurogenesis in phylogenetically older vertebrates has been associated with high regenerative capabilities, that is, species such as the salamanders which can regenerate whole limb segments exhibit wide spread adult neurogenesis in the central nervous system including the spinal cord (Kaslin et al., 2008). Similarly, adult neurogenesis is wide spread in the brain of species of the teleost fishes which exhibit continuous body and brain growth throughout life (Zupanc and Horschke, 1995; Kaslin et al., 2008). However in birds and mammals with low regenerative capabilities, adult neurogenesis is restricted to the telencephalon although it is more wide spread in the former than the latter (Kaslin et al., 2008; Barnea and Pravosudov, 2011). In mammals, adult neurogenesis occurs in two canonical regions, the subventricular zone (SVZ) of the lateral ventricle and subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Bartkowska et al., 2010; Ihunwo et al., 2016). However, other potential neurogenic areas have been reported in the mammalian brain particularly under ischemic and pathological conditions (for a review, see Kaslin et al., 2008). In birds, adult neurogenesis is widespread in

the telencephalon and is well described in the song control systems of song birds (for a review, see Barnea and Pravosudov, 2011). In addition, it has been discovered also in the telencephalon of non-song birds and other brain regions in both song and non-song birds (e.g. Boseret et al., 2007; Barnea and Pravosudov, 2011; Melleu et al., 2013). Although adult neurogenesis has been reported to vary in topography and extent in various species from different taxonomic orders, its basic mechanisms appear to be conserved across the phylogenetic tree (Cayre et al., 2002). It is universally regulated by various ecological pressures including environment, seasonal variation, animals behaviour and social interactions and also internal factors such as hormones, genetics and age (Amrein and Lipp, 2009; Sherry and Hoshooley, 2010; Barnea and Pravosudov, 2011; LaDage et al., 2011; Roth et al., 2011). Despite considerable advances towards comparative understanding of adult neurogenesis, its functional significance under normal physiologic conditions remains elusive. In song birds, adult born neurons have been found to integrate into the song control systems between the high vocal centre (HVC) and the robust nucleus of the arcopallium (RA) (Brenowitz and Larson, 2015). However, in mammals, neurons born from the subventricular zone of the lateral ventricle migrate long distances and integrate as interneurons in the olfactory bulbs whereas neurons from the subgranular zone integrate locally in the adjacent layers of the dentate gyrus (Ihunwo et al., 2016). Considering the variations and similarities in the extent and in different stages of adult neurogenesis in vertebrate species, it is important to examine adult neurogenesis in close and distantly related species across the phylogenetic tree in order to understand the basic biological processes involved and subsequently translate the findings towards development of stem cell therapies for neurodegenerative diseases. Based on this proposition, the current study examined adult neurogenesis in the species of paleognathae birds, the common ostrich (*Struthio camelus*) and the emu (*Dromaius novaehollandiae*) using the proliferating cell nuclear antigen (PCNA) antibody for dividing cells and doublecortin (DCX) antibody for immature neurons. The common ostrich belongs to

the family of the Struthionidae and the order Struthioniformes. The emu is the only extant member of the genus *Dromaius* belonging to the family of Dromaidae and the order Casuariformes. The ostrich and emu belong to the diverse group of ratite which consists of large and flightless birds and classified under the infraclass paleognathae. Both species are omnivorous and insectivorous and they cover vast tracks of land during foraging.

## **6.2. Experimental procedures**

### **6.2.1. Animals and tissue processing**

Two brains of adult male common ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*) were used in the current study. Birds were purchased from a local farm in South Africa. The birds were captured and perfused on site during spring (01-20 November) in 2015. The birds were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (clearance no: 2013/05/02B), which parallel those of the National Institute of Health (NIH) for the care and use of animals in scientific experimentation. Five minutes prior to being euthanized, both birds were given an intramuscular dose of Heparin, 5000 units (1 ml) to prevent blood clotting. Animals were euthanized with an intraperitoneal injection of Euthapent (1ml/kg). The birds were perfused and fixed through the carotid arteries, initially with a rinse of 0.9% saline solution, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) solution. The brains were carefully removed from the skull, and post fixed overnight in 4% paraformaldehyde in 0.1M PB. The brains were then weighed (common ostrich average brain mass = 26.5g; emu, average brain mass = 21.2g) and placed in a 30% sucrose in 0.1 M PB solution at 4°C for 3 days, and then transferred to an antifreeze solution for storage at -20°C. Before sectioning, the tissue was allowed to re-equilibrate in 30% sucrose in 0.1M PB solution at 4°C for 4 days. The brains were then frozen in dry ice and sectioned in the coronal plane, at 50 µm thickness, on a sliding microtome. A one in twenty series of sections were taken and three series stained

for Nissl substance, PCNA and DCX respectively. The remaining series of sections were placed back in an antifreeze solution and are stored at -20°C for future use. The series of sections used for Nissl staining were mounted on 0.5% gelatine coated slides, dried overnight, cleared in a 1:1 mixture of 100% ethanol and 100% chloroform and stained with a 1% Cresyl Violet solution.

### **6.2.2. Immunohistochemistry for PCNA and DCX**

The second and third series of sections were used for free floating PCNA and DCX immunohistochemistry. These sections were initially treated for 30 min at room temperature under gentle shaking with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1M PB: 1.6% of 30% H<sub>2</sub>O<sub>2</sub>), followed by three 10 min rinses in 0.1 M PB. To block non-specific binding sites the sections were then preincubated for 2 h, at room temperature under gentle shaking, in a blocking buffer solution consisting of 3% normal horse serum (NHS) for PCNA sections or 3% normal rabbit serum (NRS) for DCX sections, 2% bovine serum albumin, and 0.25% Triton X-100 in 0.1 M PB. Following preincubation the primary antibodies were added to the blocking buffer solution and the sections were incubated for 48 h at 4°C under gentle shaking (PCNA - 1: 500 dilution of mouse anti-PCNA, NCL-L-PCNA Leica Biosystems, Newcastle, United Kingdom; DCX - 1:300 dilution of goat anti-DCX antibody, C-18, Santa Cruz Biotechnology, Dallas, Texas, U.S.A) under gentle agitation. The primary antibody incubation was followed by three 10-min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (PCNA sections - 1:1000 dilution of biotinylated anti-mouse IgG [BA-2001, Vector Labs] in 3% normal horse serum and 2% bovine serum albumin in 0.1 M PB; DCX sections - 1:1000 dilution of anti-goat IgG [BA-5000, Vector Labs] in 3% normal rabbit serum and 2% bovine serum albumin in 0.1 M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin biotin solution (1:125 in 0.1 M PB; Vector Labs, Burlingame,

California, USA), followed by three 10-min rinses in 0.1 M PB. Sections were then transferred to a solution consisting of 0.05% diaminobenzidine tetrahydrochloride in 0.1M PB for 5 min at room temperature, after which 3.3 µl of 30% H<sub>2</sub>O<sub>2</sub>/ml of solution was added. With the aid of a low power stereomicroscope the progression of the staining was visually followed and allowed to continue until a level was reached where the background staining could assist in architectonic matching to the Nissl stained sections without obscuring the immunopositive structures. The tissue was then rinsed twice more in 0.1M PB before being mounted on glass slides coated with 0.5% gelatine and allowed to dry overnight. Once dry, the slides were placed in a solution of 70% ethanol for 2 h and then dehydrated, cleared in xylene and coverslipped with Depex. To test for non-specific staining of the immunohistochemical protocol, the primary and secondary antibodies were omitted from random sections and no staining was evident. The observed immunostaining patterns support the specificity of the antibodies and are compatible with observations made in pigeons, parakeets and quails (Charvet and Striedter, 2008; Charvet and Striedter, 2009; Melleu et al., 2013).

### **6.2.3. Analysis**

The Nissl stained sections were examined with a low power stereomicroscope and the architectonic borders traced using a camera lucida. The PCNA and DCX immunostained sections were then matched to the drawings from the Nissl stained sections and the location of immunopositive soma marked on the drawings. Selected drawings were then scanned and redrawn using the Canvas 8 Software (Deneba Software, Miami, Florida, USA). High power microscopic observation allowed for the determination of the relative densities of stained structures throughout the various regions of the brain. The relative densities of immunostained structures were visually compared and recorded on a scale ranging from low (+), moderate (++) to high (+++). A second observer was used to eliminate observer bias.



The brain regions were identified and named in accordance with the stereotaxic atlases of the brain of the domestic chicken (*Gallus gallus domesticus*) (Van Tienhoven and Juhasz, 1962), Japanese jungle crow (*Corvus macrorhynchos*) (Izawa and Watanabe, 2007), budgerigar (*Melopsittacus undulatus*) (Brauth et al., 2011) and pigeon (*Columba livia*) (Karten and Hodos, 1967) using the nomenclature recommended by the Avian Brain Nomenclature Forum (Reiner et al., 2004). Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software (Zeiss, South Africa). No pixilation adjustments or manipulation of the captured images were undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

### **Abbreviations**

3V – third ventricle

4V – fourth ventricle

A – arcopallium

A10 – ventral tegmental area

A9 – substantia nigra, pars compacta

APH – area parahippocampalis

BC – brachium conjunctivum

CA – commissura anterior

Cb – cerebellum

CbL – nucleus cerebellaris lateralis

CbM – nucleus cerebellaris intermedius

CO – chiasma opticum

DCX – doublecortin

DLA – nucleus dorsolateralis anterior thalami

DLP – nucleus dorsolateralis posterior

DMA – nucleus dorsomedialis anterior thalami

DMP – nucleus dorsomedialis posterior

E – entopallium

EM – nucleus ectomamillaris

EPL – external plexiform layer of the olfactory bulb

FLM – fasciculus longitudinalis medialis

FPL – fasciculus prosencephali lateralis

GCt – substantia grisea centralis

GL – glomerular layer of olfactory bulb

GLv – nucleus geniculatus lateralis, pars ventralis

GP – globus pallidus

HA – hyperpallium apicale

HD – hyperpallium densocellulare

HI – hyperpallium intercalatus

HM – medial habenular nucleus

Hp – hippocampus

ICo – nucleus intercollicularis

IGrL – internal granular layer of the olfactory bulb

Imc – nucleus isthmi, pars magnocellularis

Ipc – nucleus isthmi, pars parvocellularis

LHy – hypothalamus pars lateralis

Il – lateral layer of the ventral hippocampus

LSt – striatum laterale

LV – lateral ventricle

M – mesopallium

MCL – mitral cell layer of the olfactory bulb

ml – medial layer of ventral hippocampus

ML – nucleus mamillaris lateralis

MLd – nucleus mesencephalicus lateralis, pars dorsalis

MPV – nucleus mesencephalicus profundus, pars ventralis

MSt – striatum mediale

N – nidopallium

nBOR – nucleus Opticus BasalisT–

NC – nidopallium caudale

NI – nidopallium intermoderate

nIV – nucleus nervi trochlearis

nVII – nucleus nervi facialis

OB – olfactory bulb

OM – tractus occipito-mesencephalicus

ON – olfactory nerve

OV – nucleus ovoidalis

PCNA – proliferating cell nuclear antigen

PL – nucleus pontis lateralis

PM – nucleus pontis medialis

PMH – nucleus medialis hypothalami posterioris

POA – nucleus preopticus anterior

POM – nucleus preopticus medialis

PrV – nucleus sensorius principalis nervi trigemini

PVM – nucleus periventricularis magnocellularis

QF – tractus quintofrontalis

R – nucleus rephes

RPgc – nucleus reticularis pontis caudalis, pars gigantocellularis

Rt – nucleus rotundus

Ru – nucleus ruber

SCE – stratum cellulare externum

SCI – stratum cellulare internum

SL – nucleus septalis lateralis

SLu – nucleus semilunaris

SM – nucleus septalis medialis

SN – substantia nigra

SNc – substantia nigra, pars compacta

SP– nucleus subpretectalis

SPC – nucleus superficialis parvocellularis

SpL – nucleus spiriformis lateralis

SpM – nucleus spiriformis medialis;

TeO – tectum opticum

TIO – tractus isthmo-opticus

TnA – nucleus taeniae amygdalae

tr – triangular area of ventral hippocampus

TrO – tractus opticus

TT – tractus tectothalamicus

Tu– nucleus tuberis

V – ventricle

VeL – nucleus vestibularis lateralis

VeM – nucleus vestibularis medialis

## **6.3. Results**

### **6.3.1. General observations**

In the current study we examined adult neurogenesis using histological and immunohistochemical methods in the brain of adult common ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*). PCNA immunoreactive cells were observed in similar brain regions, but the pattern and density of these cells differed in the two species. PCNA immunoreactive cells were observed in the olfactory bulbs, SVZ of the lateral, third, fourth and tectal ventricles and the cerebral aqueduct, telencephalon, diencephalon, mesencephalon and rhombencephalon of both species. The density of PCNA immunoreactive cells was less exuberant in the telencephalon of the emu compared to the common ostrich. DCX immunoreactivity was observed in similar brain regions to PCNA immunoreactivity except in some nuclei and regions in the mesencephalon and rhombencephalon where indistinct punctate-like cells and fibres were observed. In the cerebellum of both species, distinct immature DCX immunoreactive cells were observed in higher numbers in the cortical layers. These results are summarised (Table 6.1) and diagrammatically represented using the common ostrich as the model animal (Fig. 6.1, 6.2).

### **6.3.2. Distribution of PCNA immunoreactive cells**

#### **6.3.2.1. Olfactory bulb**

In the common ostrich, PCNA immunoreactive cells were observed in higher density in the inner three layers; the internal granular layer (IGrL), mitral cell layer (MCL) and external plexiform layer (EPL) but at a lower density in the glomerular layer (GL) and the olfactory nerve (ON) (Fig. 6.1A, 6.3A, 6.3E). The olfactory bulbs in the emu showed a distinctive five layered structure under PCNA immunostaining and the PCNA immunoreactive cells were observed at high density in the second and fourth layers corresponding to mitral cell layer (MCL) and the glomerular layer (GL) respectively, but at a

low density in the internal granular (IGrL), external plexiform (EPL) and the olfactory nerve (ON) layers (Fig. 6.3C). The SVZ of the olfactory ventricle exhibited a higher density of PCNA immunoreactive cells in the common ostrich whereas a low density of PCNA immunoreactive cells was observed in the emu (Fig. 6.3A– C).

#### **6.3.2.2. Subventricular zone**

The subventricular zone (SVZ) of the lateral wall of the lateral ventricle exhibited a higher density of PCNA immunoreactive cells in both common ostrich and the emu (Fig. 6.1C – M). The medial wall in both species had a low density of PCNA immunoreactive cells. The lateral wall of the lateral ventricle in the emu contains a flexure before the dorsal pole, the SVZ before the flexure showed a high density of PCNA immunoreactive cells whereas that after the flexure exhibited a moderate to low density similar to the medial wall of the lateral ventricle (Fig. 6.4C). Accumulations of proliferating cells (hot spots) were observed in the dorsal and ventral poles of the lateral ventricle at rostral and middle levels along the rostro-caudal extent of the lateral ventricle in the common ostrich but only at rostral levels in the emu (Fig. 6.4A, 6.4C, 6.5A, 6.5C). A column of PCNA immunoreactive cells was observed extending from dorsal pole of the lateral ventricle into the hyperpallium apicale (HA) at rostral levels in the both species (Fig. 6.6A, 6.6C). The SVZ of the third, fourth and tectal ventricles and the cerebral aqueduct showed a moderate density of PCNA immunoreactive cells in both species.

#### **6.3.2.3. Hyperpallium, mesopallium, nidopallium and entopallium**

The pallial subdivisions of the hyperpallium apicale (HA) and densocellulare (HD), mesopallium (M) and nidopallium (N) exhibited the highest density of PCNA immunoreactive cells in the common ostrich (Fig. 6.1A – H) but in varying degrees whereas in the emu a much lower density of PCNA immunoreactive cells was observed in all the

cortical regions. In the common ostrich the HA and HD regions exhibited a high density of PCNA immunoreactive cells in the medial and lateral regions but a low density in the core regions (Fig. 6.1A – J). The mesopallium and the nidopallium showed varied distribution of PCNA immunoreactive cells in the common ostrich. The mesopallium (M) showed a higher density of PCNA immunoreactive cells in the medial and lateral regions but a moderate density in the core region. A similar trend was observed in the nidopallium (N) where the medial regions had moderate density of PCNA immunoreactive cells, much lower density in the core region and a higher density in the lateral region (Fig. 6.1C – J) . At middle levels along the rostro-caudal axis of the brain, the nidopallium intermoderate (NI) exhibited a higher density of PCNA immunopositive cells in the medial and lateral regions and a lower density in the core areas. The nidopallium caudale (NC) had the highest density of PCNA immunoreactive cells particularly in regions adjacent to the SVZ of the lateral ventricle which encircles it whereas the core regions showed a low density of PCNA immunoreactive cells (Fig. 6.1K – N). In the emu slight increases in the density of PCNA immunoreactive cells were observed in the NC but were much lower than those observed in the common ostrich. The entopallium (E) showed a moderate density of PCNA immunoreactive cells in the common ostrich (Fig. 6.1H) but a low density to absent of PCNA immunoreactive cells in the emu.

#### **6.3.2.4. Hippocampus, area parahippocambalis, dorsolateral cortical area and piriform cortex**

The hippocampus (Hp) was divided into dorsal and ventral portions according to the descriptions by Atoji and Wild (2004). In the common ostrich, the ventral hippocampus showed a low density of PCNA immunoreactive cells in medial layer (ml), a high density in the triangular layer (tr) and a moderate density in the lateral layer (ll) (Fig. 6.7A). In the emu, the situation was slightly different as follows: a low density of PCNA immunoreactive cells

observed in the medial and lateral layers (ml and ll) and a moderate density in the triangular layer (tr) (Fig. 6.7C). The dorsal hippocampus (Fig. 6.8A, 6.8C), area parahippocampalis (APH), dorsolateral cortical area (CDL) and the piriform cortex (CPi) exhibited a moderate density of PCNA immunoreactive cells in the common ostrich but a low density in the emu.

#### **6.3.2.5. Striatum, septum and arcopallium**

The medial and lateral striatum (MSt and LSt) of both the common ostrich and the emu showed a low density of PCNA immunoreactive cells but slight increases were observed in areas adjacent to the ventral pole of the lateral ventricle at rostral levels (Fig. 6.1E – J). The medial septum (SM) of both species exhibited a moderate density of PCNA immunoreactive cells. The lateral septum (SL) of the common ostrich showed a high density of PCNA immunoreactive cells whereas a moderate density was observed in the emu. A higher density of PCNA immunoreactive cells was observed in the arcopallium (A) of the common ostrich but the emu showed a low density in this brain region (Fig. 6.1J – L).

#### **6.3.2.6. Diencephalon**

The diencephalon had intensely stained PCNA immunoreactive regions in both species and these were of high density in the dorsal and dorsolateral regions in the medial habenular nucleus (HM) and thalamic nuclei including nucleus dorsomedialis anterior (DMA), nucleus dorsolateralis anterior thalami (DLL) and nucleus dorsolateralis posterior thalami (DLP), the medial regions of the hypothalamus including nucleus medialis hypothalami posterioris (PMH), nucleus preoticus medialis (POM), and nucleus periventricularis magnocellularis (PVM) and ventrally in the nucleus tuberis (Tu) (Fig. 6.1I – L). In the common ostrich, vertical columns of PCNA immunoreactive cells were observed in the medial hypothalamus adjacent to the SVZ of the third ventricle (Fig. 6.1I, 6.1J). The optic



chiasm (OC) showed a moderate density cluster of PCNA immunoreactive cells below the ventral pole of the third ventricle in both species (Fig. 6.1I, 6.9A, 6.9C).

#### **6.3.2.7. Mesencephalon, rhombencephalon and cerebellum**

Most of the nuclei in the midbrain, pons and medulla oblongata exhibited intensely stained PCNA immunoreactive cells. In the midbrain, some of the nuclei that exhibited intense PCNA immunoreactivity include, the nucleus rotundus (Rt), nucleus ruber (Ru), substantia nigra, pars compacta (SNc), substantia grisea centralis (GCt), nucleus ectomamillaris (EM), nucleus isthmi, pars parvocellularis and pars magnocellularis (Ipc and Imc), nucleus mesencephalicus lateralis, pars dorsalis (MLd) and nucleus spiriformis medialis (SpM) (Fig. 6.1L – N). The inner layers of the optic tectum (TeO) contained a higher density of PCNA immunoreactive cells in both species (Fig. 6.1K – O). Various nuclei of the pons and medulla oblongata showed intense PCNA immunoreactivity (Fig. 6.1M – P). The median raphe of the pons and the medulla oblongata showed intense PCNA immunoreactivity in both species. In the cerebellum of the common ostrich, a high density of large PCNA immunoreactive cells was observed in the Purkinje cell layer but the granule and molecular cell layers exhibited a low density (Fig. 6.10A). In the emu the Purkinje cell layer showed a moderate density of PCNA immunoreactive cells while the granule and molecular layers were similar to those in the common ostrich (Fig. 6.10C). The medial and lateral nuclei of the cerebellum (CbM and CbL) showed mild PCNA immunoreactivity in both species.

#### **6.3.3. Distribution of DCX immunoreactive cells and fibres**

##### **6.3.3.1. Olfactory bulb**

The olfactory bulbs exhibited a high density of DCX immunoreactive cells and fibres in both species. In the common ostrich, DCX immunoreactive cells and fibres were observed at a high density in the inner four layers of the olfactory bulb including the IGrL, MCL, EPL

and GL but moderate density in the outer olfactory nerve layer (ON) (Fig. 6.2A, 6.3B). The olfactory bulb in the emu showed distinct and well developed layers and DCX immunoreactive cells and fibres were observed at a high density in the inner three layers including IGrL, MCL and EPL while the outer layers (GL and ON) showed a moderate to low density of these cells and fibres respectively (Fig. 6.3D, 6.3F). The subventricular zone (SVZ) of the olfactory ventricles in both species showed a high density of DCX immunoreactive structures and cells with a unipolar morphology could be seen migrating into the hyperpallial divisions (Fig. 6.3B, 6.3D, 6.3F).

#### **6.3.3.2. Subventricular zone**

DCX immunoreactive cells and fibres were observed at a high density in the subventricular zone of the lateral wall of the lateral ventricle compared to the moderate density in the subventricular zone of the medial wall in both species (Fig. 6.2C – M). The lateral wall of the lateral ventricle in the emu contains a flexure before the dorsal pole, the SVZ before the flexure showed a high density of DCX immunoreactive cells whereas that after the flexure exhibited a moderate density similar to the medial wall of the lateral ventricle (Fig. 6.4D). Bundles of DCX immunoreactive cells and fibres were observed extending from the SVZ of the lateral wall of the lateral ventricle into the parenchyma at three distinct points at the dorsal and ventral poles of the lateral ventricle and at the middle part of the lateral wall in both species (Fig. 6.2B – G). The cells comprised a mixture of unipolar and bipolar cells with a leading process typical of migrating cells. Cells from the dorsal pole, middle part and ventral pole of the lateral ventricle migrated into the hyperpallial divisions of HA, HD and HI; the parenchyma of the nidopallium (N) and the striatum complex respectively. The majority of DCX immunoreactive cells in the SVZ of the lateral ventricle in both species were oriented parallel to the wall of the lateral ventricle suggestive of tangential migration. The dorsal and ventral hot spots were observed at rostral levels on the lateral ventricles in both species but

the common ostrich showed additional concentrations in the middle sections at the level of the anterior commissure (AC) (Fig 6.4B, 6.4D, 6.5B, 6.5D). The subventricular zones of the third, fourth and tectal ventricles and the cerebral aqueduct exhibited a moderate density of DCX immunoreactive cells and fibres.

#### **6.3.3.3. Hyperpallium, mesopallium, nidopallium and entopallium**

The telencephalon exhibited the highest density of DCX immunoreactive cells and fibres compared to other brain regions, although the density of immunopositive structures varied across various regions of the pallium. In the common ostrich and the emu, the hyperpallium apicale (HA) is a relatively large brain structure and it presented with a high density of DCX immunoreactive cells, particularly in the medial and lateral regions but a low density in the core regions (Fig. 6.2A – H). In both species the density of the DCX immunoreactive cells in the HA declined in the rostro-caudal direction until the level of the anterior commissure. The hyperpallium densocellulare and intercalatum (HD and HI) contained a high density of DCX immunoreactive cells in both species and they received a bundle of migrating neuroblasts from the dorsal pole of lateral ventricle at rostral levels (Fig. 6.6B, 6.6D).

A moderate density of DCX immunoreactive cells and fibres was observed in the mesopallium (M) of both species particularly in the medial and lateral regions, but very low density in the core regions. Similar to the HA, the density of the DCX immunoreactive cells and fibres in the M declined noticeably in the rostro-caudal direction up to the anterior commissure (Fig. 6.2B – J).

The density of the DCX immunoreactive cells in the nidopallium varied along the rostro-caudal axis in both species. At rostral levels, a moderate density of DCX immunoreactive cells was observed in the nidopallium frontale (NF) (Fig. 6.2B – H). A much higher density of DCX immunoreactive cells was observed in the medial and lateral regions

but low to absent in the core regions. Bundles of migrating neuroblasts were observed extending from the subventricular zone of the lateral ventricle into the parenchyma of the nidopallium at rostral levels in both species. At middle levels around the anterior commissure, the nidopallium intermoderate (NI) exhibited a low density of DCX immunoreactive cells in the medial and lateral regions. The core region at this level did not show any DCX immunoreactive cells in both species (Fig. 6.2I). The nidopallium caudale (NC) showed the highest density of DCX immunoreactive cells particularly in regions adjacent to the SVZ of the lateral ventricle (Fig. 6.2K – M). The lateral ventricle completely encircles the NC in both species. The core regions showed a moderate density of DCX immunoreactive cells. A very low density of DCX immunoreactive cells was observed in the entopallium (E) in both species (Fig. 6.2H).

#### **6.3.3.4. Hippocampus, area parahippocampalis, dorsolateral cortical area and piriform cortex**

The ventral hippocampus showed a moderate density of the DCX immunoreactive cells in both the common ostrich and emu. In both species, the medial and lateral layers (ml and ll) of the ventral hippocampus contained a low and moderate density of DCX immunoreactive cells respectively. However, the triangular layer (tr) of the ventral hippocampus in the common ostrich exhibited a moderate density of DCX immunoreactive cells while in the emu it showed a high density (Fig. 6.7B, 6.7D). A moderate density of DCX immunoreactive cells was observed in the dorsal hippocampus and area parahippocampalis (APH) in both the common ostrich and the emu (Fig. 6.8B, 6.8D). In both species the dorsolateral cortical area (CDL) and piriform cortex (CPi) showed a high density of DCX immunoreactive cells.

#### **6.3.3.5. Striatum, septum and arcopallium**

The medial striatum (MSt) in both species exhibited a moderate density of DCX immunoreactive cells. Slight increases in the density of DCX immunoreactive cells were observed in the medial and ventral regions of the medial striatum (MSt) (Fig. 6.2E – H). Migrating neuroblasts were observed extending from the ventral pole of the SVZ of the lateral ventricle into the medial striatum at rostral levels in both species (Fig. 6.2E – H, 6.5B, 6.5D). The lateral striatum (LSt) in the emu showed a moderate density of DCX immunoreactive cells whereas in the common ostrich it showed a low density (Fig. 2H – J). Both subdivisions of the striatum showed a rostro-caudal decline in the density of DCX immunoreactive cells in both species. The medial and lateral septum (SM and SL) showed a moderate density of DCX immunoreactive cells in both species. No DCX immunoreactivity was observed in the arcopallium (A) in both the common ostrich and the emu.

#### **6.3.3.6. Diencephalon**

A moderate density of DCX immunoreactive cells was observed in medial hypothalamus adjacent to the subventricular zone of the third ventricle in both species (Fig. 6.2I – K). The dorsolateral thalamic nuclei including DMA, DLL and DLM and the SPC nuclei exhibited a low density of DCX immunoreactive fibres and indiscernible cells in both the common ostrich and the emu. In these regions the majority of DCX immunoreactive structures were made up of fibres and punctate cell-like structures with short or absent cellular processes. The habenular nuclei and the nucleus tuberis (Tu) contained a high density of intensely stained unipolar DCX immunoreactive cells and fibres in both species (Fig. 6.2K). There was no DCX immunoreactivity in the lateral and core regions of the diencephalon. In the common ostrich, the optic chiasm (OC) exhibited an accumulation of a moderate density of DCX immunoreactive cells and fibres adjacent to the SVZ of the ventral pole of the third

ventricle, but a similar region in the emu showed a low to absent density of DCX immunoreactive fibres (Fig. 6.9B, 6.9D).

#### **6.3.3.7. Mesencephalon, rhombencephalon and cerebellum**

In the midbrain of both species, a moderate density of DCX immunoreactive cells and fibres was observed in subventricular zones of the cerebral aqueduct and some few nuclei including the GCt, Imc and Ipc. The optic tectum showed a moderate density of DCX immunoreactive fibres and cell like structures, but no clear cell boundaries were observed in both species (Fig. 6.2K – N). In the pons and medulla oblongata, DCX immunoreactive cells and fibres were observed in the median raphe and the subventricular zone of the fourth ventricle (Fig. 6.2L – P). In both the common ostrich and the emu, a moderate density of DCX immunoreactive cells was observed the cortical layers of the cerebellum including the molecular, Purkinje and the granule cell layers (Fig. 6.10B, 6.10D). The majority of DCX immunoreactive cells in these layers were unipolar and bipolar cells. The medial and lateral cerebellum nuclei (CbM and CbL) also showed a moderate density of DCX immunoreactive cells in both species.

**Table 6.1.** Summary of qualitative distribution and density of PCNA and DCX

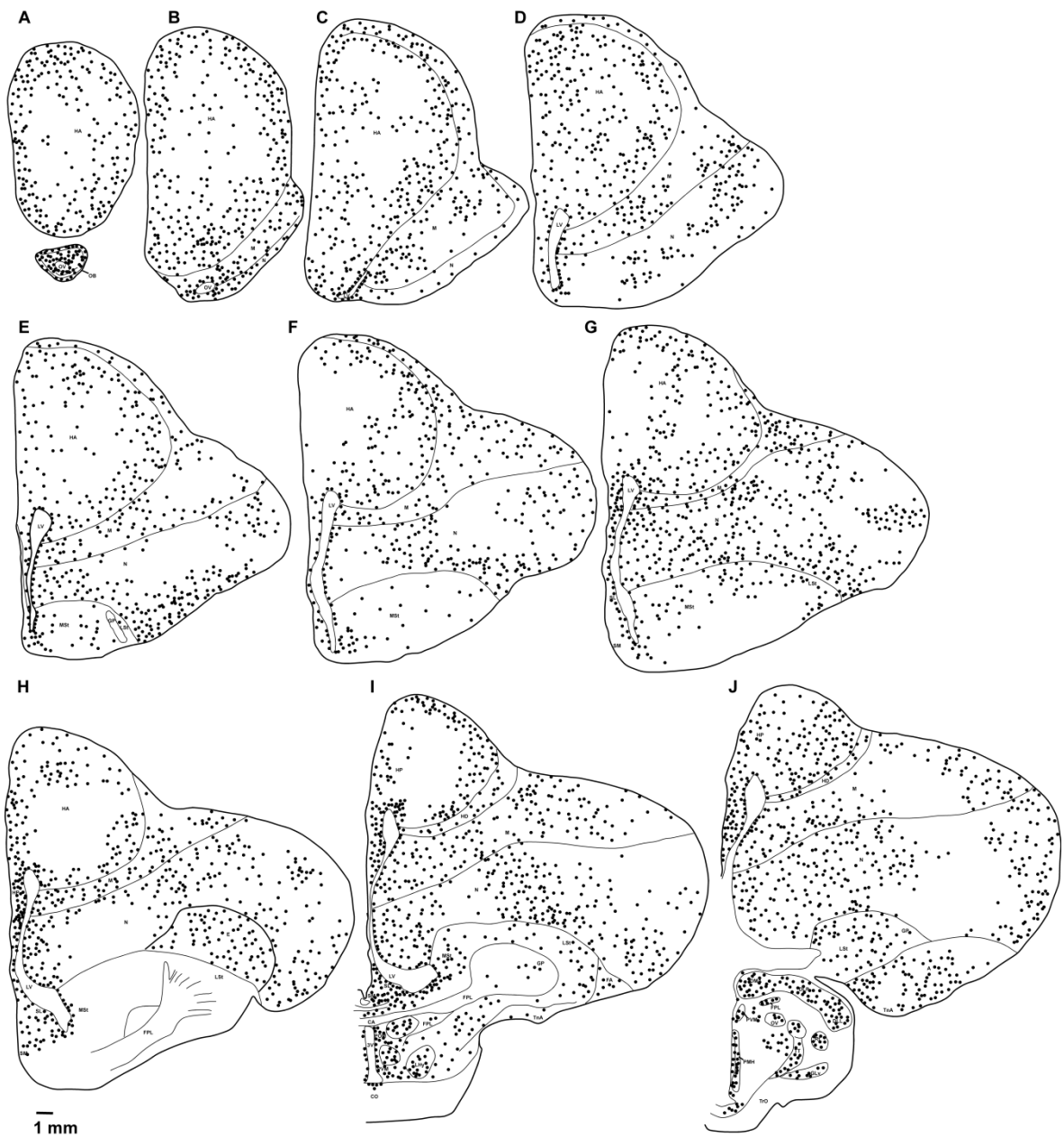
immunoreactive cells in the brain of the common ostrich and emu.

– absent, + low, ++ moderate, +++ high density of PCNA and DCX immunoreactive structures.

| Brain region    |                   | Common ostrich |     | Emu  |     |
|-----------------|-------------------|----------------|-----|------|-----|
|                 |                   | PCNA           | DCX | PCNA | DCX |
| SVZ             |                   | +++            | +++ | +++  | +++ |
| OB              |                   | +++            | +++ | ++   | ++  |
| Telencephalon   | HP                | ++             | ++  | +    | ++  |
|                 | HA                | ++             | ++  | +    | ++  |
|                 | HI                | +++            | +++ | ++   | +++ |
|                 | HD                | +++            | +++ | ++   | +++ |
|                 | M                 | +++            | ++  | +    | ++  |
|                 | N                 | ++             | ++  | +    | ++  |
|                 | NC                | +++            | +++ | +    | +++ |
|                 | E                 | ++             | +   | +    | +   |
|                 | A                 | +++            | —   | +    | —   |
|                 | MSt               | +              | ++  | +    | ++  |
|                 | LSt               | +              | +   | +    | ++  |
|                 | SM                | ++             | ++  | ++   | ++  |
|                 | SL                | ++ +           | ++  | +    | ++  |
| Diencephalon    |                   | ++             | ++  | ++   | +   |
| Mesencephalon   | Pretectum         | ++             | +   | ++   | +   |
|                 | Optic tectum      | ++             | ++  | ++   | ++  |
| Rhombencephalon | Cerebellum        | ++             | ++  | +    | ++  |
|                 | Pons              | +              | +   | +    | +   |
|                 | Medulla oblongata | +              | +   | +    | +   |



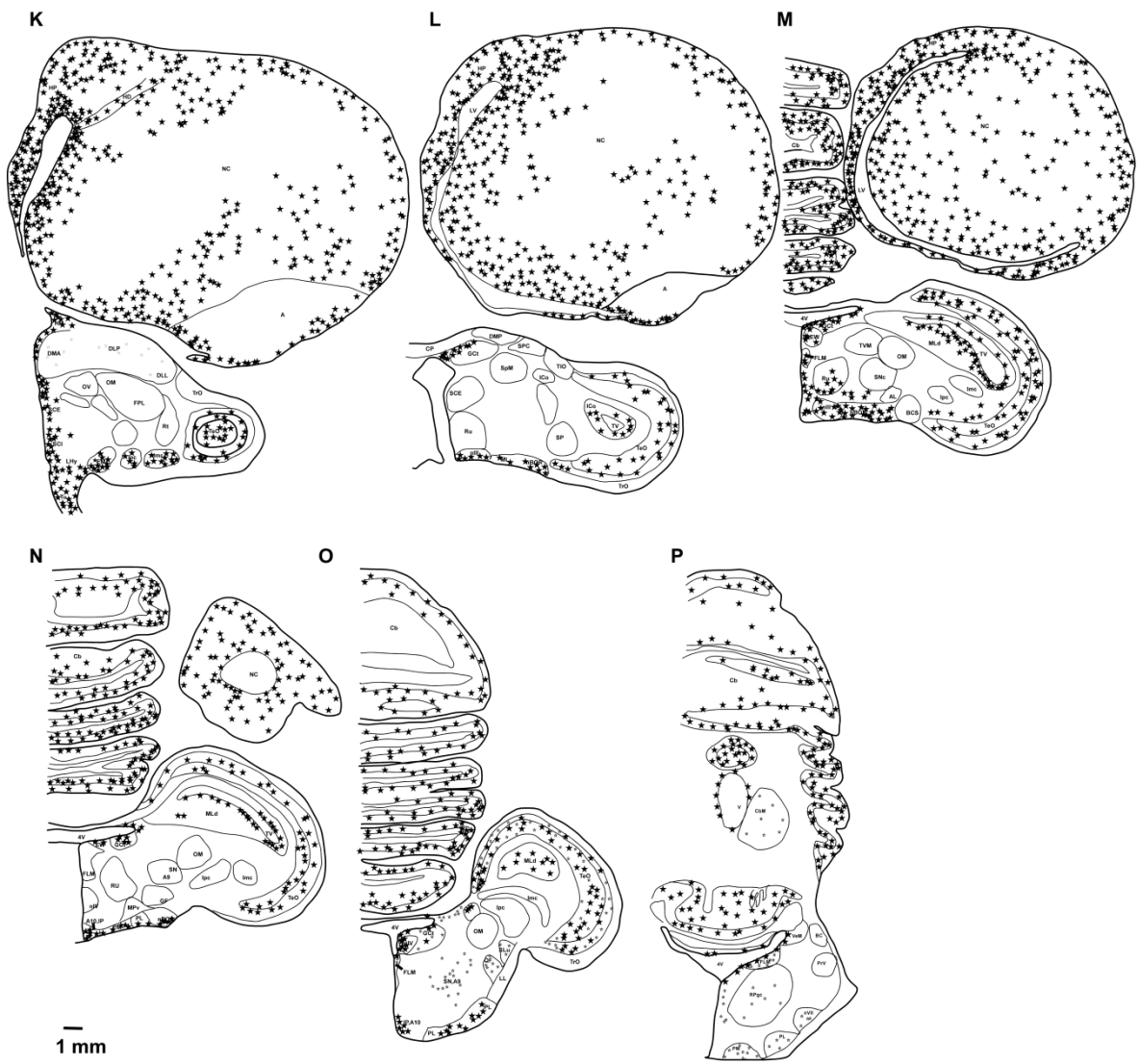
**Figure 6.1:** Diagrammatic reconstructions of coronal sections through one half of the brain of the common ostrich (*Struthio camelus*), illustrating the distribution of PCNA immunoreactive cells. A single black dot indicates a single immunolabelled cell. Drawing **A** represent the most rostral section, **P** the most caudal. Each drawing is approximately 3000 µm apart. See list for abbreviations.



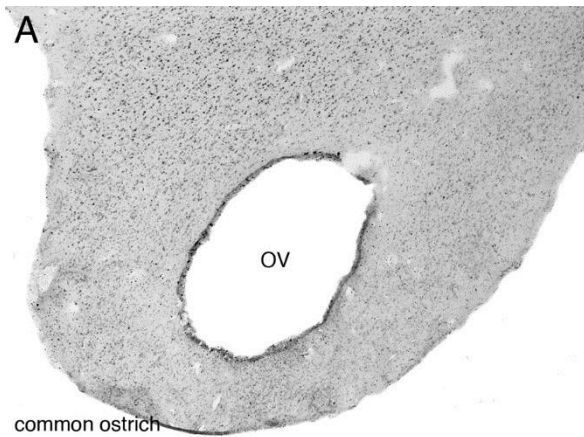


**Figure 6.2:** Diagrammatic reconstructions of coronal sections through one half of the brain of the common ostrich (*Struthio camelus*), illustrating the distribution of DCX immunoreactive cells. A single solid black star indicates a single cell body and a single open star represents a weakly stained cell body. Drawing **A** represent the most rostral section, **P** the most caudal, with each drawing matching those in Figure 1. Each drawing is approximately 3000  $\mu\text{m}$  apart. See list for abbreviations.

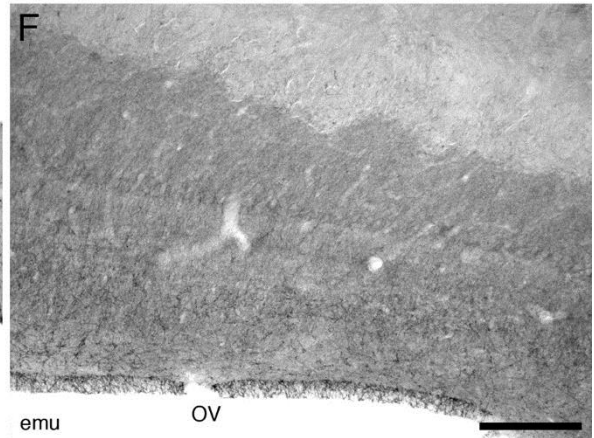
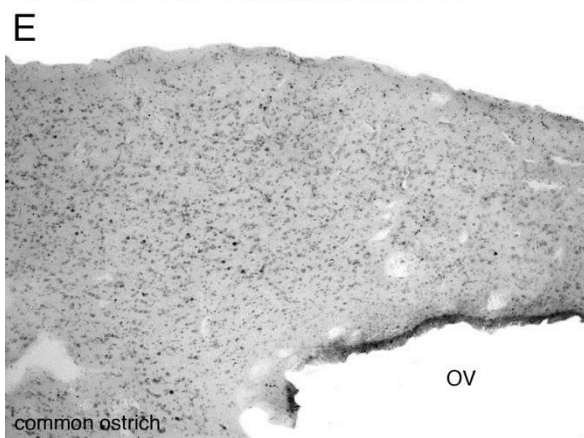
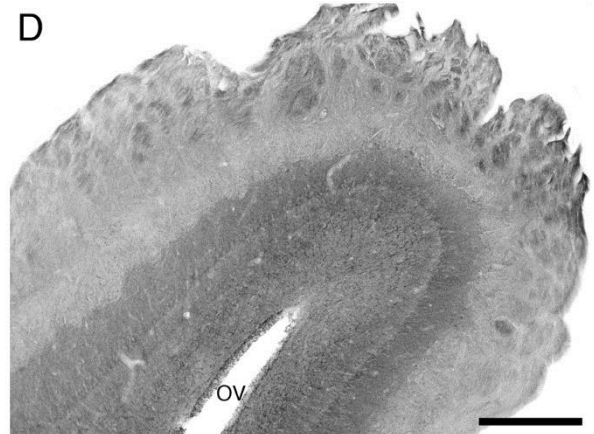
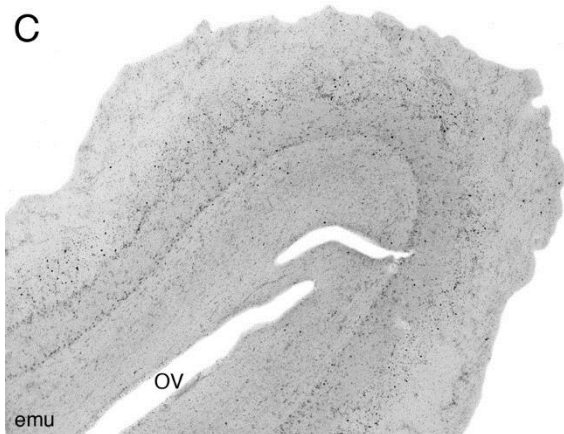
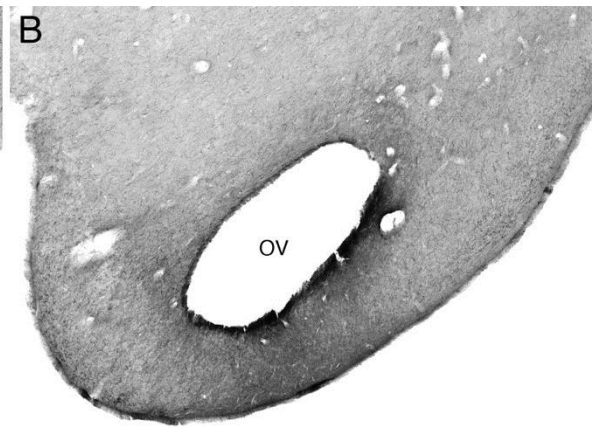




**Figure 6.3.** Photomicrographs of PCNA - (**A, C, E**) and DCX (**B, D, F**) - immunostained coronal sections through the olfactory bulb (OB) of the two ratite species – **A, B, E** – common ostrich (*Struthio camelus*), **C, D, F** – emu (*Dromaius novaehollandiae*). In all images dorsal is to the top and medial to the right. (**A, E**) PCNA immunoreactivity in the olfactory bulb of the common ostrich revealed a high number of PCNA- immunoreactive cells in the IGrL, MCL and EPL layers of the olfactory bulb and low numbers in the outer GL and ON layers. (**C**) PCNA immunoreactivity in the olfactory bulb of the emu revealed a high number of PCNA- immunoreactive cells in the MCL and GL but low numbers in the IGrL, EPL and ON layers. (**E**) A higher magnification image of PCNA immunoreactive cells in the olfactory bulb of the common ostrich. (**B**) DCX immunoreactivity in the olfactory bulb of the common ostrich revealed a high density of DCX- immunoreactive cells and fibres in all layers of the olfactory bulb (IGrL, MCL, EPL and GL). (**D, F**) DCX immunoreactivity in the olfactory bulb of the emu revealed a high density of DCX- immunoreactive cells and fibres in the IGrL, MCL and EPL layers and a moderate to low density in the GL and ON layers. (**F**) A high magnification image of the DCX immunoreactive cells in the olfactory bulb of the emu. Scale bar in D = 500 um and applies to **A** to **D**. Scale bar in F = 250 um and applies to **E** and **F**. See list for abbreviations.

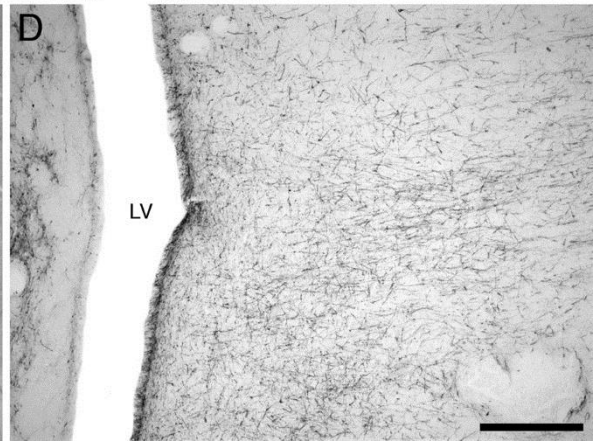
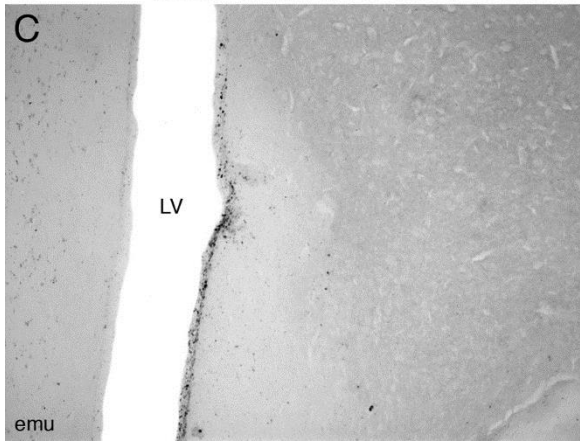
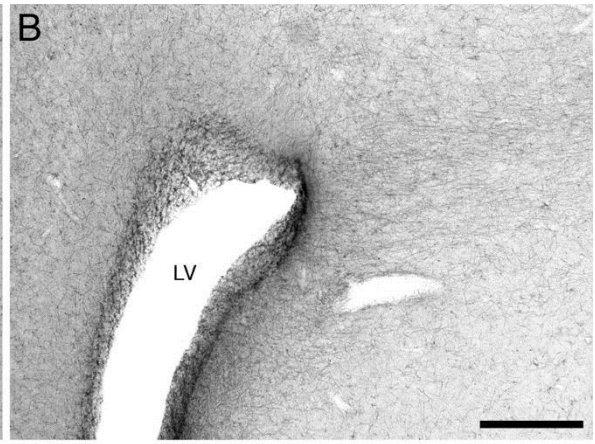
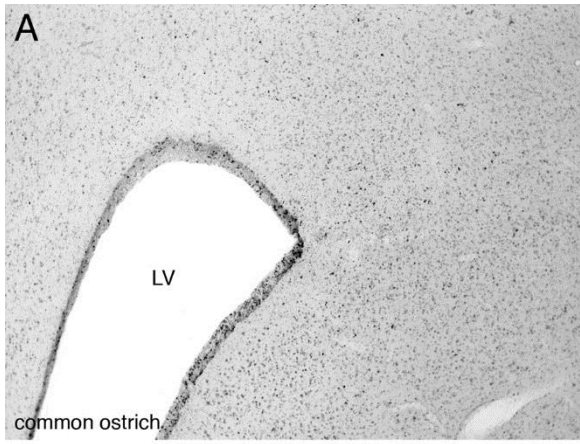


common ostrich

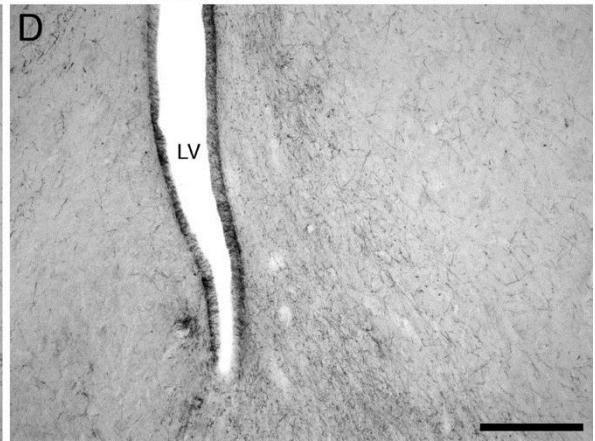
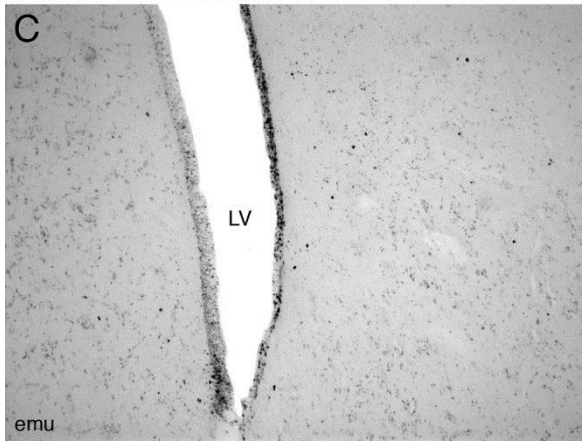
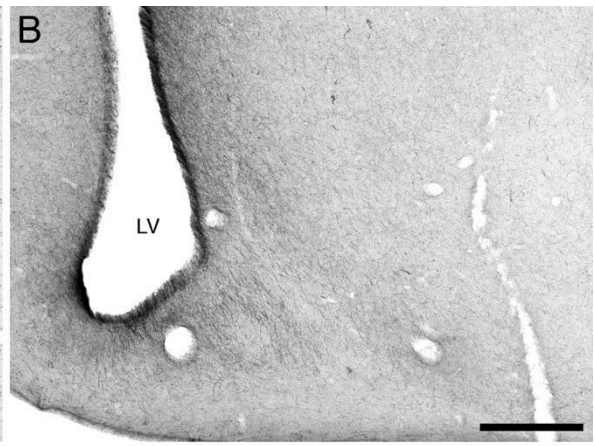
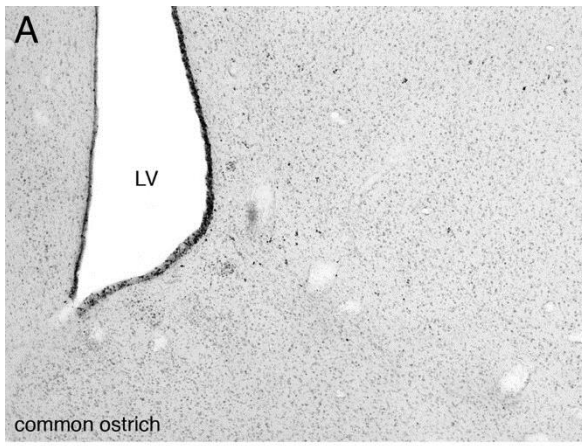




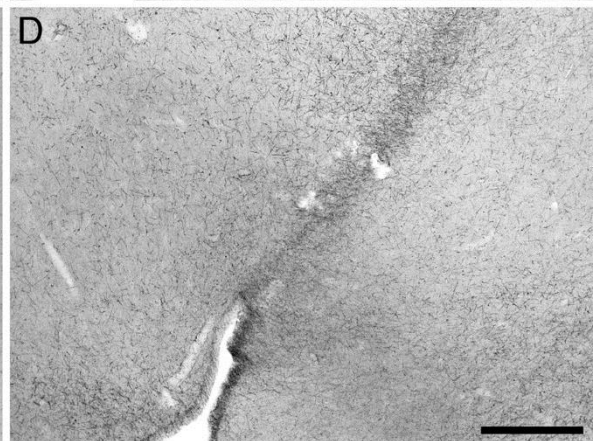
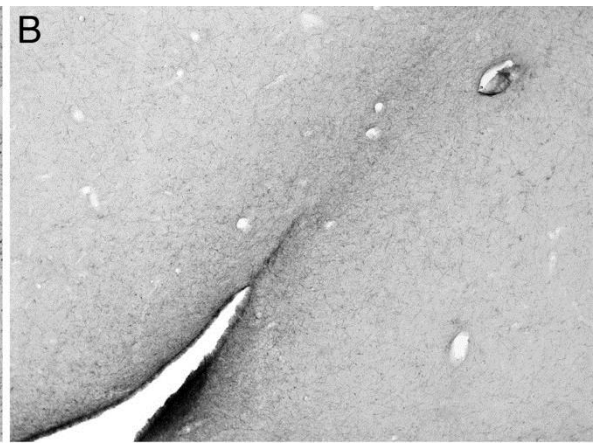
**Figure 6.4.** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the dorsal pole of the subventricular zone (SVZ) of the lateral ventricle in the brain of the two ratite species –**A, B** – common ostrich (*Struthio camelus*), **C, D** – emu (*Dromaius novaehollandiae*). PCNA immunoreactivity revealed aggregates of proliferating cells (hot spots) in the SVZ of the dorsal pole of the lateral ventricle in both common ostrich and emu (**A, C**). DCX immunoreactivity revealed aggregates of DCX immunoreactive cells and fibres in the SVZ of the dorsal pole of the lateral ventricle in both common ostrich and emu (**B, D**). Note the orientation of fibres indicating the direction of migrating cells into the pallium in both species (**B, D**). In all images dorsal is to the top and medial to left of the image. Scale bar in **B** = 500 um and applies to **A** and **B**. Scale bar in **D** = 250 um and applies to **C** and **D**. **LV** – lateral ventricle.



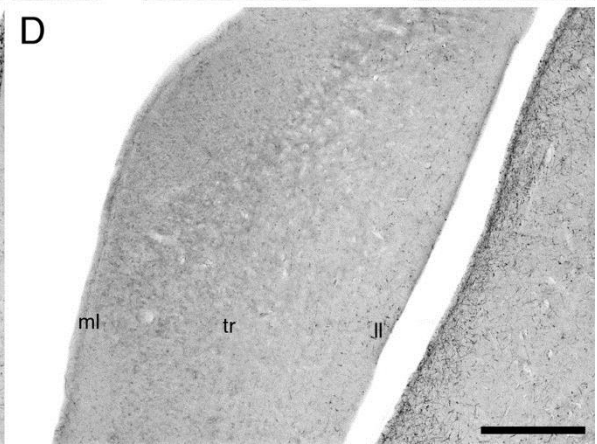
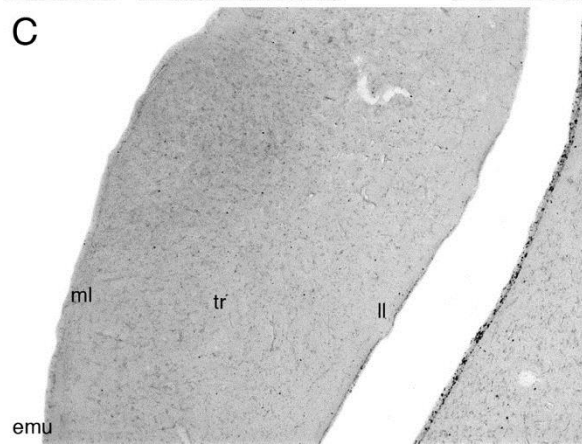
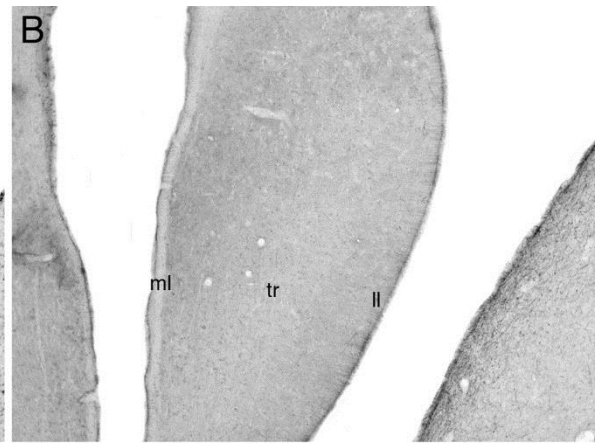
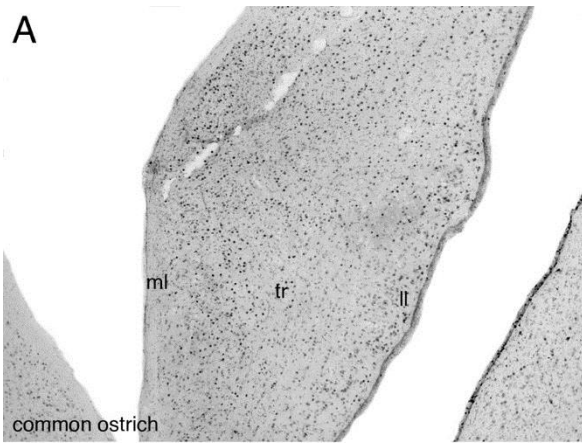
**Figure 6.5.** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the ventral pole of the subventricular zone (**SVZ**) of the lateral ventricle in the brain of the two ratite species –**A, B** – common ostrich (*Struthio camelus*), **C, D** – emu (*Dromaius novaehollandiae*). PCNA immunoreactivity revealed aggregates of proliferating cells (hot spots) in the SVZ of the ventral pole of the lateral ventricle in both common ostrich and emu (**A, C**). DCX immunoreactivity revealed aggregates of DCX immunoreactive cells and fibres in the SVZ of the ventral pole of the lateral ventricle in both common ostrich and emu (**B, D**). Note the orientation of fibres indicating the direction of migrating cells into the medial striatum in both species (**B, D**). In all images dorsal is to the top and medial to left of the image. Scale bar in **B** = 500 um and applies to **A** and **B**. Scale bar in **D** = 250 um and applies to **C** and **D**. **LV** – lateral ventricle.



**Figure 6.6.** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the dorsal pole of the subventricular zone (SVZ) of the lateral ventricle at rostral levels in the brain of the two ratite species – **A, B** – common ostrich (*Struthio camelus*), **C, D** – emu (*Dromaius novaehollandiae*). PCNA immunoreactivity revealed columns of proliferating cells from the SVZ of the dorsal pole of the lateral ventricle in both common ostrich and emu extending towards the hyperpallium (**A, C**). DCX immunoreactivity revealed bundles of migrating DCX immunoreactive cells and fibres from the SVZ of the dorsal pole of the lateral ventricle in both common ostrich and emu extending into the hyperpallium (**B, D**). In all images medial is to the left and dorsal to the top of the image. Scale bar in **D** = 500 um and applies to all. In all images medial is to the left and dorsal to the top of the image.



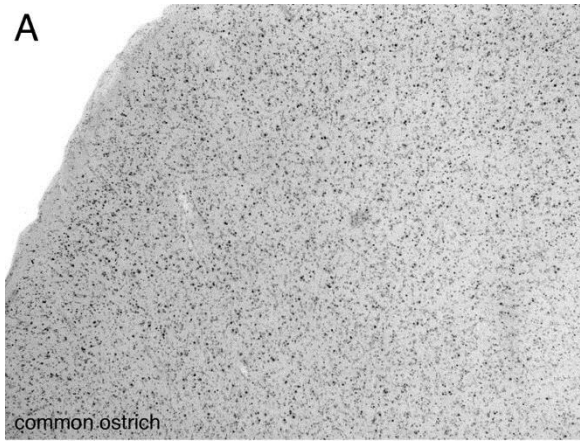
**Figure 6.7.** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the ventral hippocampus in the brains of the two ratite species – **A, B** – common ostrich (*Struthio camelus*), **C, D** – emu (*Dromaius novaehollandiae*). (**A, C**) PCNA immunoreactive cells in the ventral layers of the hippocampus revealed a high density of PCNA immunoreactive cells in the triangular and lateral layers (**tr** and **ll**) in the common ostrich (**A**) and a low density in corresponding layers in the emu (**C**). Note the medial layer (**ml**) of the ventral hippocampus showed a very low density of PCNA immunoreactive cells in both species (**A, C**). (**B, D**) DCX immunoreactive cells in the ventral layers of the hippocampus in both species revealed a moderate density in the triangular and lateral layers (**tr** and **ll**) and a very low density in the medial layer (**ml**). In all images dorsal is to the top and medial to the left. Scale bar in **D** = 500 um and applies to all.



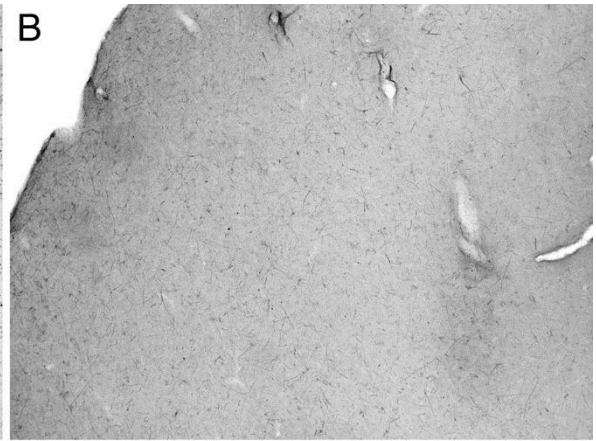


**Figure 6.8.** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the dorsal hippocampus in the brain of the two ratite species –**A, B** – common ostrich (*Struthio camelus*), **C, D** – emu (*Dromaius novaehollandiae*). (**A, C**) PCNA immunoreactive cells in the dorsal hippocampus revealed higher density of PCNA immunoreactive cells in the common ostrich and a low density in the emu. (**B**) DCX immunoreactive cells in the dorsal hippocampus revealed a moderated density of DCX immunoreactive cells and fibres in both species. In all images dorsal is to the top and medial to the left of the image. Scale bar in **D** = 500 um and applies to all.

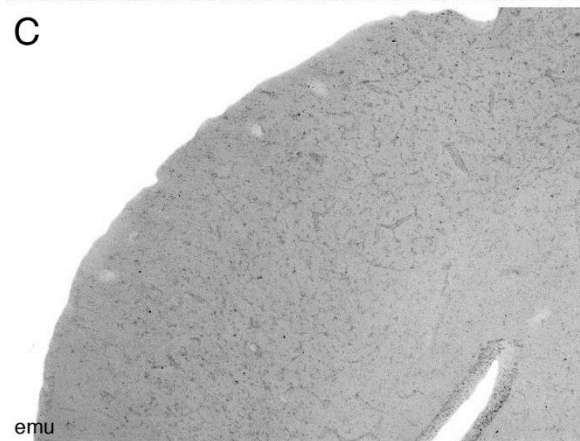
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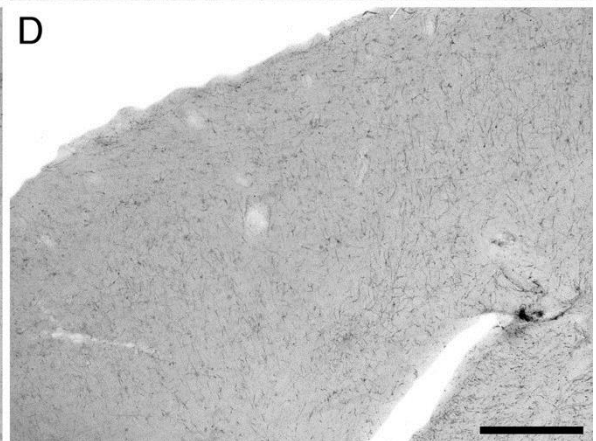
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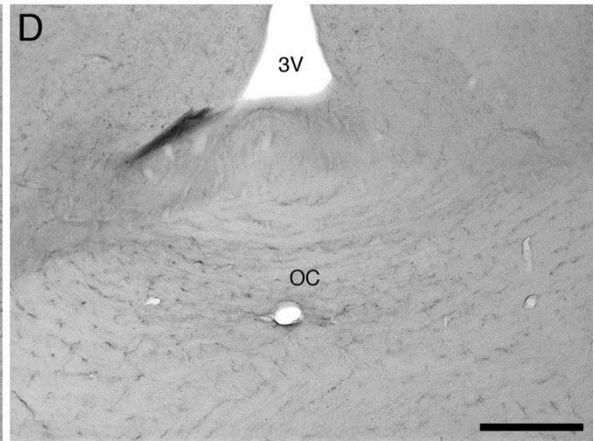
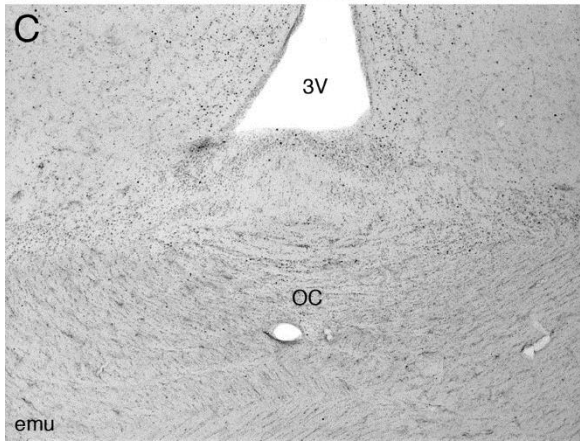
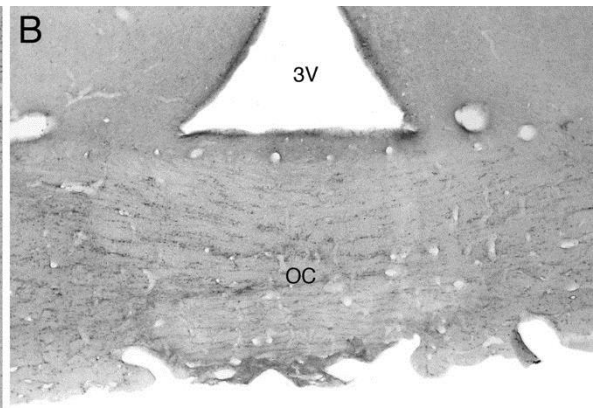
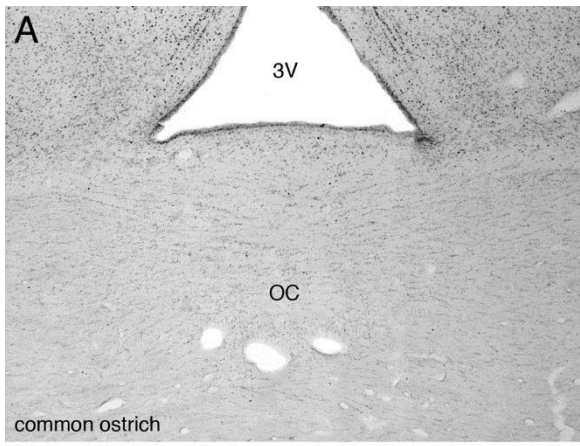
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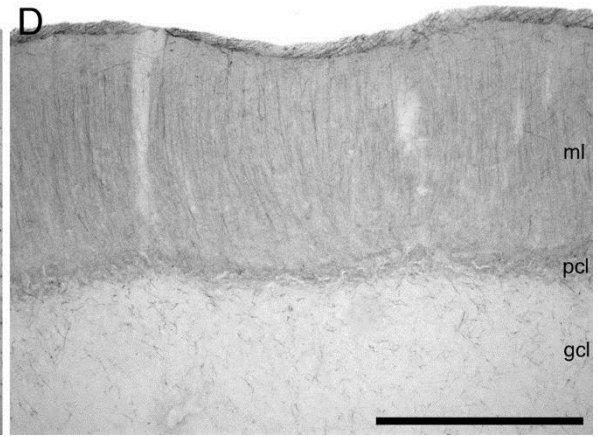
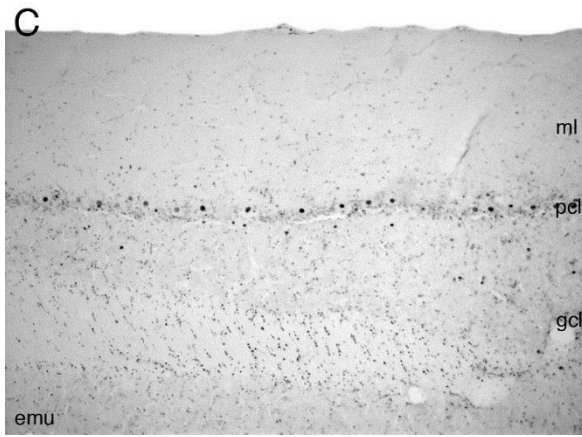
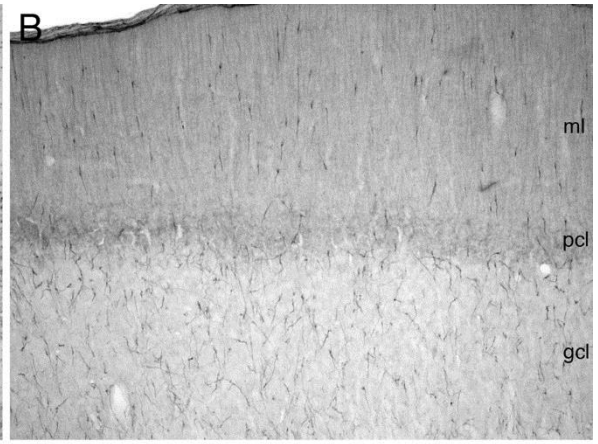
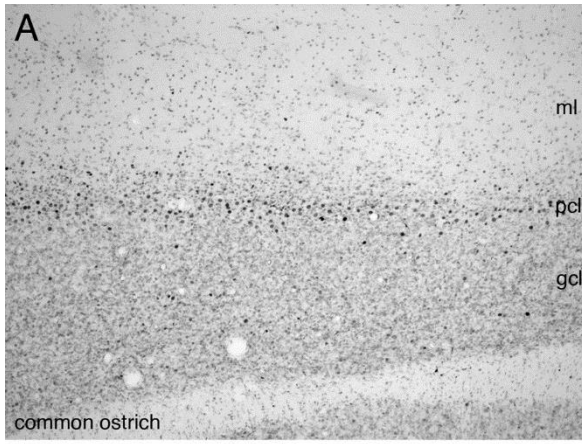
D



**Figure 6.9.** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the optic chiasm (**OC**) ventral to the hypothalamus (**Hyp**) in the brain of the two ratite species –**A, B** – common ostrich (*Struthio camelus*), **C, D** – emu (*Dromaius novaehollandiae*). (**A, C**) Accumulation of PCNA immunoreactive cells in the optic chiasm observed in both species. (**B**) Concentration of DCX immunoreactive fibres in the optic chiasm in the common ostrich. Note a low to absent density of DCX immunoreactive structures in the optic chiasm of the emu (**D**). These images depict a potential neurogenic zone at the midline of the optic chiasm. In both images the midline is the centre of the image and dorsal to the top of the image. Scale bar in **D** = 500 um and applies to all. **3V** – third ventricle.



**Figure 6.10.** Photomicrographs of PCNA (**A, C**) and DCX (**B, D**) immunostained coronal sections through the cerebellar cortex in the brains of the two ratite species –**A, B** – common ostrich (*Struthio camelus*), **C, D** – emu (*Dromaius novaehollandiae*). (**A, C**) PCNA immunoreactive cells in the layers of the cerebellar cortex revealed high density in the common ostrich and moderate density in the emu. (**B, D**) A moderate density of DCX immunoreactive structures in the cerebellar cortex sin both species. Note large and intensely stained PCNA immunoreactive cells in the Purkinje cell layer (**pcl**). In all images dorsal is to the top and medial to the left. Scale bar in **D** = 500 um and applies to all. **gcl** – granule cell layer, **ml** – molecular cell layer.



## **6.4. Discussion**

### **6.4.1. General considerations**

In the current study we examined putative adult neurogenesis in the brains of the two species of paleognathous birds the common ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*) using PCNA and DCX immunohistochemistry. The PCNA immunoreactive cells were distributed heterogeneously in all brain regions including the olfactory bulb, telencephalon, diencephalon, mesencephalon and rhombencephalon, while DCX immunoreactive structures were dense in the olfactory bulb, telencephalon and some regions of the diencephalon, but far less dense in some parts of the diencephalon, the mesencephalon and rhombencephalon in both species. The density of PCNA immunoreactive cells in the emu was comparably less pronounced than in the common ostrich. To a large extent our results conform and are comparable to the wide spread distribution of adult neurogenesis reported in the neognathous birds (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014; Mazenganya et al., 2017). The abundance of PCNA and DCX immunoreactive structures in the telencephalon compared to the subtelencephalic regions suggests that adult neurogenesis corresponds to the demands of cognitive brain functions and may be responsible for plastic changes necessary for certain behavioural repertoires in different species.

### **6.4.2. Cell proliferation in the brain of adult common ostrich and emu**

In the current study we observed cell proliferation throughout the brains of both the common ostrich and the emu. Proliferating cells as labelled by the PCNA antibody were observed in the olfactory bulbs, telencephalon, diencephalon, midbrain, hindbrain and the cerebellum in both species. These observations corroborate findings in the song bird, the canary (*Serinus canaria*) and non-song bird, the ring dove (*Streptopelia risoria*) where bromodeoxyuridine (BrdU) and <sup>3</sup>H-Thymidine positive cells were found throughout the

brains respectively (Ling et al., 1997; Vellema et al., 2010). Additionally, similar findings were observed in a recent study in the breeds of adult domestic pigeons using PCNA antibody as a marker for cell proliferation (Mazenganya et al., 2017). In the telencephalon of both the common ostrich and emu, high numbers of PCNA immunoreactive cells were observed in the SVZ of the lateral ventricle particularly in its lateral wall. Similarly, in song and non-song birds, the lateral wall of the lateral ventricle harbours a higher number of proliferating cells in the adult species (Goldman and Nottebohm, 1983; Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1990b; Ling et al., 1997; Vellema et al., 2010; Mazenganya et al., 2017). In two separate studies utilising  $^3\text{H}$ -Thymidine and BrdU as cell birth markers in adult ring doves and canaries respectively, a high number of proliferating cells was found in the SVZ of the lateral wall of the lateral ventricles, but few cells in the adjacent parenchyma 24hrs after initial injections suggesting that the SVZ of the lateral wall was the primary source of proliferating cells (Ling et al., 1997; Vellema et al., 2010). In addition Goldman and Nottebohm (1983) observed numerous cells labelled with  $^3\text{H}$ -Thymidine in the SVZ compared to the rest of the telencephalon 1 to 2 days after initial injection.

Although cell proliferation was present throughout the walls of the lateral ventricle, the SVZ of the lateral wall was not homogenous, occasional aggregates of proliferating cells were observed along the rostro-caudal axis of the lateral ventricle in both species. These aggregates of proliferating cells are consistent with proliferative hot spots observed in the canary brain (Alvarez-Buylla et al., 1990b; Vellema et al., 2010). The precise location of the proliferative hot spots varies in different species of birds along the rostro-caudal extent of the lateral ventricle (Ling et al., 1997; Vellema et al., 2010; Melleu et al., 2013; Vellema et al., 2014; Mazenganya et al., 2017). In the current study, proliferative hot spots were observed in the dorsal and ventral poles of the lateral wall of the lateral ventricle at rostral levels in both the common ostrich and the emu, but at middle sections at the level of the anterior commissure in the common ostrich. However, in song birds (canaries) varied reports exist as



to the location of proliferative hot spots. In two separate but similar studies in the song birds proliferative hot spots were found in the dorsal and ventral poles of the lateral ventricle at rostral sections in one and only in the ventral pole of the lateral ventricle at middle sections and extending into the caudal striatum in the other (Alvarez-Buylla et al., 1990b; Vellema et al., 2010). Additionally, in a study in the non-song bird, the ring dove (*Streptopelia risoria*), proliferative hot spots we observed only in the ventral pole of the lateral ventricle at rostral levels (Ling et al., 1997). The proliferative hot spots contain large quantities of radial glia cells indicating that these cells are the neuronal progenitors undergoing mitosis to generate new neurons and glia in the adult brains (Alvarez-Buylla et al., 1988; Alvarez-Buylla et al., 1990b; Alvarez-Buylla and García-Verdugo, 2002).

Apart from the SVZ of the lateral wall of the lateral ventricle, proliferating cells were observed in the other brain regions in both song and non-song birds including in the current study, but in varying degrees (Ling et al., 1997; Vellema et al., 2010; Mazenganya et al., 2017). In the current study higher numbers of PCNA immunoreactive cells were observed in the common ostrich in the olfactory bulbs and the pallium; moderate in the subpallium and brainstem regions including the cerebellum whereas comparably lower numbers were observed in equivalent brain regions in the emu. In canaries, lower numbers of BrdU positive cells were observed in the pallium, striatum, thalamus, midbrain, hindbrain, cerebellum and subventricular zones of the third, fourth and tectal ventricles 24 hours after initial BrdU injection (Vellema et al., 2010). Similarly, in ring doves few  $^3\text{H}$ -Thymidine labelled cells were found in the parenchyma throughout the brain after 24 hours of initial injection, but, no  $^3\text{H}$ -Thymidine labelled cells were observed in the SVZ of the third, fourth and tectal ventricles (Ling et al., 1997). The proliferating cells found in the brain parenchyma and other brain regions showed a similar morphology to those in SVZ of the lateral ventricle indicating that they are of the same origin and or at the same stage of differentiation (Vellema et al., 2010). In addition, the presence of BrdU and  $^3\text{H}$ -Thymidine cells in the brain parenchyma and

subtelencephalic regions shortly after initial injection suggests that new neurons are produced from local/ resident progenitors undergoing mitosis.

#### **6.4.3. Distribution of immature neurons in the brain of adult common ostrich and emu**

DCX immunoreactive cells and fibres were widespread throughout the brain of the common ostrich and the emu. The pattern of distribution of the DCX immunoreactivity was almost identical in the brain regions of the two species including the telencephalon, diencephalon, mesencephalon, rhombencephalon and cerebellum. Strong DCX immunoreactive staining was observed in the telencephalon particularly in the SVZ of the lateral wall of the lateral ventricle, hyperpallium subdivisions (HI, HD, and NC) and olfactory bulbs but less pronounced in the subtelencephalic regions except in the medial regions of the hypothalamus, optic tectum and the cortex of the cerebellum. These findings corroborates results from numerous studies which utilized DCX as a marker of immature and migrating neurons in avian brains (Capes-Davis et al., 2005; Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2010; Mezey et al., 2012; Vellema et al., 2014). Similarly, autoradiography and BrdU injection studies found that neuronal recruitment is abundant in the telencephalon than in the subtelencephalic regions of adult birds and so neuron recruitment is limited to the telencephalon in adult birds (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1994; Barnea and Nottebohm, 1994; Absil et al., 2003).

The SVZ of the lateral wall of the lateral ventricle is a well-studied neurogenic niche across avian species. Newly born neurons have been observed in higher numbers in hot spots located in the dorsal and ventral poles of the lateral ventricle in various avian species including canaries (Alvarez-Buylla et al., 1990b; Vellema et al., 2010; Vellema et al., 2014), zebra finches, (Kim et al., 2006), rock pigeons (Melleu et al., 2013; Melleu et al., 2015), ring doves (Ling et al., 1997), domestic chickens and pigeons (Mezey et al., 2012; Mazenganya et

al., 2017) and also in the current study. The majority of the DCX immunoreactive cells in the SVZ showed a unipolar, fusiform and bipolar morphology with a leading neurite suggestive of neuronal migration (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990). The neurites of the majority of these cells were oriented parallel to the wall of the lateral ventricle indicating tangential migration whereas the remaining few were oriented orthogonal to the ventricular wall suggestive of radial migration into the parenchyma (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Vellema et al., 2014).

During radial migration, immature neurons employ radial glia as scaffolds and are transported from the SVZ into the depth of the parenchyma (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990). This association between radial glia and immature neurons may explain the observation of higher numbers of DCX immunoreactive cells in the SVZ and regions abutting the medial regions of the pallial subdivisions in various avian species including in the current study (Capes-Davis et al., 2005; Kim et al., 2006; Boseret et al., 2007; Vellema et al., 2010; Melleu et al., 2013; Vellema et al., 2014; Mazenganya et al., 2017). In a study conducted in song birds, the canaries, mapping the distribution of radial glial cells, results confirmed that radial glia span a few micrometres from the SVZ into the parenchyma, therefore radial migration through radial scaffolds is only permissible for a short distance from the SVZ (Alvarez-Buylla et al., 1988). However, in mammals, radial glia spans across the whole telencephalon and facilitate neuronal migration across the cerebrum (Rakic, 1990; Rakic, 2003). Despite this anatomical disposition of radial glia in avian species, a common observation including in the current study is that DCX immunoreactive cells are found deep in the parenchyma and in high numbers in the lateral regions of the telencephalon far away from the SVZ and ranges of radial glia suggesting a possibility for another mode of neuronal migration being utilised by migrating cells (Kim et al., 2006; Boseret et al., 2007; Mezey et al., 2012; Melleu et al., 2013; Melleu et al., 2015; Mazenganya et al., 2017). Vellema et al. (2010) described random mode of migration whereby immature neurons reach

far and deep regions of the telencephalon through undefined routes and means. Conversely, it is possible that resident progenitor cells divide and generate new neurons in the parenchyma particularly in animals with large brains (Paredes et al., 2016).

In adult avian species, distinct migratory routes of newly formed neurons are not well defined particularly the equivalent of the well-known rostral migratory stream in mammals. Very few studies have alluded to the presence of a rostral migratory stream in birds, although with no clear delineation (Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013; Vellema et al., 2014). Herein, we observed three distinct bundles of migrating neuroblasts from the SVZ of the lateral wall of the lateral ventricle at rostral sections of the brain in both species at the dorsal and ventral poles and the middle portion of the lateral wall and the cells were directed into the parenchyma of the hyperpallium, medial striatum and nidopallium respectively. Apart from the SVZ of the lateral ventricles and the regions neighbouring it, DCX immunoreactive cells were found in the SVZ of the third, fourth and tectal ventricles in the current study and in also domestic pigeons and canaries (Vellema et al., 2014; Mazenganya et al., 2017), but in low densities. However, it remains unclear whether these subventricular zones contribute to new neurons in the subtelencephalic regions of the brain.

In the olfactory bulbs, DCX immunoreactive cells and fibres were found in high density in the inner layers particularly the IGrL, EPL, and MCL and less in the GL and ON layers in both species. Olfactory neurogenesis has been reported in few avian species and in those species the olfactory bulbs have been regarded as highly neurogenic including pigeons (Melleu et al., 2013; Mazenganya et al., 2017), domestic chicks and canaries (Vellema et al., 2010; Mezey et al., 2012; Vellema et al., 2014). The observation of olfactory neurogenesis including its associated cortices of the piriform cortex (CPi) and dorsolateral cortical area (CDL) warrants further investigations into the functional significance of adult olfactory neurogenesis. The olfactory sense in birds has been found to mediate various behaviours such as feeding, social recognition, reproduction and homing (Papi et al., 1972; Balthazart and

Taziaux, 2009). Therefore generation and recruitment of new neurons in the olfactory bulb in adult species may be vital to species adaptation to external environments and survival. For example, olfactory sense may be helpful to the common ostrich and emu which feed on varied diets including insects and seeds and have to forage large tracks of land for food.

The hippocampus and the parahippocampal areas overall showed a moderate density of DCX immunoreactive cells and fibres in the common ostrich and emu. However, the ventral hippocampus contained varied densities of DCX immunoreactivity from low to high with a highest density being observed in the triangular region (tr) in the emu. Similar findings were reported in the domestic pigeons (Mazenganya et al., 2017), rock pigeons (Melleu et al., 2013), marsh tits (Patel et al., 1997), zebra finches (Kim et al., 2006), canaries (Boseret et al., 2007; Balthazart et al., 2008). Various reports have been reported on the degree and spread of neuronal recruitment in the adult avian hippocampus. High levels of hippocampal neuronal recruitment was observed in species relying on spatial memory such as food caching and migration (Hoshooley and Sherry, 2007; LaDage et al., 2009; Sherry and Hoshooley, 2009; LaDage et al., 2010; Sherry and Hoshooley, 2010; LaDage et al., 2011). Very low levels of hippocampal neuronal recruitment were reported in the domestic chickens and canaries (Mezey et al., 2012; Vellema et al., 2014). A separate study in canaries found no evidence of neuronal recruitment in the hippocampus (Vellema et al., 2010). In food caching birds, adult hippocampal neurogenesis varies according to the time of the year. It is highest in the autumn when food sources are abundant and well reduced during periods of low food supplies in winter (Sherry and Hoshooley, 2009, 2010). It is therefore unclear whether neuronal recruitment in hippocampus of non-food caching species conforms to this seasonal variation, hence the need to examine and compare adult hippocampal neurogenesis in various species of birds during different times of the year.

Recruitment of neurons in adult bird species in the pallial regions generally shows a non-uniform pattern of distribution. In song birds, the pallial regions house the song control

nuclei and most of the immature neurons are found within and in the vicinity of these song control nuclei more than other regions (Vellema et al., 2010). However in both song and non-song birds, observations of higher numbers of immature neurons in the medial and lateral regions of the pallial subdivisions and less in the core regions seems to be a common feature (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013; Vellema et al., 2014; Mazenganya et al., 2017). Herein, the pallial subdivisions of the hyperpallium intercalatum and densocellulare, mesopallium, nidopallium and the nidopallium caudale had densest DCX immunoreactive cells and fibres in the medial and lateral regions in both species. In rostral sections, bundles of migrating neuroblasts could be seen extending into the hyperpallium, nidopallium and medial striatum regions indicating preferential recruitment into these pallial subdivisions. The pallial and subpallial subdivisions of the avian telencephalon participate in higher order/ executive functions of the brain; hence the presence of high numbers of immature neurons may be complimentary. The nidopallium caudale compares with the mammalian prefrontal cortex and is involved in the execution of higher order functions in birds such as the decision making (Güntürkün, 2005). The striatum and hyperpallium apicale constitutes an association prefrontal cortex and they are involved in memory and learning, motor control and visual processing respectively (Luo et al., 2001; Kuenzel et al., 2011; Melleu et al., 2013). Surprisingly, in many species of birds examined to date, DCX immunoreactivity is less pronounced in the core regions of the pallial subdivision particularly the hyperpallium apicale, nidopallium and mesopallium which are important centres for some of the cognitive functions including somatosensory and visual processing (core of hyperpallium apicale) and auditory (core of mesopallium) (Luo et al., 2001; Kao et al., 2005; Melleu et al., 2013). The arcopallium showed no DCX immunoreactive structures in both the ostrich and emu. This observation corroborates findings from numerous studies which reported that the arcopallium lacks neuronal recruitment in birds (Boseret et al., 2007; Balthazart et al., 2008;

Vellema et al., 2010; Vellema et al., 2014). However this is topographically improbable as the arcopallium lies very close to the caudo-ventral pole of the lateral ventricle, a region with high concentrations of both proliferating and immature neurons. Vellema et al. (2010) suggested that the arcopallium may possess some repelling mechanism which prohibits the migration and eventual recruitment of new neurons.

In the common ostrich and emu, the subtelencephalic regions of the brain contained low density of weakly stained DCX immunoreactive cells and fibres except in the paraventricular regions of the third ventricle within the medial hypothalamus and the cerebellar cortex. Neuronal recruitment in the subtelencephalic regions of the adult avian brain is generally low under normal physiologic conditions (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2010; Vellema et al., 2014; Mazenganya et al., 2017). However, substantial amounts of new neurons were observed in pathological and ablation studies particularly in the diencephalon (Cao et al., 2002; Chen et al., 2006). The weak DCX immunoreactivity in the subtelencephalic regions was associated with dendritic and axonal remodelling instead of neuronal recruitment (Nacher et al., 2001; Vellema et al., 2010; Vellema et al., 2014). The presence of moderately dense DCX immunoreactivity in the cerebellar cortex of the common ostrich and emu may be complementary to the demands of bipedal locomotion and posture which relies entirely on balance. Conversely, the common ostrich and emu belong to the phylogenetically old paleognathe group of birds; hence the presence of a pronounced DCX immunoreactivity in the cerebellum can be compared to other evolutionary old vertebrates such as teleost fishes where the cerebellum is a well pronounced neurogenic niche (Zupanc and Horschke, 1995; Zupanc et al., 2005).

In conclusion, the results of the current study corroborates earlier findings on birds from the neognathous group showing that adult neurogenesis is widespread throughout the brains of adult birds and also suggest that the process may be vital to learning, environmental adaptation, cognition, reproduction and survival of species. The exception being the

pronounced DCX immunoreactivity in the cerebellum related to the locomotor function in these species.



## **CHAPTER SEVEN: Discussion**

### **7.1. Overview**

The studies in the current thesis examined the topographical distribution of neurogenic niches in the avian brains from various taxonomic orders. Specifically the studies focused on the distribution of cell proliferation, identification of migratory routes of neuroblasts and mapping of the recruitment sites of newly generated neurons throughout the brains of the birds from various orders namely, the Columbiformes (domestic pigeons; racing homer and utility carneau pigeons); Psittaciformes (Congo African grey and Timneh grey parrots); Passeriformes (pied crow); Struthioniformes (common ostrich) and Casuariiformes (emu). Free floating immunohistochemical methods against proliferating cell nuclear antigen (PCNA) which labels proliferating cells and doublecortin (DCX) which stains immature neurons were used to determine the regions of cells proliferation, migratory routes of immature neurons and recruitment sites as final destination in the brains of the birds to see if these processes are associated with variations in behavioural repertoires and/or show phylogenetic relations.

In summary the results showed that: 1) cell proliferation is widespread throughout the brains of all the birds studied with high levels of cell proliferation in the olfactory bulbs and the SVZ of the lateral ventricles in the telencephalon; 2) the SVZ of the lateral ventricle is the primary neurogenic zone contributing new neurons in the adult telencephalon; 3) immature neurons were localised in high density in the olfactory bulb and the subdivisions of the telencephalon, but in low densities in the subtelencephalic regions except in the ratite birds (common ostrich and emu) where moderate densities were observed in the cortex of the cerebellum; 4) the distribution of the immature neurons showed region specificity within the subdivisions of the telencephalon across all species with high densities in the medial and lateral regions of the pallial and subpallial regions, but devoid in the core areas; 5) species-specific distribution of immature neurons was also observed in the hippocampal formations

and song control systems of the pied crow and cerebellum of the ratite birds indicating behavioural specialisations and 6) migratory routes of immature neurons were not clearly defined across species except in the ratites birds where three distinct bundles of migrating neuroblasts were observed in the telencephalon. In analysing these principal findings many questions emerged regarding the behavioural and functional implications of adult neurogenesis across these avian species. The sections below present possible explanations towards species diversity and functional implications of adult neurogenesis on species behaviour and adaptation for survival.

## **7.2. Distribution of cell proliferation in the brains of adult birds**

In the current study, proliferating cells as labelled by the PCNA antigen were distributed heterogeneously throughout the brains of all the species examined. Proliferating cells were observed in the olfactory bulb, telencephalic subdivisions of pallium and subpallium, diencephalon, mesencephalon, rhombencephalon and the cerebellum. The distribution of proliferating cells in the brain of adult birds has been characterized in various species of song and non-song birds exhibiting diverse behavioural repertoires using autoradiography and bromouridine (BrdU) immunohistochemistry (Goldman and Nottebohm, 1983; Nottebohm, 1985; Alvarez-Buylla et al., 1990b; Alvarez-Buylla and Kim, 1997; Ling et al., 1997). Results from these numerous studies revealed that cell proliferation is common in the brains of adult birds, but differences exist within and between species on the topographical distribution of proliferating cells. Pioneering studies on adult neurogenesis in birds concurred that cell proliferation is limited to the telencephalon, particularly in the subventricular zone of the lateral ventricle (Nottebohm, 1981; Goldman and Nottebohm, 1983; Nottebohm, 1985; Walton et al., 2012). However, more recent studies showed similar findings to the current study that cell proliferation is widely distributed throughout the brains of adult birds (Ling et al., 1997; Vellema et al., 2010; Mazenganya et al., 2017).

The subventricular zone of the lateral wall of the lateral ventricle showed a high density of PCNA immunoreactive cells in all the species examined in the current study indicating high levels of cell proliferation. Similarly, numerous studies have shown that the SVZ of the lateral ventricle harbours large quantities of proliferating cells and is considered a primary source (neurogenic niche) of neurons in adult birds (Nottebohm, 1985; Alvarez-Buylla et al., 1990b; García-Verdugo et al., 2002; Mezey et al., 2012; Melleu et al., 2013). Cell proliferation happens throughout the entire subventricular of the lateral ventricle, but it is found in higher rates at intervals along the wall of the lateral ventricles in regions referred to as the proliferative hot spots (Alvarez-Buylla et al., 1990b; Vellema et al., 2010). In birds, proliferative hot spots are located in the dorsal and ventral reaches of the SVZ of the lateral wall of the lateral ventricle (Alvarez-Buylla et al., 1990b; Vellema et al., 2010). The ventral hot spot contributes more cells than the dorsal counterpart and both contain large quantities of radial glia cells indicating that radial glia are the primary neural progenitors in the SVZ (Alvarez-Buylla et al., 1988; Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1998; Alvarez-Buylla and García-Verdugo, 2002). The location of proliferative hot spots varies along the rostro-caudal axis of the lateral ventricle in different species of birds examined to date (Alvarez-Buylla et al., 1990b; Ling et al., 1997; Vellema et al., 2010; Melleu et al., 2013; Vellema et al., 2014). Consistent with earlier researchers, we found proliferative hot spots at different levels in the species of birds examined in the current study. Proliferative hot spots were observed in the dorsal and ventral poles of the lateral ventricle at rostral levels in the Congo African grey parrot, Timneh grey parrot (only in the dorsal pole), domestic pigeons, pied crow, common ostrich and emu. Additional hot spots were observed at middle telencephalic levels located in the dorsal and ventral poles of the lateral ventricle in the common ostrich. Another intriguing finding was that the locations of the hot spots varied in closely related species, as shown for example within the two species of the African grey parrots and in ratite birds (common ostrich and emu). The subventricular zones of the third,

fourth and tectal ventricles also evinced some heterogenous distribution of proliferating cells with highest levels in the pied crow and domestic pigeons and moderate levels in the ratites and parrots species. Correspondingly varied and low levels of cell proliferation in the SVZ of the tectal, third and fourth ventricles were observed in separate studies in the species of canaries (Alvarez-Buylla et al., 1990b; Vellema et al., 2010). Conversely, a study in ring doves did not find any proliferating cells in the above mentioned regions indicating species specific differences among avian species (Ling et al., 1997). According to Alvarez-Buylla et al. (1990b) the subventricular zone of the tectal ventricle contributes less than one percent of the total proliferating cells in the avian brain; however, it is unclear where cells from the regions contribute to the pool of adult born neurons.

Apart from the SVZ of the lateral ventricles, high levels of cell proliferation were observed in the olfactory bulbs of all birds examined in the current study, particularly in the inner three layers except in the emu which showed high densities of proliferating cells in the second and fourth layers. A review of literature found few studies reporting on the presence and distribution of proliferating cells in the olfactory bulb of avian species (Vellema et al., 2010; Mezey et al., 2012; Vellema et al., 2014; Mazenganya et al., 2017). In these few studies it was shown that the olfactory bulb exhibits high levels of cell proliferation in comparison to other regions of the brain (Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013; Vellema et al., 2014; Mazenganya et al., 2017). The paucity of information on cell proliferation in the olfactory bulbs can be attributed in part to tissue loss during dissection and experimental procedures since the olfactory bulbs of most avian species are very small. Evidence of PCNA immunoreactivity in the olfactory bulbs of adult birds indicates the presence of local progenitors in the olfactory bulbs which are capable of cell division contributing to a local pool of neurons that possibly integrate in local circuits (Vellema et al., 2010; Vellema et al., 2014). However in mammals, new neurons to the olfactory bulbs are born in distant regions in the SVZ of the lateral ventricle and then migrate long distances

through the rostral migratory stream (Gould and Gross, 2002; Chawana et al., 2013; Patzke et al., 2013; Ihunwo et al., 2016).

Cell proliferation was also observed in varying densities in the parenchyma of the pallial and subpallial subdivisions of the telencephalon, diencephalon, midbrain and hindbrain including the cerebellum in all species examined in the current study. These findings are in agreement with separate studies in ring doves and canaries where proliferating cells were found in the parenchyma of the brain away from the SVZ 24 hours after [ $^3\text{H}$ ]-thymidine and BrdU injection (Ling et al., 1997; Vellema et al., 2010). The results suggest the proliferative potential of local neuronal progenitors deep in the parenchyma of the telencephalon of adult birds contradicting the phenomenon that the SVZ is the sole and primary source of neurons. Resident progenitors were found to contribute to local neurogenic niches, particularly in mammalian species with larger brains (Vellema et al., 2010; Paredes et al., 2016), hence birth dating and lineage studies need to be carried out in avian species with larger brain to body ratios, such as African grey parrots and pied crows to confirm this phenomenon. The proliferating cells in the parenchyma of the telencephalon were found to exhibit a similar morphology to proliferating cells in the subventricular zones of the lateral ventricles implying that these cells may be from the same neurogenic niche/source or they may be at similar levels of maturation (Vellema et al., 2010).

Proliferation in the hippocampal formation was observed across all species examined in the current study. The dorsal hippocampus and area parahippocampalis evinced similar levels of proliferation across species while the ventral part of hippocampus presented with varied degrees of proliferation. With reference to the divisions of the ventral hippocampus (Atoji and Wild, 2004), the lateral and triangular layers of the ventral hippocampus showed high levels of cell proliferation than the medial layer in all species. Of all the examined species, the pied crow showed low levels of cell proliferation in the ventral hippocampus while highest levels of cell proliferation were observed in the common ostrich.

Various reports have been put forward on the extent of cell proliferation and neurogenesis in the hippocampus of both song birds and non-song birds (Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013; Vellema et al., 2014; Melleu et al., 2015; Mazenganya et al., 2017). Adult hippocampal neurogenesis is affected by a number of factors including seasonal variations, environmental complexity, social environment, physical activity, stress, steroid hormones, disease and age (Barnea and Nottebohm, 1994, 1996; Barnea et al., 2006; Barkan et al., 2007; Barnea and Pravosudov, 2011). Functionally, hippocampal neurogenesis is associated with learning and memory, particularly in spatial memory dependant tasks such as food caching and retrieval and navigation (Hoshooley and Sherry, 2007; LaDage et al., 2009; LaDage et al., 2010; Sherry and Hoshooley, 2010; LaDage et al., 2011).

In all birds examined, cell proliferation was observed in the subtelencephalic regions including the diencephalon, mesencephalon, rhombencephalon and the cerebellum. The identity and fate of proliferating cells in these regions is yet to be understood. According to Ling et al. (1997), the [<sup>3</sup>H]-thymidine labelled cells in the subtelencephalic regions had a small soma and a darker nucleus and failed to co-express neuron specific markers suggesting that they are of glial lineage. Generation of neurons in the subtelencephalic regions has been demonstrated in lesioned experiments particularly in the preoptic region of the diencephalon indicating the potential of reactive neurogenesis in adult avian species (Cao et al., 2002; Chen et al., 2006). Within the cerebellum, cell proliferation was conspicuous in the Purkinje cell layer of all the birds examined. The cerebellum in phylogenetically old species, particularly teleost fishes is a large neurogenic niche contributing about 75% of all adult generated cells in the brain (Zupanc and Horschke, 1995; García-Verdugo et al., 2002; Zupanc et al., 2005; Zupanc, 2006). The recapitulation and preservation of this niche in adult birds indicates phyletic relationships among vertebrates.

Generally, cell proliferation in birds is affected by both intrinsic and extrinsic factors (Barnea and Pravosudov, 2011). In ring doves, the level of cell proliferation was negatively

affected by age, but its distribution patterns remained unchanged throughout the telencephalon of adult birds (Ling et al., 1997). Inferring this to the current study, all the animals examined were adult breeding pairs just over puberty and the results were consistent with age matched groups in the ring doves except in the emu which showed generally reduced levels of PCNA immunoreactivity. This can be explained in two parts; first the relative age of adulthood in the emu cannot be compared to other species and secondly the PCNA antibody may not exclusively pick proliferating cells in the emu.

### **7.3. Neuronal migration and recruitment in adult birds**

DCX immunoreactive structures were distributed heterogeneously throughout the brain in regions including the olfactory bulb, telencephalon, diencephalon, mesencephalon, rhombencephalon and the cerebellum in all the studied animals. Similar distribution patterns have been described in both song birds and non-song birds examined to date (Capes-Davis et al., 2005; Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Mezey et al., 2012; Mazenganya et al., 2017). Strong and intense DCX immunoreactivity was observed in the olfactory bulb and the telencephalon of all the examined species herein. The olfactory sense in birds is vital in the regulation of behaviours such as feeding, parenting, reproduction, social recognition, detection of danger and migration (Papi et al., 1972; Balthazart and Taziaux, 2009; Akers et al., 2010). Newly born granule neurons in the olfactory bulbs integrate as interneurons contributing to plasticity of local olfactory circuits (Akers et al., 2010). Despite this remarkable contribution to species survival, adult olfactory neurogenesis has not received much attention in birds and to date very few studies have examined the potential of adult olfactory neurogenesis (Vellema et al., 2010; Mezey et al., 2012; Vellema et al., 2014; Mazenganya et al., 2017).

The majority of DCX immunoreactive cells in the telencephalon were located within and around regions abutting the SVZ of the walls of the lateral ventricle medially, lateral

regions and few in the core areas in all species examined. Most of the DCX immunoreactive cells in the regions abutting the SVZ of the lateral wall of the lateral ventricle were unipolar, fusiform and or bipolar in morphology, depicting that they were migrating cells (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Lois and Alvarez-Buylla, 1994). Few cells showed a multipolar morphology and these cells were found mainly in the lateral cortical regions far from the SVZ. Migrating cells possess a leading neurite orientated towards the intended direction (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990). The most common migratory route in all birds examined was parallel to the lateral wall of the lateral ventricle. A second migratory route observed was orthogonal to the lateral wall of the lateral ventricle with migrating neuroblasts oriented towards the parenchyma of the telencephalon. The former is referred to as the tangential migration while the latter is the radial migration (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990). Very few studies in birds have identified tangential migration of neurones out of the SVZ of the lateral ventricles extending into the olfactory bulb (Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013; Vellema et al., 2014). In these few studies, the exact rostral migratory stream is not clearly defined as it is depicted in mammalian species. The possible explanation to this may be twofold: (1) the olfactory bulb in birds houses an abundance of proliferating cells as observed in the current study, particularly in regions adjacent to the olfactory ventricle and these cells might be contributing to new neurons which will subsequently integrate into local circuits diminishing the need for neurons born in distant places, and (2) the sectioning method employed in most avian studies including the current study, where the brain was sectioned along the coronal plane instead of the sagittal plane made it difficult to view the rostral migratory route (Vellema et al., 2010; Mezey et al., 2012; Melleu et al., 2013; Vellema et al., 2014).

Radial migration of neuroblasts is guided by radial glia cells as scaffolds leading immature neurons into the parenchyma (Alvarez-Buylla and Nottebohm, 1988; Alvarez-



Buylla, 1990; Rakic, 1990; Vellema et al., 2010). In birds, radial glia extend very short distances into the parenchyma suggesting that migration of immature neurons through this route is only permissible for a very few micrometres into the parenchyma (Alvarez-Buylla et al., 1988; Alvarez-Buylla, 1990; Vellema et al., 2010; Vellema et al., 2014). However in mammalian species, radial glia spans across the whole cerebrum and are responsible for the migration of neuroblasts to distant locations in the cortex (Rakic, 1990). With this information, it becomes problematic to explain the common observation in birds where new neurons are found deep into the parenchyma away from the subventricular zones and into the lateral regions of the telencephalon. Vellema et al. (2010) described random migration of multipolar DCX immunoreactive cells and suggested that cells with a mature like phenotype migrate in an undefined route to reach deeper regions of the telencephalon. In adult neurogenesis, multipolar cells are regarded as mature, non-migratory and integrating cells; hence the notion that these cells are capable of migration raises more questions than answers. To support this concept, studies in song bird canary found that DCX positive multipolar cells failed to co-express neuron specific markers for mature neurons indicating that these cells are still immature and are capable of migration (Vellema et al., 2010; Vellema et al., 2014). Another possibility to explain the presence of multipolar DCX cells deep in the parenchyma and in the lateral regions of the telencephalon can be that local/resident progenitors divide and contribute to the local pool of neurons as suggested by (Paredes et al., 2016) regardless of the brain size. In the current study we observed both PCNA and DCX immunoreactive cells in the parenchyma and in the lateral regions of the telencephalon. It is however not clear if PCNA immunoreactive cells gave rise to the DCX immunoreactive cells observed. Explanations to such observations can be provided by designing double labelling immunohistochemistry studies whereby PCNA and DCX markers are co-labelled to determine the lineage of proliferating cells and their destiny. In the current study, clearly defined migratory routes/bundles of migrating cells into the parenchyma of the telencephalon were observed in the

ratite birds, the common ostrich and emu. The bundles of migrating cells could be seen at three distinct points extending from the lateral wall of the lateral ventricle into the hyperpallium, mesopallium/nidopallium regions and the medial striatum at rostral levels of the telencephalon. Migration of new neurons into the hyperpallium and the medial striatum has been observed in a variety of avian species, receiving new neurons from the dorsal and ventral hot spots (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1990b; Melleu et al., 2013).

Recruitment of neurons as depicted by the localisation of DCX immunoreactive cells was wide spread in the telencephalon of all birds examined in the current study. The distribution pattern of DCX immunoreactive structures showed region/area specificity within individual species and species-specificity differences among various orders of species examined. DCX immunoreactivity was abundant and strong in the olfactory bulbs and the telencephalon, but weak in the subtelencephalic regions in all groups of birds examined herein. Within the telencephalon, DCX immunoreactivity was concentrated in the medial and lateral regions of the pallial and subpallial regions and devoid in the core regions. Similarly, several studies utilising DCX as a marker of adult born neurons have described this pattern of distribution of immature neurons in adult birds (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2010; Melleu et al., 2013; Vellema et al., 2014; Melleu et al., 2015; Mazenganya et al., 2017). This distribution pattern raises concerns on the functional implications of adult neurogenesis in birds considering that the core regions of most pallial subdivisions participate in sensorimotor activities (Melleu et al., 2013; Melleu et al., 2015). Functions such as vision, auditory, and motor activities are carried out in the core of hyperpallium apicale, mesopallium, and the entopallium respectively (Luo et al., 2001; Kuenzel et al., 2011; Melleu et al., 2013; Melleu et al., 2015). Conversely the lack of recruitment of new neurons in these regions may suggest that the functional integrity needs to be preserved throughout the life of the animal. In the pied crow, species-specific neuronal

recruitment was observed in the song control nuclei whereby high densities of DCX immunoreactive structures were evinced including in the high vocal centre (HVC), nucleus mesencephalicus profundus (MAN) and area X but not in the robust nucleus of the arcopallium (RA). The distribution pattern of immature neurons is typical of all song birds examined to date; however, the functional implications of adult born neurons in the pied crow song nuclei remains elusive since the song produced by the pied crows is not as elaborate as it is in the majority of song birds (Wang et al., 2009). The hippocampus, particularly the ventral hippocampus showed varied distribution of immature neurons across the studied species of birds. A moderate density of DCX immunoreactive cells was observed in the triangular and lateral regions of the ventral hippocampus in the domestic pigeons, African grey parrots and the ratite birds but very low densities in the pied crows. The ventral hippocampus is the equivalent of the dentate gyrus in mammalian species (Atoji and Wild, 2004). The observation of less dense DCX immunoreactivity in the ventral hippocampus of pied crows is surprising considering that close species in the genus *corvus* demonstrate remarkable spatial memory dependant tasks such as food caching and retrieval (Emery and Clayton, 2004). Specific information about spatial memory dependent tasks is lacking in the pied crow, hence most of the behavioural information is inferred from closely related species such as the Caledonian crows and magpies (Emery and Clayton, 2004). Another brain region which showed species-specific recruitment of neurons is the cortex of the cerebellum in ratites. This region contained a moderate density of DCX immunoreactive cells; hence functionally this can be associated with balance and bipedal locomotion which are fundamental to the survival of these species, particularly during foraging. Conversely the presence of DCX immunoreactive cells and fibres in the cortex of the cerebellum in ratite species may indicate the recapitulation of this neurogenic niche from evolutionarily old vertebrate species such as teleost fishes where the cerebellum is a robust neurogenic region indicating phylogenetic relationships among vertebrates species. In all birds examined, apart from the cerebellum in

ratite, all the subtelencephalic regions exhibited low levels of DCX immunoreactivity. DCX immunoreactivity in these regions has been associated with dendritic and synaptic reorganisation rather than adult neurogenesis (Nacher et al., 2001; Brown et al., 2003). Considerable levels of adult neurogenesis in these regions are only noticed during ablation and or pathological states whereby neuronal regeneration is compensatory (Cao et al., 2002; Cheng et al., 2004; Chen et al., 2006).

#### **7.4. Limitations of the study**

Limitations to the current study were entirely methodological. The reliance on PCNA antibody as a marker of proliferating cells has its own disadvantages. The PCNA antibody is expressed in all stages of the cell cycle and also in cells undergoing DNA replication without cell division (Kurki et al., 1988; Hall et al., 1990). These characteristics do lead to misrepresentation of the actual cells undergoing cell division and hence over estimation of the potential of cell proliferation. However, the PCNA antibody remains a reliable marker of cell proliferation, particularly in avian species where common proliferation markers such as Ki-67 are not detected and also in cancer studies (Balthazart and Ball, 2014a). In the current study, the PCNA antibody was the only available endogenous marker of cell proliferation which was compatible with avian species. In the current study another potential limitation arose from the use of only male birds eliminating comparisons between males and females to show the effect of sex hormones on adult neurogenesis. The study also utilised two animals in each experiment which are below the minimum of three recommended especially when conducting quantitative studies in order to have an appropriate statistical significance. However the current study was deliberately designed to evaluate qualitatively the potential of adult neurogenesis in seven species of birds. Lastly the study compared animals from the wild with domesticated/farm animals on the potential of adult neurogenesis. Domestication of animals is

viewed as a selection pressure that can exert profound effects on adult neurogenesis and this may manifest in the varied states of expression of the markers used in the study.

### **7.5. Recommendations to future studies**

Future studies must employ double labelling and lineage markers in order to have a clear understanding of the degree of cell proliferation and neuronal recruitment in the brains of adult birds and to make resolute conclusions on the possible functional and behavioural implications of adult neurogenesis. Additional lineage studies require coupling with quantitative methods, particularly unbiased stereology to determine the exact quantities of proliferating cells and immature neurons. This will enable comparative assessments of adult neurogenesis in diverse species with various behavioural repertoires and also to determine the extent of adult neurogenesis in different brain regions within and among species in order to understand the brain function and behaviour. Lastly, adult neurogenesis is influenced by a number of environmental factors including social arrangements and domestication. Most birds live in large social groups which might impact on adult neurogenesis; hence the need to design experiments which consider the implications of social environmental variables on adult neurogenesis to understand its effect on group dynamics, social isolation and partnerships and on overall brain functions. The influence of domestication on adult neurogenesis is not well understood. Designing a study focusing on the impact of domestication and generations in captivity will help to understand the influence of various associated exogenous factors on adult neurogenesis.

### **7.6. Conclusions**

The following conclusions can be drawn from the current thesis:

- i. Cell proliferation and neuronal recruitment are widespread in the brain of adult birds and the processes are concentrated in similar brain regions of closely related species
- ii. Neuronal recruitment is conspicuous in the telencephalon and olfactory bulbs of adult birds
- iii. Adult neurogenesis is a conserved trait occurring in all seven avian species investigated.
- iv. Recruitment of neurons in brain of adult birds shows area specificity and species-specific variations which could be associated with behaviours such as navigation, foraging, song production and cognitive abilities.
- v. Neuronal recruitment in the cerebellum especially in the ostrich and emu might complement bipedal locomotion and or show phylogenetic relationships and such will need further investigation.
- vi. Antibody specificity, particularly the PCNA antibody immunoreactivity varies in closely related species.

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## Appendix



**STRICTLY CONFIDENTIAL**

**ANIMAL ETHICS SCREENING COMMITTEE (AESC)**

**CLEARANCE CERTIFICATE NO.** 2013/05/02B

**APPLICANT:** Mr P Mazenganya

**SCHOOL:** Anatomical Sciences

**DEPARTMENT:**

**LOCATION:**

**PROJECT TITLE:** A comparative survey of adult neurogenic sites in avian species

**Number and Species**

6 each of owl, ostrich, emu, vulture, crow, weaver, Congo and Timneh grey parrots, racing and utility pigeons

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20130319. This approval remains valid until 20150318.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: \_\_\_\_\_

(Chairperson, AESC)

Date: \_\_\_\_\_

6/5/13

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: \_\_\_\_\_

(Registered Veterinarian)

Date: \_\_\_\_\_

6/5/13

cc: Supervisor: Professor A Ihunwo  
Director: CAS

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# Putative adult neurogenesis in two domestic pigeon breeds (*Columba livia domestica*): racing homer versus utility carneau pigeons

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## Abstract

Generation of neurons in the brains of adult birds has been studied extensively in the telencephalon of song birds and few studies are reported on the distribution of PCNA and DCX in the telencephalon of adult non-song learning birds. We report here on adult neurogenesis throughout the brains of two breeds of adult domestic pigeons (*Columba livia domestica*), the racing homer and utility carneau using endogenous immunohistochemical markers proliferating cell nuclear antigen (PCNA) for proliferating cells and doublecortin (DCX) for immature and migrating neurons. The distribution of PCNA and DCX immunoreactivity was very similar in both pigeon breeds with only a few minor differences. In both pigeons, PCNA and DCX immunoreactivity was observed in the olfactory bulbs, walls of the lateral ventricle, telencephalic subdivisions of the pallium and subpallium, diencephalon, mesencephalon and cerebellum. Generally, the olfactory bulbs and telencephalon had more PCNA and DCX cells than other regions. Two proliferative hotspots were evident in the dorsal and ventral poles of the lateral ventricles. PCNA- and DCX-immunoreactive cells migrated radially from the walls of the lateral ventricle into the parenchyma. In most telencephalic regions, the density of PCNA- and DCX-immunoreactive cells increased from rostral to caudal, except in the mesopallium where the density decreased from rostral to middle levels and then increased caudally. DCX immunoreactivity was more intense in fibres than in cell bodies and DCX-immunoreactive cells included small granular cells, fusiform bipolar cells, large round and or polygonal multipolar cells. The similarity in the distribution of proliferating cells and new neurons in the telencephalon of the two breeds of pigeons may suggest that adult neurogenesis is a conserved trait as an ecological adaptation irrespective of body size.

**Key Words:** nerve regeneration; proliferating cell nuclear antigen; doublecortin; immunohistochemistry; avian brain; racing homer; utility carneau; brain evolution; neural regeneration

## Introduction

The process of adult neurogenesis occurs in both invertebrates and vertebrates, including humans (Eriksson et al., 1998; Bartkowska et al., 2010; Barnea and Pravosudov, 2011). In birds, generation of new neurons is limited to the dorsal and ventral reaches of the subventricular zone of the lateral ventricles (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1998). Along these areas of the subventricular zone, proliferating cells form aggregates referred to as proliferative hotspots (Alvarez-Buylla et al., 1990a). From the walls of the lateral ventricles, new neurons migrate to various areas of the telencephalon which includes, but are not limited to, the high vocal centre, area X, the nidopallium caudale in song birds, and the hippocampus in both non-song birds and song birds (Paton and Nottebohm, 1984; Nottebohm, 1985; Alvarez-Buylla et al., 1994; Lipkind et al., 2002; Sherry and Hoshoooley, 2010; Melleu et al., 2013).

In adult song birds, such as canaries, and food caching birds, such as the black capped chickadees, seasonal variations in the recruitment of new neurons in the high vocal centre and hippocampus, respectively, have been observed (Kirn and Nottebohm, 1993; Barnea and Nottebohm, 1994).

There is evidence that adult neurogenesis varies in the members of the same species from different populations, for example in black capped chickadees (Chancellor et al., 2011) and mice (Kempermann et al., 1997).

Adult neurogenesis has been reported in the telencephalon of song birds including canaries (Alvarez-Buylla and Nottebohm, 1988; Balthazart et al., 2008) and zebra finches (Kim et al., 2006) and non-song birds such as chickens (Mezey et al., 2012), black capped chickadees (Sherry and Hoshoooley, 2010), Japanese quails (Balthazart et al., 2010), ring doves (Ling et al., 1997) and rock pigeons (Melleu et al., 2013, 2016). Domestic pigeons (*Columba livia domestica*) of the order Columbiformes are descendants of the wild rock pigeon through domestication and selective breeding (Levi, 1986; Stringham et al., 2012). The racing homer possesses characteristics such as increased flight speed, large home ranges, improved spatial memory, and larger hippocampal formations and olfactory bulbs than other pigeon breeds (Bingman et al., 2006; Rehkämper et al., 2008; Mehlhorn and Rehkämper, 2009). Utility pigeons were selectively bred for their fast growth and large body size desirable for meat production. These different characteristics in the two breeds of domestic pigeons allow

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examining and comparing the process of adult neurogenesis in closely related species with different behaviour repertoires.

Examination of multiple species with phylogenetically diverse traits and also in closely related species, or breeds, in different ecological niches may facilitate our understanding of the functions of adult neurogenesis and the factors contributing to variances in this neural trait amongst species (Jarvis et al., 2005; Amrein and Lipp, 2009; Bartkowska et al., 2010; Ihunwo and Olaleye, 2014). Based on this premise, we examined putative adult neurogenesis in the brains of two breeds of the adult domestic pigeon (*Columba livia domestica*), the racing homer pigeons and the utility carneau pigeons using the markers proliferating nuclear cell antigen (PCNA) and doublecortin (DCX) which label proliferating cells and immature neurones respectively (Hall et al., 1990; Brown et al., 2003; Melleu et al., 2013).

## Materials and Methods

### Animals and tissue processing

Four adult male domestic pigeon brains, two of each of racing homer and utility carneau pigeons were purchased from a local breeder and used in this study. The animals were bred in isolated cages according to the breeds in large social groups consisting of both males and females. The animals were supplied with water and food *ad libitum*.

The pigeons were 8 months old and sexually mature (Ling et al., 1997) and sex was determined by inspecting gonads during dissection. The animals were purchased and sacrificed during spring (November 1–8) in 2013. Animals were transported from the aviary to the University of the Witwatersrand Central Animal Services (CAS) in well ventilated, roomy plastic boxes. Animals were housed in the CAS and allowed to acclimatize for a period of 7 days with food and water supplied *ad libitum*. All animals were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (approval No. 2013/05/02B), which parallel those of the NIH guide for the care and use of animals in scientific experimentation.

Five minutes prior to being euthanized, all animals were given an intramuscular dose of heparin (0.5 mL) to prevent blood clotting. Animals were euthanized with an intraperitoneal injection of Euthapent (0.5–1 mL/kg) and the average body mass was  $316.00 \pm 23.33$  g for racing homer pigeon and  $542.35 \pm 7.00$  g for utility carneau pigeons. Animals were transcardially perfusion-fixed, initially with a rinse of 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) solution. Brains were carefully removed from the skull, post fixed overnight in 4% paraformaldehyde in 0.1 M PB. The brains were then weighed  $2.20 \pm 0.00$  g for racing homer pigeons and  $2.80 \pm 0.14$  g for utility carneau pigeons, cryopreserved in 30% sucrose in 0.1 M PB at 4°C for 3 days and then stored in an antifreeze solution at –20°C. Before sectioning, the tissue was allowed to equilibrate in 30% sucrose in 0.1 M PB at 4°C. The brains were frozen in dry ice and sectioned along the coronal plane into 50 µm thick sections, on a sliding microtome (Microm HM 430, Thermo Scientific, Schaumburg, IL, USA). One of three series of sections from each animal were taken and stained for Nissl substance, PCNA and DCX.

### Nissl staining for cytoarchitecture

The first series of sections for Nissl staining were mounted

on 0.5% gelatine coated slides, dried overnight, cleared in a 1:1 mixture of 100% ethanol and 100% chloroform and stained with a 1% cresylviolet solution (Sigma-Aldrich).

### Immunohistochemistry for PCNA and DCX

The second and third series of sections from each animal were used for free floating PCNA and DCX immunohistochemistry. The sections were incubated in a solution containing 1.6% H<sub>2</sub>O<sub>2</sub>, 49.2% methanol and 49.2% 0.1 M PB, for 30 minutes to reduce endogenous peroxidase activity, which was followed by three 10-minute rinses in 0.1 M PB. To block unspecific binding, the sections were then pre-incubated for 2 hours, at room temperature under gentle shaking, in a blocking buffer solution consisting of 3% normal horse serum (for PCNA sections) or 3% normal rabbit serum (for DCX sections), 2% bovine serum albumin, and 0.25% Triton X-100 in 0.1 M PB. Following pre-incubation, the primary antibodies were added to the blocking buffer solution and the sections were incubated for 48 hours at 4°C under gentle shaking (PCNA, 1:500 dilution of mouse anti-PCNA, NCL-L-PCNA Leica Biosystems, Newcastle, United Kingdom; DCX, 1:300 dilution of goat anti-DCX antibody, C-18, Santa Cruz Biotechnology, Dallas, TX, USA) under gentle agitation. The primary antibody incubation was followed by three 10-minute rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution [PCNA sections, 1:1,000 dilution of biotinylated anti-mouse IgG (BA-2001, Vector Laboratories, Burlingame, CA, USA)] in 3% normal horse serum and 2% bovine serum albumin in 0.1 M PB; DCX sections, 1:1,000 dilution of anti-goat IgG (BA-5000, Vector Laboratories) in 3% normal rabbit serum and 2% bovine serum albumin in 0.1 M PB) for 2 hours at room temperature. This was followed by three 10-minute rinses in 0.1 M PB, after which sections were incubated for 1 hour in an avidinbiotin solution (1:125 in 0.1 M PB; Vector Laboratories), followed by three 10-minute rinses in 0.1 M PB. Sections were then transferred to a solution consisting of 0.05% diaminobenzidine tetrahydrochloride in 0.1 M PBs for 5 minutes at room temperature, after which 3.3 µL 30% H<sub>2</sub>O<sub>2</sub>/mL solution was added. With the aid of a low power objective lens on a stereomicroscope (Leica MZ 7.5, Meyer instruments, Houston, TX, USA), the progression of the staining was visually followed and allowed to continue until a level was reached where the background staining could assist in analysis by matching to architectonic borders from Nissl stained sections without obscuring the immunopositive structures. The tissue was then rinsed twice more in 0.1 M PB before being mounted on glass slides coated with 0.5% gelatine and allowed to dry overnight. Once dry, the slides were placed in a solution of 70% ethanol for 2 hours and then dehydrated, cleared in xylene, and coverslipped with DPX mountant. To test for non-specific staining of the immunohistochemical protocol, the primary and secondary antibodies were omitted from random sections and no specific staining was evident. The observed immunostaining patterns support the specificity of the antibodies and are compatible with observations made in columbiformes and other avian species (Boseret et al., 2007; Charvet and Striedter, 2008; Melleu et al., 2013, 2016).

### Data analysis

The Nissl stained sections were examined under a low power

objective lens on a stereomicroscope (Leica MZ 7.5, Meyer instruments) and the architectonic borders were traced using a camera Lucida fitted on the stereomicroscope (Leica MZ 7.5, Meyer instruments). The PCNA and DCX immunostained sections were then matched to the drawings from the Nissl stained sections and the location of immunopositive soma marked on the drawings. Selected drawings were then scanned and redrawn using the Canvas 8 Software (Deneba Software, Miami, FL, USA). The brain regions were identified and named in accordance with the stereotaxic atlas of the brain of the pigeon (Karten and Hodos, 1967) using the nomenclature recommended by the Avian Brain Nomenclature Forum (Reiner et al., 2004). Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss, South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software (Axioskop 2 plus). No pixilation adjustments or manipulation of the captured images were undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7. The distribution of PCNA and DCX-immunoreactive cells was described qualitatively using a low power ( $\times 5$ ) objective lens on a light microscope (AxioStar plus, Zeiss, South Africa) and the densities of immunostained cells were visually compared and recorded on a scale ranging from low (+), moderate (++) to high (+++). A second observer was used to eliminate observer bias.

## Results

### General observations

We examined putative adult neurogenesis throughout the brains of the racing homer and utility carneau pigeon breeds using immunohistochemical techniques for the endogenous markers PCNA and DCX. The distribution of PCNA and DCX immunoreactivity was almost identical in both breeds, but a few minor differences were observed (Table 1). Due to this extensive similarity, we depicted only the mapping of the distribution of the PCNA and DCX-immunoreactive cells in the racing homer pigeons (Figure 1).

In both pigeons, PCNA and DCX immunoreactivity was observed in the olfactory bulbs, subdivisions of the pallium (hippocampus, hyperpallium apicale, hyperpallium intercalatum, hyperpallium densocellulare, mesopallium, nidopallium, entopallium, arcopallium, dorsolateral cortical area, piriform cortex and the temporo-parieto-occipital area), subpallium (medial striatum, lateral striatum and the globus pallidus), diencephalon, mesencephalon (pretectum and tectum), and cerebellum. Generally, the telencephalic regions had a higher density of PCNA- and DCX-immunoreactive cells than other brain regions in both breeds of pigeons examined, while the lowest density of cells immunoreactive to both markers was observed in the cerebellum. In the majority of the telencephalic regions, the density of PCNA- and DCX-immunoreactive cells increased from rostral to caudal in both pigeon breeds, except in the mesopallium where the staining density of cells first decreased from rostral to middle levels and then increased at caudal levels. DCX immunoreactivity was more intense in fibres than in cell bodies. DCX-immunoreactive cells included small rounded cells, fusiform bipolar cells, and large round and/or polygonal multipolar cells in terms of cell shapes.

**Table 1 Qualitative distribution and density of PCNA and DCX-immunoreactive structures in the brains of the racing homer and utility carneau pigeons**

| Brain region                | Racing homer pigeon |     | Utility carneau pigeon |     |
|-----------------------------|---------------------|-----|------------------------|-----|
|                             | PCNA                | DCX | PCNA                   | DCX |
| Subventricular zone         | +++                 | ++  | +++                    | ++  |
| Olfactory bulb              | +++                 | +++ | +++                    | +++ |
| Telencephalon               |                     |     |                        |     |
| Hippocampus                 | ++                  | ++  | ++                     | ++  |
| Hyperpallium apicale        | +++                 | ++  | +++                    | +++ |
| Hyperpallium intercalatum   | ++                  | ++  | ++                     | ++  |
| Hyperpallium densocellulare | +                   | ++  | +                      | ++  |
| Mesopallium                 | ++                  | ++  | ++                     | ++  |
| Nidopallium                 | ++                  | ++  | ++                     | ++  |
| Entopallium                 | ++                  | ++  | +++                    | +   |
| Arcopallium                 | +++                 | ++  | +++                    | ++  |
| Striatum                    | ++                  | +++ | ++                     | +++ |
| Pallidum                    | ++                  | ++  | ++                     | ++  |
| Diencephalon                | ++                  | ++  | ++                     | ++  |
| Mesencephalon               |                     |     |                        |     |
| Pretectum                   | ++                  | +   | ++                     | ++  |
| Tectum                      | ++                  | ++  | ++                     | +   |
| Metencephalon               |                     |     |                        |     |
| Cerebellum                  | +                   | ++  | +                      | +   |

+, ++, +++: Low, moderate, and high density of DCX-immunoreactive structures, respectively. PCNA: Proliferating cell nuclear antigen; DCX: doublecortin.

### Distribution of PCNA-immunoreactive cells

#### Olfactory bulbs

The olfactory bulbs contained the highest density of PCNA-immunoreactive cells of all regions of the brain in both pigeon breeds. PCNA-immunoreactive cells were observed at high density in the internal granular layer, mitral cell layer and external plexiform layer, while they were found at a lower density in the glomerular layer and olfactory nerve layer (Figure 2A and C).

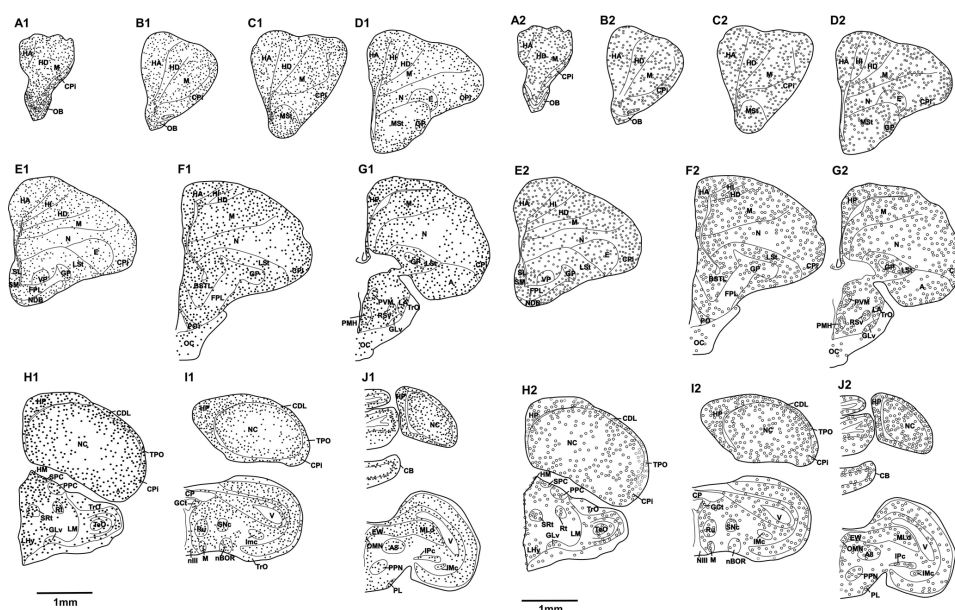
#### Subventricular zones of the lateral, third, fourth, and tectal ventricles

PCNA-immunoreactive cells at a high density were observed in the walls of the lateral, third, fourth and tectal ventricles, forming an uninterrupted layer, one to four cells thick, of cells with dark-stained nuclei in both pigeon breeds. Proliferative hotspots were observed in both dorsal and ventral poles of the lateral ventricle, mostly in rostral levels of the brain (Figure 3A and C). PCNA-immunoreactive cells at a high density was observed in the racing homer pigeon associated with the walls of the lateral ventricle abutting the medial striatum, nidopallium, nidopallium caudale and hyperpallium apicale when compared to the utility carneau pigeon.

#### Hyperpallium, mesopallium, nidopallium, and entopallium

In the pallial regions (hyperpallium apicale, hyperpallium intercalatum, hyperpallium densocellulare, mesopallium and nidopallium), PCNA-immunoreactive cells were observed in high density along the cortical margins and regions adjacent





**Figure 1** Reconstruction of coronal sections through one half of the brain of the racing homer pigeon showing areas of the detected PCNA-immunoreactive cells and DCX-immunoreactive immature and migrating neurons.

(A1–J1 and A2–J2) Black dots represent proliferating cells (PCNA-immunoreactive cells) and black circles indicate DCX-immunoreactive cells. Each drawing of the coronal section is approximately 1,500  $\mu\text{m}$  apart, with A1 and A2 being the most rostral section and J1 and J2 being the most caudal. All architectonic drawings were traced and drawn from selected Nissl stained sections using camera Lucida mounted on the stereomicroscope (Leica MZ 7.5, Meyer instruments, Houston, TX, USA). The PCNA- and DCX-immunostained sections were then matched to the drawings from the Nissl-stained sections and the location of immunopositive soma marked on the drawings. The drawings were then scanned with a color document scanner (DS-50000, Epson, South Africa) and reproduced using the Canvas 8 software (Deneba Software, Miami, FL, USA). A: arcopallium; BSTL: lateral part of the bed nucleus of the stria terminalis; CB: cerebellum; CDL: dorsolateral cortical area; CP: posterior commissure; CPI: piriform cortex; E: entopallium; EW: nucleus of Edinger-Westphal; FPL: fasciculus prosencephali lateralis; GCt: griseum centrale; GLv: nucleus geniculatus lateralis, pars ventralis; GP: globus pallidus; HA: hyperpallium apicale; HD: hyperpallium densocellulare; HI: hyperpallium intercalatum; HM: nucleus habenularis medialis; HP: hippocampus; IMc: nucleus isthmi, pars magnocellularis; IPc: nucleus isthmi, pars parvocellularis; LA: nucleus lateralis anterior thalami; LHv: lateral hypothalamus; LM: nucleus lentiformis; LSt: lateral striatum; Mm: mammillary region; M: mesopallium; N: nidopallium; MLd: nucleus mesencephalicus lateralis, pars dorsalis; MSt: medial striatum; nBOR: nucleus of the optic root; NC: nidopallium caudale; NDB: nucleus diagonalis Broca; nIII: oculomotor nerve; OB: olfactory bulb; OC: optic chiasm; OMN: oculomotor nucleus; PL: nucleus pontis lateralis; PMH: nucleus medialis hypothalamic posterioris; PO: preoptic region; PPC: nucleus principalis precommissuralis; PPN: pedunculopontine tegmental nucleus; PVM: nucleus periventricularis magnocellularis; R5v: nucleus reticularis superior, pars ventralis; Rt: nucleus rotundus; Ru: nucleus ruber; SL: lateral septum; SM: medial septum; SNC: substantia nigra, pars compacta; SPC: nucleus superficialis parvocellularis; SRt: nucleus subrotundus; TeO: optic tectum; TPO: temporo-parieto-occipital area; TrO: optic tract; V: ventricle; VP: ventral pallidum.

to the lateral wall of the lateral ventricle, but this density was obviously reduced in the core regions of hyperpallium densocellulare, mesopallium and nidopallium in both pigeon breeds (**Figure 1**). At caudal levels, the nidopallium caudale exhibited an increased density of PCNA-immunoreactive cells at the regions adjacent to the walls of the lateral ventricle and also towards the lateral cortex. The core of the NC had scattered PCNA-immunoreactive cells. In the entopallium, there was a higher density of PCNA-immunoreactive cells rostrally, but the density gradually decreased towards the caudal levels of the telencephalon in all pigeons. In all these telencephalic regions, the density of PCNA-immunoreactive cells appeared greater in the racing homer pigeons than in the utility carneau pigeons.

#### *Hippocampus, piriform cortex, and dorsolateral cortical area*

The hippocampus in the pigeons studied herein was defined according to the descriptions provided by Atoji and Wild (2004). PCNA-immunoreactive cells were sparsely distributed throughout the medial layer of the ventral hippocampus, while a medium density of PCNA-immunoreactive cells was observed in the triangular area (Tr), the lateral layer (**Figure 4A and D**), and the dorsomedial region in both pigeons. The dorsolateral region of the hippocampus exhibited the highest density of PCNA-immunoreactive cells in both pigeon breeds. Throughout dorsolateral region of the hippocampus, the PCNA-immunoreactive cells were homogeneously distributed,

and these cells were observed to continue into the dorsolateral cortical area. The dorsolateral cortical area exhibited a high density of PCNA-immunoreactive cells, especially in areas adjacent to the dorsolateral wall of the lateral ventricles in the caudal levels of this region. Ventrolaterally from dorsolateral cortical area towards the temporo-parieto-occipital cortex, the density of the PCNA-immunoreactive cells gradually decreased to a moderate density. The piriform cortex exhibited a high density of PCNA-immunoreactive cells throughout the rostro-caudal axis of the telencephalon in both pigeon breeds.

#### *Striatum, septum, and arcopallium*

In the striatum of both pigeonbreeds, a moderate density of PCNA-immunoreactive cells was observed in the medial striatum adjacent to the ventrolateral wall of the lateral ventricle, while the lateral striatum exhibited a low density of PCNA-immunoreactive cells. Generally in the striatum, there was a rostro-caudal decline in the density of PCNA-immunoreactive cells. In the racing homer pigeon, the medial septum housed a medium density of PCNA-immunoreactive cells, the density of which lessened towards the most medial margin of this nucleus. The SM of the utility carneau pigeon had very densely packed PCNA-immunoreactive cells with large nuclei, which was prominent at the level of the anterior commissure (**Figure 5A, C and D**). In the lateral septum of both pigeonbreeds, a higher density of PCNA-immunoreactive cells was

observed when compared to the medial septum, and within this nucleus there was a rostrocaudal decline in PCNA-immunoreactive cellular density. The arcopallium in both pigeon-breeds exhibited a moderate density of PCNA-immunoreactive cells along the cortical margins and towards the ventral parts of the nidopallium caudale, while the central areas had a very low to absent density of PCNA-immunoreactive cells.

#### *Diencephalon, optic chiasm, and anterior commissure*

The diencephalon exhibited a high density of PCNA-immunoreactive cells in the various nuclei compared to the surrounding tissues. This was observed in the paraventricular nuclei which include nucleus preopticus medialis, periventricular magnocellular nucleus and nucleus medialis hypothalamic posteriolis medially, the dorsal margin in the nucleus dorsomedialis anterior thalami, nucleus dorsolateralis anterior thalami, pars lateralis nuclei and the lateral margin in the nucleus rotundus and nucleus geniculatus lateralis, pars ventralis. Further laterally the optic tract housed a moderate density of PCNA-immunoreactive cells. In the core regions of the hypothalamus, PCNA-immunoreactive cells were either absent or in a low density. The optic chiasm of both pigeon breeds exhibited a cluster of PCNA-immunoreactive cells which increased slightly in density adjacent to the ventral pole of the subventricular zone of the third ventricle (**Figure 6A and C**). There were few scattered PCNA-immunoreactive cells in the anterior commissure.

#### *Pretectum, tectum, posterior commissure, and cerebellum*

The prepectum in both pigeon breeds exhibited a high density of PCNA-immunoreactive cells in the dorsal margins and ventrally in the nucleus subpretectalis. Laterally, the layers of the optic tectum had a low density of PCNA-immunoreactive cells, and in the core regions only a few PCNA-immunoreactive cells could be observed. Within the optic tectum, a high density of PCNA-immunoreactive cells was observed in the paraventricular nuclei closer to the fourth ventricle medially, the layers of the optic tectum laterally in areas adjacent to the tectal ventricle and the nucleus of the basal optic tract ventrally in both pigeon breeds. In the utility carneau pigeons, the nucleus principalis precommissuralis near the subventricular zone of the fourth ventricle had a high density of PCNA-immunoreactive cells that exhibited very large cell bodies, but these were not observed in the racing homer pigeons. PCNA-immunoreactive cells were scattered in the central nuclei of the optic tectum, such as the nucleus spiriformis lateralis, but were absent in the nucleus subpretectalis. In the posterior commissure of both pigeon breeds, there were a few PCNA-immunoreactive cells that were intercalated with the commissural fibres. In the cerebellum of both pigeon breeds, PCNA-immunoreactive cells were observed in high density in the Purkinje cell layer and the occasional PCNA-immunoreactive cells were observed in the molecular and granule cell layers (**Figure 7A and C**).

#### **Distribution of DCX-immunoreactive cells**

DCX-immunoreactive cells were identified throughout the brains of the two adult pigeon breeds and were generally small fusiform shaped, bipolar and round multipolar cells with slight variations in size in the different brain regions. Any size

and/or shape variations are noted in relevant regions.

#### *Olfactory bulb*

The olfactory bulb was the region that contained the highest density of DCX-immunoreactive structures in both pigeon breeds. DCX-immunoreactive cells and fibres were seen in high density in the inner layers of the olfactory bulb, but at a lesser density in the outer two layers (**Figure 2B and D**). DCX-immunoreactive cells appeared to be in the process of migrating from the olfactory bulbs into the pallial regions of hyperpallium apicale and hyperpallium densocellulare.

#### *Subventricular zone*

The subventricular zone of the lateral and third ventricles evinced 3 to 4 layers of DCX-immunoreactive cells in both breeds, which intermittently formed cell clusters of intensely stained cells at high density. This arrangement was more apparent on the lateral wall of the lateral ventricle than on the medial wall. The DCX-immunoreactive fibres emanating from the subventricular zone were oriented either parallel or orthogonal to the ventricular wall (**Figure 3B and D**). The fourth and tectal ventricles of the racing homer pigeon exhibited a continuous layer of DCX-immunoreactive cells without cell clustering, whereas in the utility carneau pigeon, the tectal ventricles had more DCX-immunoreactive fibres rather than cells.

#### *Hyperpallium, mesopallium, nidopallium, and entopallium*

There was a moderate density of DCX-immunoreactive cells in the divisions of the hyperpallium (hyperpallium apicale, hyperpallium intercalatum and hyperpallium densocellulare), with these cells and fibres being of higher density at the periphery and very low density in central areas (**Figure 1**). At more rostral levels, the hyperpallium densocellulare appeared to receive migrating neuroblasts from the olfactory bulb. A similar group of apparently migrating neuroblasts was observed at caudal levels, towards the anterior commissure, where they appeared to migrate from the walls of the lateral ventricle into the hyperpallium apicale. The mesopallium and nidopallium exhibited a moderate density of DCX-immunoreactive cells and fibres, with higher densities of DCX-immunoreactive structures observed in the peripheral areas adjacent to the lateral wall of the lateral ventricle medially, the dorsoventral wall of the lateral ventricle dorsolaterally and piriform cortex ventrolaterally (**Figure 1**). At the caudal level, posterior to the anterior commissure, the nidopallium caudale exhibited a similar density of DCX-immunoreactive cells and fibres as seen in the nidopallium and mesopallium. Higher densities of DCX-immunoreactive cells and fibres were observed near the walls of the lateral ventricle both medially and dorsolaterally in the nidopallium caudale, the piriform cortex ventrolaterally and the arcopallium ventrocaudally. The core areas of mesopallium, nidopallium and nidopallium caudale had a low to absent density of DCX-immunoreactive structures, but the DCX-immunoreactive cells in these areas were characterised by large round or polygonal somata and thick branching fibres. The entopallium contained a moderate density DCX-immunoreactive cells and fibres in the racing homer pigeon but very low density of DCX-immunoreactive cells and fibres in the

utility carneau pigeon. Generally, there was a rostral-caudal increase in the density and morphological complexity of the DCX-immunoreactive cells in the nidopallium caudale.

#### *Hippocampus, piriform cortex, and dorsolateral cortical area*

The medial region of the ventral hippocampus had sparsely distributed DCX-immunoreactive cells and fibres, while the triangular and the lateral regions exhibited a medium density of DCX-immunoreactive cells and fibres in both pigeon breeds (**Figure 4B, C, E and F**). In the dorsomedial region of the hippocampus, DCX-immunoreactive cells were observed at a higher density in the central area and towards the dorsomedial wall of the lateral ventricle. There were low-density DCX-immunoreactive cells and fibres in the dorsolateral region of the hippocampus, close to the border with dorsomedial region medially. The density of DCX-immunoreactive cells in the dorsolateral region gradually increased towards the dorsolateral cortical region.

In the dorsolateral cortical region, there were moderate-density DCX-immunoreactive cells and fibres. At the caudal level, the dorsolateral cortical region had higher density DCX-immunoreactive cells and fibres in areas adjacent to the dorsolateral margin of the lateral ventricle. The piriform cortex had higher density intensely stained DCX-immunoreactive cells and fibres in both pigeon breeds.

#### *Striatum, septum, and arcopallium*

In the striatum, the medial striatum exhibited a medium density of DCX-immunoreactive structures with a higher density of stained structures in areas abutting the ventral parts of the lateral wall of the lateral ventricle, while the density of immunoreactive structures was less in the core regions. Ventral to the striatum, there was a high density of DCX-immunoreactive cells in the olfactory tubercle and nucleus of the fasciculus diagonalis Brocae. The lateral striatum had moderate to low density of DCX structures in both pigeon breeds, but a high density of DCX-immunoreactive structures was observed in the globus pallidus and interpeduncular nucleus at levels caudal to the anterior commissure.

In the racing homer pigeon, the septal nuclear complex exhibited a low density of DCX-immunoreactive structures in the medial septum, whereas the lateral septum had a moderate density of DCX-immunoreactive structures, although this density was reduced at the caudal level. In the utility carneau pigeon, the medial septum exhibited a moderate density of DCX-immunoreactive cells and fibres (**Figure 5B**). There was a moderate density of DCX-immunoreactive cells and fibres in the arcopallium, especially along the ventral margins in both pigeon breeds, but the core areas had less dense staining in the utility carneau pigeon.

#### *Diencephalon, optic chiasm, and anterior commissure*

The diencephalon exhibited a moderate density of DCX-immunoreactive cells and fibres in the paraventricular regions, near the third ventricle, in the nucleus preopticus medialis, and the nucleus medialis hypothalamic posterioris (**Figure 1**). Dorsally, a moderate density of DCX-immunoreactive structures were observed in the stria medullaris and some dorsal thalamic nuclei, including the nucleus dorsolateralis anterior,

pars medialis and nucleus dorsolateralis, pars lateralis, and laterally in the thalamus, a low density of DCX-immunoreactive structures were evident in the nucleus geniculatus lateralis, pars ventralis, nucleus lateralis anterior thalami and nucleus rotundus. The subthalamic nuclei were devoid of DCX-immunoreactive. The optic chiasm of both pigeon breeds exhibited a cluster of DCX-immunoreactive cells and fibres that spread lateral to the optic tract (**Figure 6B and D**). In the utility carneau pigeon, the anterior commissure exhibited a few scattered DCX-immunoreactive cells and fibres, but this was not evident in the racing homer pigeon.

#### *Pretectum, tectum, posterior commissure, and cerebellum*

In the mesencephalic pretectum, a moderate density of DCX-immunoreactive structures were observed in the tegmentum in the supraoptic decussation. In the tectal region of the mesencephalon, a moderate density of DCX-immunoreactive structures were observed medially in the nucleus ruber, griseum centralis, oculomotor nucleus and the Edinger-Westphal nucleus, dorsally in the nucleus habenularis medialis (HM) and nucleus spiriformis lateralis, and ventrally in the nucleus tuberis, pedunculopontine tegmental nucleus and the nucleus of the basal optic root. The Edinger-Westphal nuclei and nucleus ruber contained large round DCX-immunoreactive cells. DCX-immunoreactive structures were scattered in the nucleus rotundus and in the layers of the optic tectum. The posterior commissure exhibited a few DCX-immunoreactive structures especially on margins adjacent to the walls of the cerebral aqueduct. In the cerebellum, the granule cell layer of the utility carneau pigeon had a moderate density of DCX-immunoreactive structures, while in both pigeon breeds, the Purkinje cell layer had a high density of DCX-immunoreactive structures. The dendrites of the DCX-immunoreactive cells projected into the molecular layer of the cerebellum (**Figure 7B and D**).

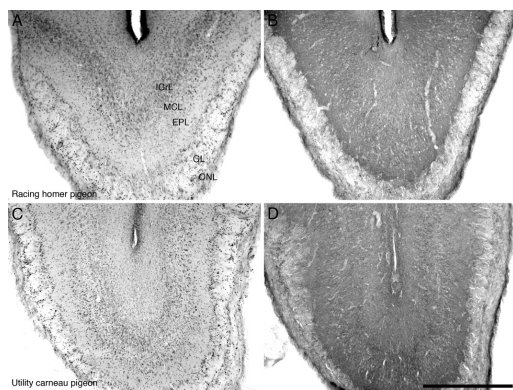
## Discussion

### General considerations regarding PCNA- and DCX-immunoreactivity in pigeons

In the current study, we observed potential adult neurogenesis in the brains of the two breeds of the adult pigeons, the racing homer and the utility carneau. Putative adult neurogenesis was demonstrated through the expression of the PCNA and DCX molecules, revealed using immunohistochemical techniques, throughout the brains of both pigeon breeds. The two antibodies were localized in similar brain regions of the pigeon breeds with putative adult neurogenesis including the olfactory bulb, telencephalic subdivisions (hyperpallium apicale, hyperpallium intercalatum, hyperpallium densocellulare, mesopallium, nidopallium, nidopallium caudale, entopallium, medial striatum, lateral striatum, lateral septum, medial septum), the subventricular zone, the diencephalon, the mesencephalon and the cerebellum.

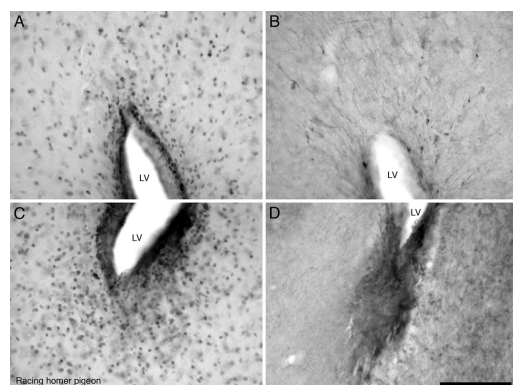
PCNA is a marker for proliferating cells that is essential for DNA replication and repair. It is synthesized during the early G1 and S phases of the cell cycle, abundant during the S phase and declines during G2/M phase (Kurki et al., 1988; Hall et al., 1990). In the two pigeon breeds studied, the density of PCNA-immunoreactive cells was higher in the olfactory bulbs, subventricular zone, nidopallium caudale and the piriform





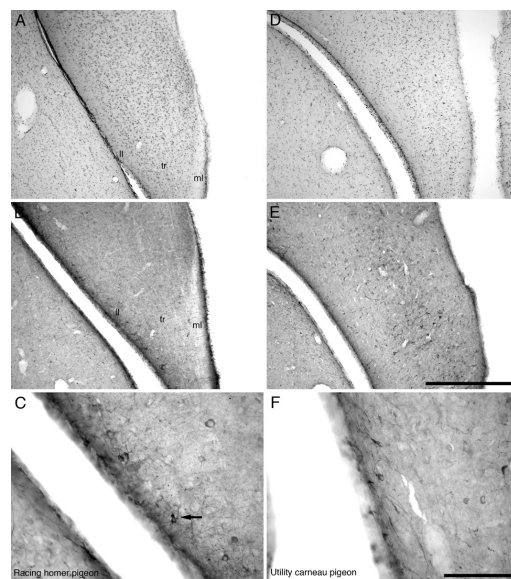
**Figure 2** Photomicrographs showing the olfactory bulbs of the racing homer pigeon (A, B) and the utility carneau pigeon (C, D).

The distribution of PCNA immunoreactivity is shown in the cellular layers of the olfactory bulb of the racing homer pigeon (A) and utility carneau pigeon (C). The distribution of DCX immunoreactivity is shown in the olfactory bulbs of the racing homer pigeon (B) and utility carneau pigeon (D). Scale bar: 500 µm. Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss, South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software (Zeiss, South Africa). IGL: Internal granular layer; MCL: mitral cell layer; EPL: external plexiform layer; GL: glomerular layer; ONL: olfactory nerve layer.



**Figure 3** Photomicrographs showing the distribution of PCNA (A, C) and DCX (B, D) immunoreactivity in the dorsal and ventral reaches of the subventricular zone of the lateral ventricle (LV) in the racing homer pigeon. Note the intense PCNA staining in the subventricular zone of the lateral ventricle and also migrating DCX-immunoreactive cells from the subventricular zone of the lateral ventricle into the parenchyma of the telencephalon. Scale bar: 100 µm. Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss, South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss) and operating on the ZEN 2010 computer software (Zeiss).

cortex, but was generally less dense in the potential adult neurogenic sites such as the diencephalon, mesencephalon and the cerebellum. This pattern of cellular staining is similar to that seen in other non-mammalian vertebrates including teleost fish (Zupanc et al., 2005), amphibians (Cerri et al., 2009), and chickens (Hannan et al., 1999). However, BrdU-positive cells in the subtelencephalic regions in canaries did not co-express neuron specific marker Hu protein suggesting that the proliferating cells in this region are of glial cell lineage (Vellema et al., 2010). In the subventricular zone of the lateral ventricles, a high density of PCNA-immunoreactive cells was observed in the ventral and dorsal borders of the ventricles. In these regions, the putative proliferating cells formed intermittent clusters that were consistent with the proliferative 'hotspots'



**Figure 4** Photomicrographs showing the distribution of proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) in the hippocampus of the racing homer and utility carneau pigeons.

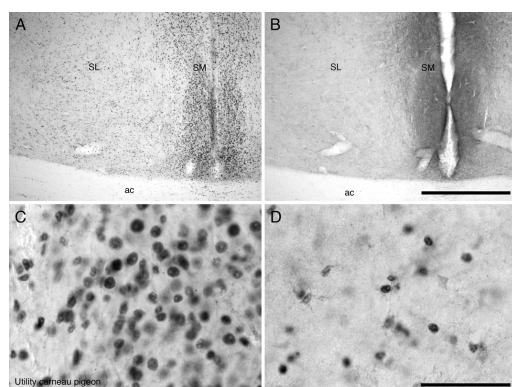
The distribution of PCNA immunoreactivity in the ventral subdivisions of the hippocampus showing mild PCNA immunoreactivity in the medial layer (ml), moderate density in the lateral layer (ll) and dense reactivity in the triangular area (tr) in the racing homer (A) and the utility carneau pigeons (D). The distribution of DCX immunoreactivity in the ventral division of the hippocampus showing a moderate concentration of DCX-immunoreactive cells and fibres in the ll and tr and mild distribution in the ml of the ventral hippocampus of the racing homer (B) and utility carneau pigeons (E). A higher magnification showing DCX-immunoreactive fibres and round unipolar cells in the ll and triangular area of the ventral hippocampus in the racing homer (C) and utility carneau pigeons (F); arrow shows a DCX-immunoreactive cell. Scale bar in E, valid for A, B, D, E: 500 µm. Scale bar in F, valid for C and F: 100 µm. Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss) mounted on the light microscope (Axioskop 2 plus, Zeiss) and operating on the ZEN 2010 computer software (Zeiss).

identified by Alvarez-Buylla et al. (1990b).

Proliferating cells were also observed throughout the telencephalon and four possible reasons can be inferred for this: (1) these putative proliferating cells may have migrated from the subventricular zone throughout the parenchyma (Almli and Wilczynski, 2007); (2) resident progenitors may be proliferating (Alvarez-Buylla et al., 1990a, b; Doetsch et al., 1999; Zupanc et al., 2005); (3) putative proliferating cells may be resident glial cells (Cerri et al., 2009); and (4) putative proliferative cells may be both neuronal and glial cells undergoing DNA replication and repair (Hall et al., 1990).

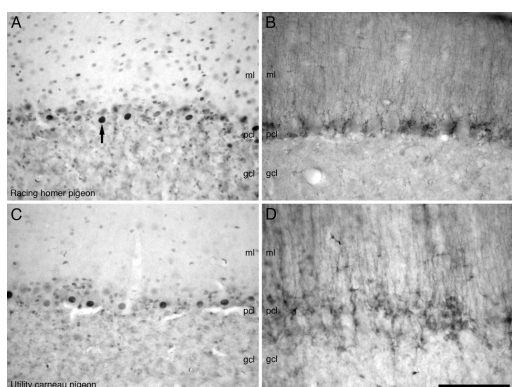
DCX is a microtubule associated protein that plays a key role in the migration of neurons during development and in post-mitotic neurons undergoing migration, remodelling of their dendritic processes and synaptogenesis in adulthood (Gleeson et al., 1999; Capes-Davis et al., 2005; Couillard-Despres et al., 2005). In this study, we observed that DCX expression was widespread and heterogeneous in the brains of the two breeds of domestic pigeons (*Columba livia domestica*). The density of DCX-immunoreactive cells and fibres was most intense in the olfactory bulbs, associated olfactory cortices, telencephalic pallial structures including the hippocampus, the striatum and the nidopallium caudale, but less dense in subtelencephalic structures such as the diencephalon, mesencephalon and cerebellum. This widespread expression of DCX





**Figure 5** Photomicrographs showing distribution of proliferating cell nuclear antigen (PCNA)- and doublecortin (DCX)-immunoreactive cells in the septum of the utility carneau pigeon.

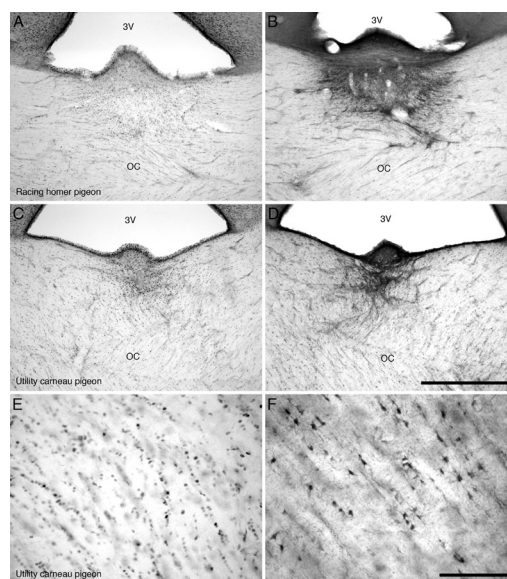
(A) Distribution of PCNA-immunoreactive cells in the septum. (B) Distribution of DCX-immunoreactive cells and fibres in the septum. (C) A higher magnification of the medial septum (SM) showing dense PCNA-immunoreactive cells. (D) A higher magnification of the lateral septum (SL) showing sparse PCNA-immunoreactive cells. Scale bar in B, valid for A and B: 500  $\mu$ m. Scale bar in D, valid for C and D: 50  $\mu$ m. Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss, South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software (Zeiss).



**Figure 7** Photomicrographs of the cerebellar cortex of the racing homer (A, B) and utility carneau pigeons (C, D).

The distribution of PCNA-immunoreactive cells in the granule cell layer (gcl), Purkinje cell layer (pcl) and the molecular cell layer (ml) of the cerebella cortex in the racing homer (A) and utility carneau pigeons (C); arrow shows a PCNA-immunoreactive cell. The distribution of DCX-immunoreactive cells and fibres in the layers of the cerebellar cortex in the racing homer pigeon (B) and utility carneau pigeon (D). Note the large and prominent PCNA immunoreactivity cells in the pcl of both pigeon breeds. Scale bar: 100  $\mu$ m. Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss, South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss) and operating on the ZEN 2010 computer software (Zeiss). PCNA: Proliferating cell nuclear antigen; DCX: doublecortin.

was consistent with observations made in other non-song birds such as the developing domestic chick brain (Capes-Davis et al., 2005), in song birds such as adult canaries (Boseret et al., 2007; Vellema et al., 2014) and in subadult zebra finches (Kim et al., 2006). Moderate intensity DCX expression in the prosencephalon has been reported in numerous studies (Kim et al., 2006; Boseret et al., 2007; Vellema et al., 2010, 2014; Mezey et al., 2012; Melleu et al., 2013). The majority of pioneering studies on adult neurogenesis in birds reported that the process was only limited to the prosencephalon (Alvarez-Buylla and Nottebohm, 1988; Kirn et al., 1994). Few studies reported the presence of DCX expression in the subtelencephalic regions in



**Figure 6** Photomicrographs of proliferating cell nuclear antigen (PCNA) (A, C, E) and doublecortin (DCX) (B, D, E) immunostained sections through the dorsal aspect of the optic chiasm (OC), at its border with the floor of the third ventricle (3V) in racing homer (A, B) and utility carneau (C, D, E, F) pigeons.

These images depict a potential neurogenic zone, or bulge, at the dorsal midline of the optic chiasm that appears to produce newly generated cells (shown by PCNA immunostaining) and appears to migrate (as shown by DCX immunostaining) into the optic chiasm, optic nerve (E, F) and optic tract of both pigeon types. Scale bar in D, valid for A–D: 500  $\mu$ m; scale bar in E and F, valid for E and F: 100  $\mu$ m. Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss, South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software (Zeiss).

pathologically normal adult species (Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014). However, increased levels of adult neurogenesis have been reported in the subtelencephalic regions under injury and pathological conditions (Cao et al., 2002; Chen et al., 2006).

We also noted that the areas with the densest expression of DCX-immunoreactive structures were adjacent to the walls of the ventricles in all brain regions, suggesting that new neurons were generated in the subventricular zone and migrated to their target regions. Migration of newly born neurons follows well defined routes in mammalian species, where they migrate from the subgranular zone of the dentate gyrus to the granule cell layer, and the subventricular zone of the lateral ventricle to the olfactory bulbs through the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Chawana et al., 2013; Patzke et al., 2013). In birds studied to date, including the current study, the migratory routes appear diffuse, with many regions of the brain appearing to incorporate new neurons. Despite this, as in mammals, a rostral migratory-like stream has been identified in rock pigeons, seen to stretch from the caudal nidopallium to the rostral mesopallium, hyperpallium apicale and densocellulare (Melleu et al., 2013). New neurons migrate from the subventricular zone into the brain parenchyma through radial glia scaffolding (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988). This phenomenon led to the identification of DCX-immunoreactive neuroblasts with the morphology typical of migrating cells (small round and fusiform bipolar cells) (Alvarez-Buylla

and Nottebohm, 1988; Alvarez-Buylla et al., 1988; Melleu et al., 2013) in the parenchyma away from the subventricular zone. However, DCX immunoreactivity was also observed in post-migratory cells of the Purkinje cell layer of the cerebellum in the current study (Capes-Davis et al., 2005) and in areas of the telencephalic structures where large round and polygonal multipolar cells with elaborate dendritic processes were also seen to express DCX. In the rock pigeons, these mature-like DCX-immunoreactive cells did not co-express neuron specific marker NeuN (Melleu et al., 2013), suggesting that these cells were immature neurons establishing synaptic contacts in local circuits (Gleeson et al., 1999). Despite this, DCX-immunoreactive cells outside the subventricular zone in the mouse brain were found to express NeuN (Yang et al., 2004), indicating that DCX is also expressed in mature neurons that undergo dendritic arborization and axonal growth (Nacher et al., 2001; Brown et al., 2003).

DCX expression has been found to persist for more than 20 days in cells in the adult canary brain (Balthazart et al., 2008), explaining its presence in morphologically distinct neurons in the cores of the hippocampus and other pallial structures as observed in the current study, and in rock pigeons (Melleu et al., 2013). This suggests that DCX-immunoreactive cells in deeper areas of the telencephalic parenchyma represent earlier generations of new-born neurons that completed migration prior to the sacrifice of the animal. In addition, the concept also explains the abundance of DCX-immunoreactive cells observed in the telencephalon of avian species in previous studies (Kim et al., 2006; Melleu et al., 2013; Vellema et al., 2014).

### Olfactory neurogenesis in pigeons

The olfactory bulbs are known to continuously incorporate new neurons in adulthood in various mammalian species (Kempermann, 2012). We found that the olfactory bulbs and associated brain areas such as the piriform cortex, olfactory tubercles and dorsolateral cortical areas exhibited high densities of PCNA- and DCX-immunoreactive structures. Similar findings were reported in other bird species such as domestic chicks and rock pigeons (Mezey et al., 2012; Melleu et al., 2013). In contrast to olfactory neurogenesis in mammals where new neurons migrate long distances from the subventricular zone, the presence of PCNA- and DCX-immunoreactive cells in the walls of the olfactory ventricle and adjacent olfactory bulb layers suggests that new neurons are generated and incorporated in the olfactory bulbs locally rather than by migration. Melleu et al. (2013) also found conspicuous BrdU labelled cells in the walls of the olfactory ventricles. Retrograde tracing studies in pigeons have shown that olfactory bulb neuronal fibres project to the piriform cortex, medial septum (Reiner and Karten, 1985) and the hyperpallium densocellulare (Patzke et al., 2011). Similar to a report by Melleu et al. (2013) in rock pigeons, we also observed a stream of DCX-immunoreactive cells that appeared to be migrating from the olfactory bulbs to the hyperpallium densocellulare. Processing of the olfactory sense varies greatly in different orders of birds studied to date (Balthazart and Taziaux, 2009). In homing pigeons, olfactory processing is used during navigation from unfamiliar places to home lofts (Patzke et al., 2010) by following familiar airborne odours (Papi et al., 1972). In other species of the order columbiformes, olfactory cues were

associated with parental behavior in ring doves (Bonadonna et al., 2003), social and reproductive behaviors in rock pigeons (Patzke et al., 2010), feeding behaviors in domestic chicken (Jones and Roper, 1997), and food localization in vultures (Graves, 1992) and sea birds (Grubb, 1972). Thus, process of adult neurogenesis and neural plasticity in birds might aid in replenishing neurons and renewing circuits to facilitate adaptation into natural environments using the olfactory sense.

### Hippocampal neurogenesis

The density of PCNA- and DCX-immunoreactive structures was lower in the hippocampus of domestic pigeons especially in the medial layer of the ventral hippocampus than in the surrounding brain parenchyma. The triangular region and the lateral layers exhibited a moderate density of PCNA- and DCX-immunoreactive structures. The dorsolateral regions of the hippocampus had a higher density of PCNA- and DCX-immunoreactive structures than the dorsomedial region. Similar differential densities were also reported in rock pigeons (Melleu et al., 2013), but domestic pigeons have been found to have a significantly larger hippocampus than the ancestral wild rock pigeons (Rehkämper et al., 2008), indicating that the similarity in the extent of adult hippocampal neurogenesis between rock and domestic (homing) pigeons may be best ascertained by quantitative methods.

In birds, adult hippocampal neurogenesis has been associated with the integration of new experiences, such as new songs, and the clearance of old memories (Nottebohm, 1981; Wilbrecht and Kirn, 2004; Kempermann, 2008), thus, the process may serve to prevent interference between old and new memories, especially in food caching birds that need to recall both old and new food caches accurately (Barnea et al., 2006; Wiskott et al., 2006; Pravosudov and Smulders, 2010). Kempermann (2008) also suggested that AHN may contribute to a neurogenic reserve that can be incorporated only when there is a need for new learning. Generally, AHN decreases with advancing age in both mammals and non-mammalian vertebrates (Barnea and Nottebohm, 1994; Kempermann et al., 1997; Eriksson et al., 1998; Kim et al., 2006); however, in some reptiles and fish, AHN appears to contribute, in part, to the continuous growth of the nervous system (Zupanc and Horschke, 1995; Ngwenya et al., 2013). Barnea and Nottebohm (1994) found that AHN varies seasonally, with more neuronal accumulation occurring in the autumn in black capped chickadees. The hippocampal formation has also been associated with spatial memory dependent tasks related to learning and acquisition of a spatial map and its operation in homing pigeons (Bingman et al., 2005), migration and food caching (Bingman and Cheng, 2005; Hoshoooley and Sherry, 2007).

### Neurogenesis in other brain areas

Our observation of wide spread DCX immunoreactivity throughout the brains of domestic pigeons is similar to that reported for adult chicken, canaries and zebra finches (Capes-Davis et al., 2005; Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008). In the pallial structures, PCNA and DCX-immunoreactive structures were apparent in the margins of the hyperpallium apicale, mesopallium, nidopallium and nidopallium caudale rather than their core areas. In addition, we observed PCNA and DCX immunoreactivity in



the septum, striatum and the entopallium in both pigeons. Expression of the DCX antibody in the entopallium was comparatively denser in racing homer pigeon than in utility carneau pigeon. Mezey et al. (2012) reported similar findings but mild DCX expression in the entopallium of chicken. The striatum complex and the entopallium participate in the processing of visual information which is vital during navigating long distances particularly by homing pigeons. Birds are heavily visual and auditory reliant during feeding, reproduction, socialisation and many other daily functions (Grubb, 1972; Papi et al., 1972; Graves, 1992; Bonadonna et al., 2003; Patzke et al., 2011), but the cores of the telencephalon that process visual and auditory information exhibited very low to absent densities of PCNA- and DCX-immunoreactive structures in the domestic pigeon breeds studied. In contrast, the diencephalic and mesencephalic regions associated with these senses exhibited moderate densities of PCNA- and DCX-immunoreactive structures for example the dorsal nuclei of the thalamus which connects with the medial striatum (MSt) and also the tectum which connects with the lateral striatum (Kuenzel et al., 2011; Shanahan et al., 2013). We also observed PCNA and DCX immunoreactivity in the optic chiasma, anterior and posterior commissures and suggested that glial cells and neuronal axons may be proliferating and remodelling respectively.

In conclusion, adult neurogenesis appears to be a conserved process in the pigeon brain and may help in continuous reinforcement and remodelling of neuronal circuits and behavior.

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# Putative Adult Neurogenesis in Old World Parrots: The Congo African Grey Parrot (*Psittacus erithacus*) and Timneh Grey Parrot (*Psittacus timneh*)

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In the current study, we examined for the first time, the potential for adult neurogenesis throughout the brain of the Congo African grey parrot (*Psittacus erithacus*) and Timneh grey parrot (*Psittacus timneh*) using immunohistochemistry for the endogenous markers proliferating cell nuclear antigen (PCNA), which labels proliferating cells, and doublecortin (DCX), which stains immature and migrating neurons. A similar distribution of PCNA and DCX immunoreactivity was found throughout the brain of the Congo African grey and Timneh grey parrots, but minor differences were also observed. In both species of parrots, PCNA and DCX immunoreactivity was observed in the olfactory bulbs, subventricular zone of the lateral wall of the lateral ventricle, telencephalic subdivisions of the pallium and subpallium, diencephalon, mesencephalon and the rhombencephalon. The olfactory bulb and telencephalic subdivisions exhibited a higher density of both PCNA and DCX immunoreactive cells than any other brain region. DCX immunoreactive staining was stronger in the telencephalon than in the subtelencephalic structures. There was evidence of proliferative hot spots in the dorsal and ventral poles of the lateral ventricle in the Congo African grey parrots at rostral levels, whereas only the dorsal accumulation of proliferating cells was observed in the Timneh grey parrot. In most pallial regions the density of PCNA and DCX stained cells increased from rostral to caudal levels with the densest staining in the nidopallium caudolaterale (NCL). The widespread distribution of PCNA and DCX in the brains of both parrot species suggest the importance of adult neurogenesis and neuronal plasticity during learning and adaptation to external environmental variations.

**Keywords:** doublecortin, proliferating cell nuclear antigen, old world parrots, Congo African grey parrot, Timneh grey parrot, adult neurogenesis, cell proliferation, cell migration

## INTRODUCTION

Adult neurogenesis encompasses the birth and maturation of new neurons that become incorporated into existing circuitry or replace old and damaged neurons under normal physiological and or pathological conditions (Lindsey and Tropepe, 2006). The process of adult neurogenesis was confirmed in a variety of species from different taxa ranging from insects to humans (for reviews, see Cayre et al., 2002; García-Verdugo et al., 2002; Lindsey and Tropepe, 2006;



Chapouton et al., 2007). Although this process has been confirmed in wide spectrum of species, the animals studied to date represent only a small fraction of the extant species, and studying further species will allow a broader understanding of the potential diversity of the process and function of adult neurogenesis (Barnea and Pravosudov, 2011). Across many species similarities in the process of adult neurogenesis have been observed, which include the origin of neurons, the phenotype of stem cells and their proliferative mechanisms, the migration and differentiation of neurons (for a review, see Doetsch and Scharff, 2001); however, they are also important differences among species in the spatial distribution of adult born neurons, their mode of migration and phenotypic diversity (Doetsch and Scharff, 2001; Ngwenya et al., 2017).

Numerous studies have revealed widespread adult neurogenesis in non-mammalian species such as fish, amphibians, reptiles, and birds when compared to mammalian species (García-Verdugo et al., 2002; Marchioro et al., 2005; Ghosh and Hui, 2016; Macedo-Lima et al., 2016; Ngwenya et al., 2017). Generally, the rate of adult neurogenesis decreases with age across vertebrate taxa (Kuhn et al., 1996; Knoth et al., 2010; Amrein et al., 2011; LaDage et al., 2011; Ngwenya et al., 2017), and the process is affected by factors such as genetics, endogenous and exogenous factors, and seasonal variation (for a review, see Barnea and Pravosudov, 2011).

**Abbreviations:** A, arcopallium; AL, ansa lenticularis; An, nucleus angularis; APH, area parahippocampalis; Bas, nucleus basorostralis; CA, commissura anterior; Cb, cerebellum; CbL, nucleus cerebellaris lateralis; CbM, nucleus cerebellaris medialis; OC, optic chiasm; CP, commissura posterior; CS, nucleus centralis superior (Bechterew); DBC, brachium conjunctivum descendens; DCX, doublecortin; DLL, nucleus dorsolateralis anterior thalami pars lateralis; DLP, nucleus dorsolateralis posterior thalami; DMA, nucleus dorsomedialis anterior thalami; DMM, magnicellular nucleus of the dorsomedial thalamus; DMP, nucleus dorsomedialis posterior thalami; E, entopallium; EM, nucleus ectomammillaris; EPL, external plexiform layer of the olfactory bulb; FPL, fasciculus prosencephali lateralis; GCT, substantia grisea centralis; GL, glomerular layer of olfactory bulb; GP, Globus pallidus; HA, hyperpallium apicale; HD, hyperpallium densocellulare; HI, hyperpallium intercalatus; HP, hippocampus; IGrL, internal granular layer of the olfactory bulb; Imc, nucleus isthmi, pars magnocellularis; IP, nucleus interpeduncularis; Ipc, nucleus isthmi pars parvocellularis; L1, lamina 1 of Field L; L2a, lamina 2a of Field L; La, nucleus laminaris; Ll, lateral layer of the ventral hippocampus; LLV, ventral nucleus of the lateral lemniscus; LoC, locus coeruleus; LSt, lateral striatum; LV, lateral ventricle; M, mesopallium; MCL, mitral cell layer of the olfactory bulb; ml, medial layer of ventral hippocampus; MPV, nucleus mesencephalicus profundus; MSt, medial striatum; N, nidopallium; NC, nidopallium caudale; NI, nidopallium intermedium; nIII, nucleus oculomotorius; NIVL, nidopallium intermedium pars ventrolateralis; NLC, central nucleus of the lateral nidopallium; nV, nucleus motorius nervi trigemini; nX, vagus nucleus; nXIIts, nucleus nervi hypoglossus pars tracheosyringalis; OI, nucleus olivaris inferioris; OM, tractus occipitomesencephalicus; ON, olfactory nerve; OV, nucleus ovoidalis; PCNA, proliferating cell nuclear antigen; PL, nucleus pontis lateralis; PM, nucleus pontis medialis; POA, nucleus preopticus anterior; POM, nucleus preopticus medialis; PrV, nucleus sensorium principalis nervi trigemini; RP, nucleus reticularis pontis; Rt, nucleus rotundus; SCv, nucleus subcoeruleus ventralis; SGC, stratum griseum centrale; SGF, stratum griseum et fibrosum; SL, lateral septum; SLu, nucleus semilunaris; SM, medial septum; SNC, substantia nigra pars compacta; SPC, nucleus superficialis parvocellularis; SpM, nucleus spiriformis medialis; SpL, nucleus spiriformis lateralis; TeO, Optic tectum; tn, nucleus taenia of the amygdala tr, triangular area of ventral hippocampus; TrO, Optic tract; TTd, nucleus et tractus descendens nervi trigemini; Tu, nucleus tuberis; VMH, ventromedial hypothalamus nucleus; VSt, ventral striatum.

African grey parrots are stocky, short tailed birds with an average body mass of 400 g. The Congo African grey parrot (*Psittacus erithacus*) is larger and lighter grey in color compared to the smaller and darker grey Timneh grey parrot (*Psittacus timneh*). The African grey parrots live a long life span ranging between 20 and 50 years in the wild, but this can increase to nearly 100 years in captivity (Carey and Judge, 2001; Schmid et al., 2006). African grey parrots lead a complex social life, coupled with long term monogamous relationships (Seibert, 2006; Emery et al., 2007). In addition to displaying complex social interactions, African grey parrots are thought to exhibit potentially advanced cognitive capabilities comparable to Apes and young humans (Pepperberg et al., 1999; Emery and Clayton, 2004; Iwaniuk et al., 2004; Pepperberg, 2006). Their relative brain size is comparable to that of primates (Olkowicz et al., 2016). Parrots feature very high neuronal densities and high total neuronal numbers particularly in the forebrain when compared primates with larger brain sizes (Olkowicz et al., 2016). These parrots display vocal learning and mimicry abilities that rival those of young humans (Iwaniuk et al., 2004; Pepperberg, 2006) and these characteristics are valuable in studies examining and comparing vocal learning and cognitive abilities in avians and primates. Parrots show cooperative problem solving and the ability to discriminate discrete and continuous variables (Al Aïn et al., 2009; Péron et al., 2011). Here we investigated the generation and maturation of new neurons throughout the brains of the two subspecies of African grey parrots, the Congo African grey parrot (*Psittacus erithacus*), and the Timneh grey parrot (*Psittacus timneh*) using PCNA and DCX immunohistochemistry.

## MATERIALS AND METHODS

### Animals and Tissue Processing

Two brains of adult male Congo African grey (*Psittacus erithacus*) and two brains of adult male Timneh grey parrots (*Psittacus timneh*) were used in the current study. The birds were purchased from a local breeder in South Africa, and were sacrificed and perfused in November 2013. The birds were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (clearance no: 2013/05/02B), which parallel those of the NIH for the care and use of animals in scientific experimentation. Five minutes prior to being euthanized, both birds were given an intramuscular dose of heparin, 2,500 units (0.5 ml) to prevent blood clotting. Animals were then injected with an intraperitoneal dose of Euthapent (1 ml/kg) and body mass recorded. The average body mass of the two Congo African grey parrots was 453.75 g, and the two Timneh grey parrots was 286.36 g. All animals were transcardially perfusion-fixed, initially with a rinse of 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). The brains were carefully removed from the skull, and post-fixed overnight in 4% paraformaldehyde in 0.1M PB. The average brain mass of the Congo African grey parrots was 10.30 g and that of the Timneh grey parrot was 7.85 g. Before sectioning, the tissue was allowed to equilibrate in a 30% sucrose in 0.1M PB solution at 4°C for 4 days. The brains were then frozen in dry

ice and sectioned in the coronal plane, 50  $\mu$ m thick sections, on a sliding microtome. A one in ten series of sections were taken and three series stained for Nissl substance, PCNA and DCX. The remaining series of sections were placed in an antifreeze solution and are stored at  $-20^{\circ}\text{C}$  for future use. The series of sections used for Nissl staining were mounted on 0.5% gelatine coated slides, dried overnight, cleared in a 1:1 mixture of 100% ethanol and 100% chloroform and stained with a 1% cresyl violet solution.

## Immunohistochemistry for PCNA and DCX

The series of sections used for free floating PCNA and DCX immunohistochemistry were initially treated for 30 min at room temperature under gentle shaking with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1M PB: 1.6% of 30%  $\text{H}_2\text{O}_2 = 0.48\%$   $\text{H}_2\text{O}_2$ ), followed by three 10 min rinses in 0.1M PB. To block non-specific binding sites the sections were then preincubated for 2 h, at room temperature under gentle shaking, in a blocking buffer solution consisting of 3% normal horse serum (NHS) for PCNA sections or 3% normal rabbit serum (NRS) for DCX sections, 2% bovine serum albumin, and 0.25% Triton X-100 in 0.1M PB. Following preincubation the primary antibodies were added to the blocking buffer solution and the sections were incubated for 48 h at  $4^{\circ}\text{C}$  under gentle shaking (PCNA – 1:500 dilution of mouse anti-PCNA, NCL-L-PCNA Leica Biosystems, Newcastle, United Kingdom; DCX – 1:300 dilution of goat anti-DCX antibody, C-18, Santa

Cruz Biotechnology, Dallas, TX, United States) under gentle agitation. The primary antibody incubation was followed by three 10-min rinses in 0.1M PB and the sections were then incubated in a secondary antibody solution (PCNA sections – 1:1000 dilution of biotinylated anti-mouse IgG [BA-2001, Vector Labs] in 3% NHS and 2% bovine serum albumin in 0.1M PB; DCX sections – 1:1000 dilution of anti-goat IgG [BA-5000, Vector Labs] in 3% NRS and 2% bovine serum albumin in 0.1M PB) for 2 h at room temperature. This was followed by three 10-min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin biotin solution (1:125 in 0.1M PB; Vector Labs, Burlingame, CA, United States), followed by three 10-min rinses in 0.1M PB. Sections were then transferred to a solution consisting of 0.05% diaminobenzidine tetrahydrochloride in 0.1M PB for 5 min at room temperature, after which 3.3  $\mu$ l of 30%  $\text{H}_2\text{O}_2$ /ml of solution was added. With the aid of a low power stereomicroscope the progression of the staining was visually followed and allowed to continue until a level was reached where the background staining could assist in architectonic matching to the Nissl stained sections without obscuring the immunopositive structures. The tissue was then rinsed twice more in 0.1M PB before being mounted on glass slides coated with 0.5% gelatine and allowed to dry overnight. Once dry, the slides were placed in a solution of 70% ethanol for 2 h and then dehydrated, cleared in xylene and coverslipped with Depex. To test for non-specific staining of the immunohistochemical protocol, the primary and secondary antibodies were omitted

**TABLE 1 |** Summary of qualitative distribution and density of PCNA and DCX immunoreactive cells in the brain of the Congo African grey parrot and Timneh grey parrot.

| Brain region    |                   | Congo African grey |     | Timneh grey |     |
|-----------------|-------------------|--------------------|-----|-------------|-----|
|                 |                   | PCNA               | DCX | PCNA        | DCX |
| SVZ             |                   | +++                | +++ | +++         | +++ |
| OB              |                   | +++                | +++ | +++         | +++ |
| Telencephalon   | HP                | ++                 | ++  | ++          | ++  |
|                 | HA                | ++                 | ++  | ++          | ++  |
|                 | HI                | ++                 | ++  | ++          | ++  |
|                 | HD                | ++                 | ++  | ++          | ++  |
|                 | M                 | ++                 | +   | ++          | +   |
|                 | N                 | +++                | ++  | +++         | ++  |
|                 | NC                | ++                 | +++ | ++          | +++ |
|                 | E                 | ++                 | +   | ++          | +   |
|                 | A                 | ++                 | +   | ++          | +   |
|                 | MSt               | ++                 | ++  | ++          | ++  |
|                 | LSt               | ++                 | ++  | ++          | ++  |
|                 | GP                | –                  | –   | –           | –   |
|                 | SM                | +                  | +   | +           | +   |
|                 | SL                | ++                 | +   | ++          | +   |
| Diencephalon    |                   | ++                 | +   | ++          | +   |
| Mesencephalon   | Pretectum         | ++                 | +   | ++          | +   |
|                 | Optic tectum      | +++                | +   | +++         | +   |
| Rhombencephalon | Cerebellum        | +                  | +   | +           | +   |
|                 | Pons              | ++                 | +   | ++          | +   |
|                 | Medulla oblongata | ++                 | +   | ++          | +   |

–, absent; +, low; ++, moderate; +++, high density of PCNA and DCX immunoreactive structures.

from random sections and no staining was evident. The observed immunostaining patterns support the specificity of the antibodies and are compatible with observations made in pigeons, parakeets, and quails (Charvet and Striedter, 2008, 2009; Melleu et al., 2013, 2015).

## Analysis

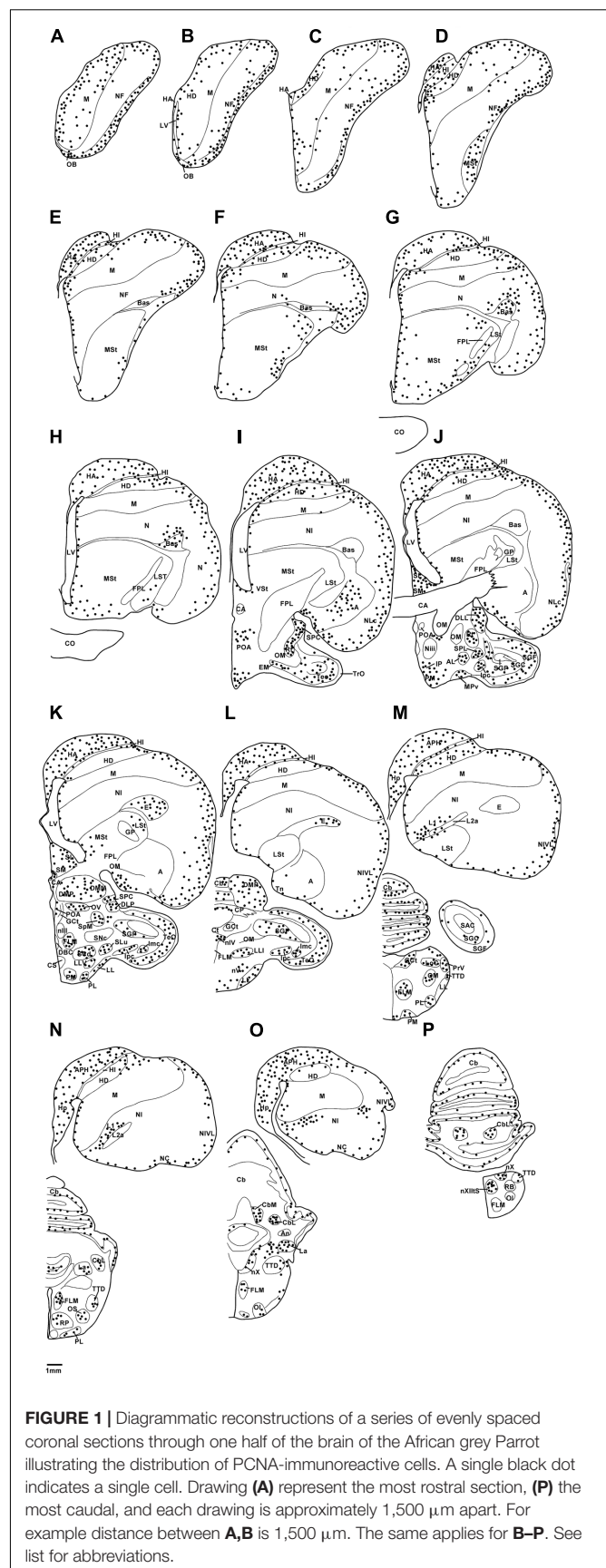
The Nissl stained sections were examined with a low power stereomicroscope and the architectonic borders traced using a camera lucida. The PCNA and DCX immunostained sections were then matched to the drawings from the Nissl stained sections and the location of immunopositive soma marked on the drawings. Selected drawings were then scanned and redrawn using the Canvas 8 Software (Deneba Software, Miami, FL, United States). High power microscopic observation allowed for the determination of the relative densities of stained structures throughout the various regions of the brain. The relative densities of immunostained structures were visually compared and recorded on a scale ranging from low (+) to moderate (++) to high (+++). A second observer was used to eliminate observer bias.

The brain regions were identified and named in accordance with the stereotaxic atlas of the brain of the budgerigar (Brauth et al., 2011) using the nomenclature recommended by the Avian Brain Nomenclature Forum (Reiner et al., 2004). Digital photomicrographs were captured using a digital camera (Axio Cam HRC, Zeiss, South Africa) mounted on the light microscope (Axioskop 2 plus, Zeiss, South Africa) and operating on the ZEN 2010 computer software (Zeiss, South Africa). No pixilation adjustments or manipulation of the captured images were undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

## RESULTS

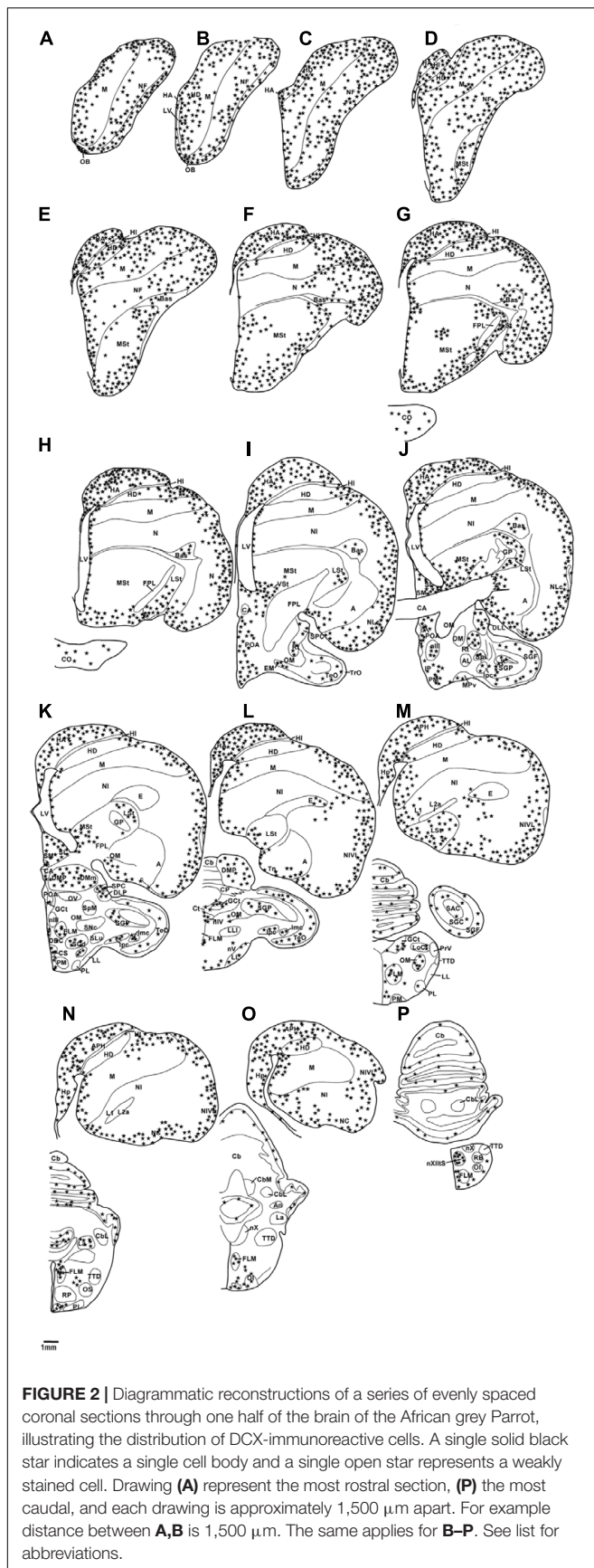
### General Observations

In the present study, we examined putative adult neurogenesis throughout the brains of the Congo African grey parrot (*Psittacus erithacus*) and the Timneh grey parrot (*Psittacus timneh*) using immunohistochemical techniques for the endogenous markers PCNA and DCX. The distribution of PCNA and DCX immunoreactivity was almost identical in both subspecies, but a few minor differences were observed (Table 1). Due to this extensive similarity we depict only the mapping of the distribution of the PCNA and DCX immunoreactive cells in the Timneh grey parrot (Figures 1, 2). In both parrots, PCNA and DCX immunoreactivity was observed in the layers of the olfactory bulbs (OBs), the subventricular zone (SVZ) of the lateral, third and fourth ventricles and the cerebral aqueduct, subdivisions of the pallium (Hp, HA, HI, HD, M, N, E, and A), subpallium (MSt, LSt, SM, and SL), diencephalon, mesencephalon, and rhombencephalon. Generally, the telencephalic regions had a higher density of PCNA and DCX immunoreactive cells than other brain regions in both subspecies of parrots examined. In the majority of the telencephalic regions, the density of PCNA and DCX



**FIGURE 1 |** Diagrammatic reconstructions of a series of evenly spaced coronal sections through one half of the brain of the African grey Parrot illustrating the distribution of PCNA-immunoreactive cells. A single black dot indicates a single cell. Drawing (A) represent the most rostral section, (P) the most caudal, and each drawing is approximately 1,500  $\mu\text{m}$  apart. For example distance between A,B is 1,500  $\mu\text{m}$ . The same applies for B-P. See list for abbreviations.





immunoreactive cells increased from rostral to caudal in both parrot subspecies. DCX immunoreactivity was more intense in fibers than in cell bodies and the majority of DCX immunopositive cells included small rounded cells, fusiform unipolar and bipolar cells.

## Distribution of PCNA Immunoreactivity

### Olfactory Bulb

Proliferating cell nuclear antigen immunoreactive cells were observed at high density in the IGrL, MCL, and EPL layer, while they were found in low density in the GL and ON layer (Figures 3A,C).

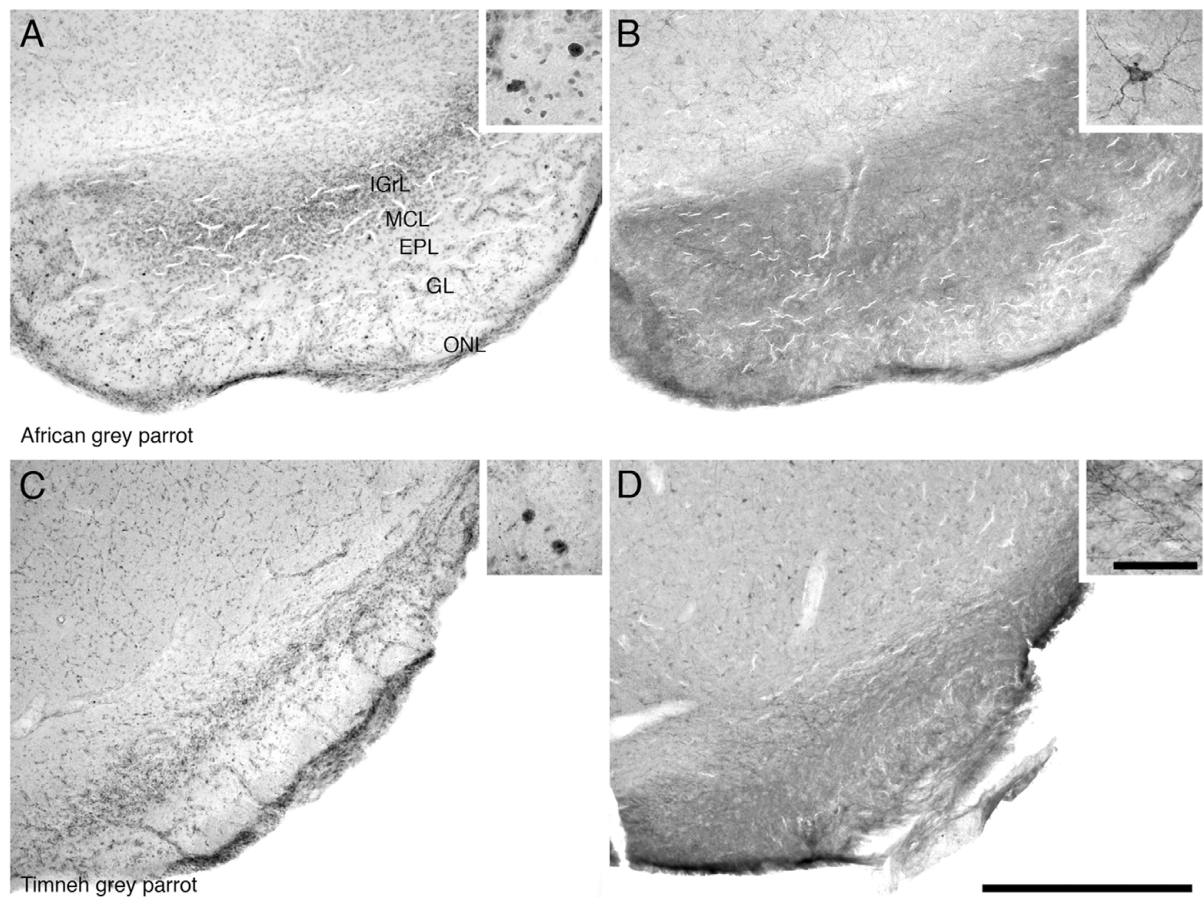
### Subventricular Zone

In both parrot species the SVZ of the lateral ventricle exhibited a high density of PCNA immunoreactivity with occasional cells clustering. There was evidence of proliferative hot spots as described by Alvarez-Buylla et al. (1990) in the dorsal and ventral poles of the lateral ventricle in the Congo African grey parrots at rostral levels, but only a dorsal accumulation of PCNA immunoreactive cells was observed in the Timneh grey parrots (Figures 4A,C). The third and fourth ventricles and the cerebral aqueduct of both parrot species exhibited a medium density of PCNA immunoreactivity without cell clustering. There was a low density of PCNA immunoreactivity in the SVZ of the tectal portion of the cerebral aqueduct in both parrot species.

### Pallial Regions

In both parrot subspecies examined the HA, HD, HP, and APH exhibited a moderate density of PCNA immunoreactive cells (Figure 5A). In the ventral hippocampus the ml showed a low density of PCNA immunoreactive cells compared to the tr and ll which presented a moderate density (Figure 5C). The HI showed a low density of PCNA immunoreactive cells in the medial and core regions but an increase in the density of PCNA immunoreactive cells was observed in its lateral regions.

The mesopallium (M) exhibited a homogenous moderate density of PCNA immunoreactive cells at rostral levels, but at caudal levels the density of PCNA immunoreactive cells were reduced to low density in core regions, but moderate density in the medial and lateral regions. The entopallium exhibited a moderate density of PCNA immunoreactive cells and their distribution decreased markedly in caudal sections in both species of African grey parrots. There was a high density of PCNA immunoreactive cells in the nidopallium frontale (NF) of both parrot subspecies. The core region of NF of the Congo African grey parrot exhibited a large cluster of PCNA immunoreactive cells. At caudal levels, the NI and NC exhibited a moderate density of PCNA immunoreactive cells in the lateral regions, while the medial and core regions showed a low density of PCNA immunoreactive cells in both parrot species. The arcopallium exhibited a moderate density of PCNA immunoreactive cells which were distributed in higher density in the ventral regions encompassing the Tn than the dorsal regions, which showed a low density to almost no PCNA immunoreactive cells.



**FIGURE 3 |** Photomicrographs of PCNA – (A,C) and DCX (B,D) – immunostained coronal sections through the olfactory bulb (OB) of the two parrot species – (A,B) – African grey parrot (*Psittacus erithacus*), (C,D) – Timneh grey parrot (*Psittacus timneh*). In all images dorsal is to the top and medial to the right. (A,C) PCNA immunoreactivity revealed a high number of PCNA-immunoreactive cells in the IGrL, MCL, and EPL layers of the olfactory bulb. Insets in (A,C) show a higher magnification images of PCNA immunoreactive cells. (B,D) DCX immunoreactivity revealed a high density of DCX-immunoreactive cells and fibers in all layers of the olfactory bulb (IGrL, MCL, EPL, and GL) except the olfactory nerve layer. Insets in (B,D) show a higher magnification of DCX immunoreactive cells and fibers. All high power images on inserts were taken from consecutive sections to low power images. Scale bar in (D) = 500  $\mu$ m and applies to all. The scale bar in the inset in (D) = 50  $\mu$ m and applies to all insets. See list for abbreviations.

### Subpallial Regions

In the striatum of both parrot species, a moderate density of PCNA immunoreactive cells was observed in MSt and LSt. PCNA immunoreactive cells were distributed in higher density in ventral regions of the MSt and also in regions adjacent to the ventrolateral wall of the lateral ventricle. No detectible PCNA immunoreactive cells were observed in the regions corresponding to the GP. Generally in the striatum, there was a rostro-caudal decline in the density of PCNA immunoreactive cells. In the septal complex of both parrots, the SM exhibited a low density of PCNA immunoreactive cells while the SL showed a moderate density of PCNA immunoreactive cells.

### Diencephalon

The diencephalon exhibited a high density of PCNA immunoreactive cells in the paraventricular nuclei, including POA and POM, the dorsal margin in the DMA, DLL, and DLP and the lateral margin in the nucleus rotundus (Rt), SPC, and

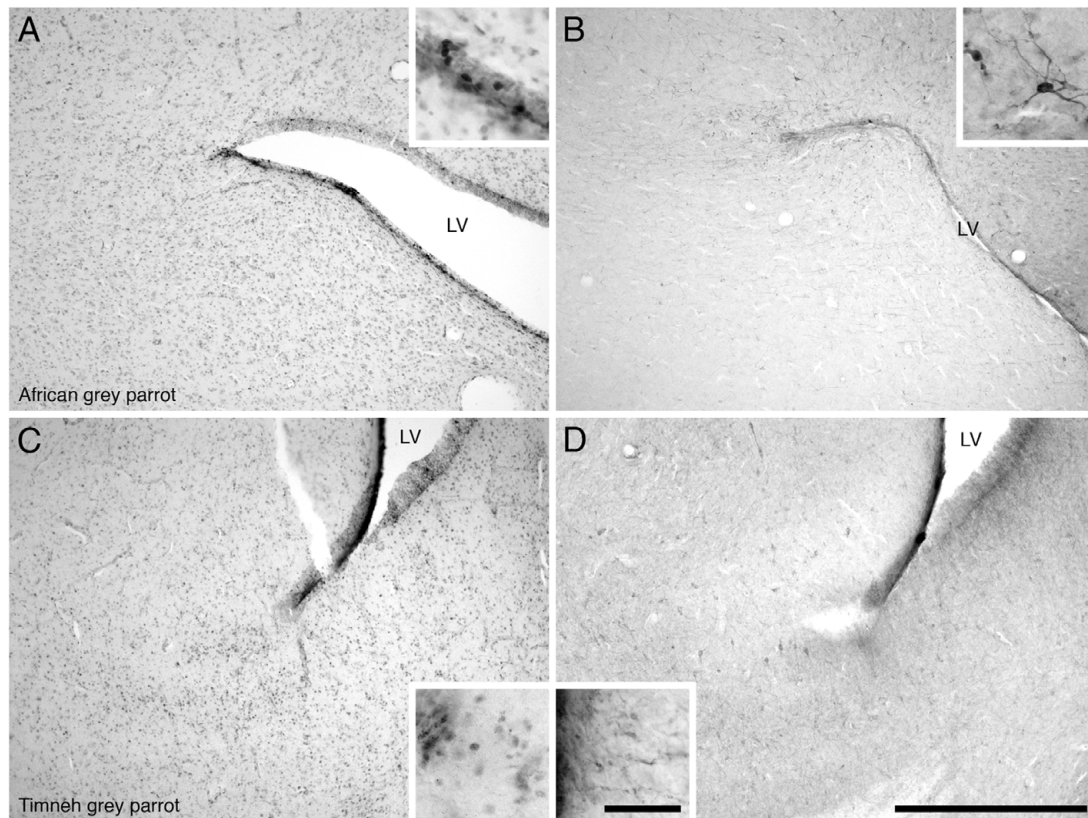
SpL. In the core regions of the diencephalon, the African grey parrot exhibited a moderate density of PCNA immunoreactive cells in OV. The VMH in the ventral hypothalamus of both parrots exhibited the highest density of PCNA immunoreactive cells. The OC showed PCNA immunoreactive cells ventral to the inferior pole of the third ventricle in both species (Figures 6A,C).

### Mesencephalon, Rhombencephalon, and Cerebellum

Most of the regions of the midbrain exhibited a moderate density of PCNA immunoreactive cells, including the nIII, GCt, IP and the isthmus region in the SLu, Ipc, and Imc. In the optic tectum in both parrots a higher density of PCNA immunoreactive cells was observed in the SGF and SGC.

In the cerebellum, a moderate density of PCNA immunoreactive cells was observed in the Purkinje cell layer and the CbM and CbL (Figures 7A,C). Occasional PCNA immunoreactive cells were observed in the molecular and granule cell layers. In the pons and the medulla oblongata, a





**FIGURE 4 |** Photomicrographs of PCNA – (A,C) and DCX (B,D) – immunostained coronal sections through the subventricular zones (SVZ) of the lateral ventricle in the brains of the two parrot species – (A,B) – African grey parrot (*Psittacus erithacus*), (C,D) – Timneh grey parrot (*Psittacus timneh*). PCNA immunoreactivity revealed aggregates of proliferating cells (hot spots) in the SVZ of the dorsal pole of the lateral ventricle in the African grey parrot (*Psittacus erithacus*) (A), and ventral pole of the lateral ventricle in Timneh grey parrot (*Psittacus timneh*) (C). Insets in (A,C) show a higher magnification images of PCNA immunoreactive cells. DCX immunoreactivity revealed aggregates of DCX-immunoreactive cells and fibers in the SVZ of the dorsal pole of the lateral ventricle in the African grey parrot (*Psittacus erithacus*) (B), and in the ventral pole of the lateral ventricle in the Timneh grey parrot (*Psittacus timneh*) (D). Insets in (B,D) show a higher magnification of DCX immunoreactive cells and fibers. All high power images on inserts were taken from consecutive sections to low power images. In all images dorsal is to the top and medial to the right. Scale bar in (D) = 500  $\mu$ m and applies to all. The scale bar in the inset in (D) = 50  $\mu$ m and applies to all insets. LV, lateral ventricle.

moderate density of PCNA immunoreactive cells was observed in some regions not limited to the LoC, medial and lateral pontine nuclei (PM and PL), PrV, TTD, and nucleus raphe (R).

## Distribution of DCX Immunoreactivity

### Olfactory Bulb

The OB was the region that contained the highest density of DCX immunoreactive structures in both parrots. DCX immunoreactive cells and fibers were seen in high density in the inner layers of the olfactory bulb, but in lower density in the outer layers (Figures 3B,D).

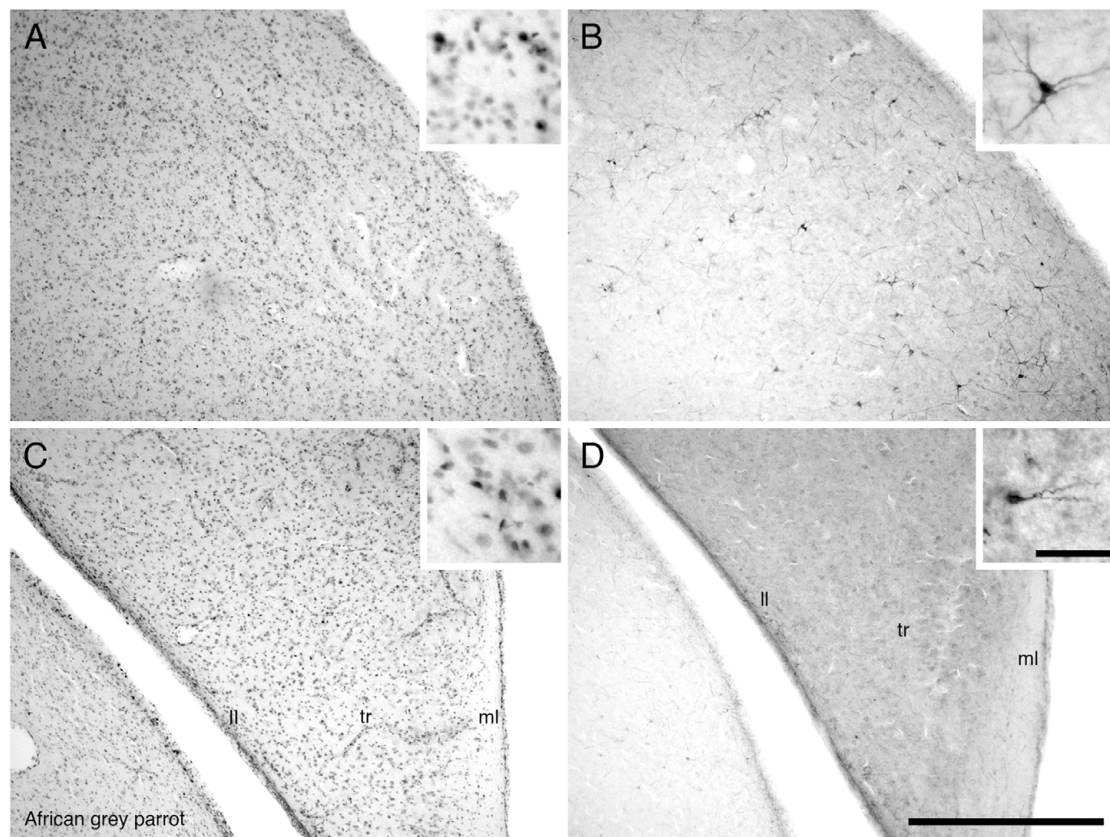
### Subventricular Zone

In both parrot subspecies, the SVZ of the lateral ventricle exhibited a high density of DCX immunoreactive cells and fibers. The majority of the DCX immunoreactive cells were orientated parallel to the walls of the lateral ventricles, depicting tangential migration, while a few DCX immunoreactive cells were oriented perpendicular to the walls of the lateral ventricle, suggesting radial migration. At some rostral levels in the Congo

African grey parrot there were occasional accumulations of DCX immunoreactive cell clusters in the dorsal and ventral poles of the lateral ventricle, similar to the dorsal and ventral 'hot spots' suggested by Alvarez-Buylla et al. (1990) (Figures 4B,D). Such accumulations of DCX immunoreactive cells were only observed in the dorsal pole of the lateral ventricle of the Timneh grey parrots. The SVZ of the third and fourth ventricles and that of the cerebral aqueduct showed a low density of DCX immunoreactive fibers with no cells clearly discernable.

### Pallial Regions

The pallial portion of the telencephalon exhibited the highest density of DCX immunoreactive cells and fibers, although the density of DCX immunopositive structures varied across different pallial regions in both parrots. Moderate densities of DCX immunoreactive cells and fibers were present in the HA, HD, medial and lateral NF, nidopallium (N), and NI. The medial and lateral regions of the HI and the M showed a low density of DCX immunoreactive cells and fibers. There was almost no DCX immunoreactivity detected in the



**FIGURE 5 |** Photomicrographs of PCNA – (A,C) and DCX (B,D) – immunostained coronal sections through the hippocampus (HP) in the brain of the African grey parrot (*Psittacus erithacus*). Distribution of PCNA immunoreactive cells in the dorsal hippocampus (A), and ventral layers of the hippocampus (C). Insets in (A,C) show a higher magnification images of PCNA immunoreactive cells. Distribution of DCX immunoreactive cells in the dorsal hippocampus (B), and ventral layers of the hippocampus (D). Insets in (B,D) show a higher magnification of DCX immunoreactive cells. All high power images on inserts were taken from consecutive sections to low power images. Note multipolar DCX immunoreactive cells in (B) compared to unipolar DCX immunoreactive cells in (D). In all images dorsal is to the top and medial to the right. Scale bar in (D) = 500  $\mu$ m and applies to (A–D). The scale bar in the inset in (D) = 50  $\mu$ m and applies to all insets. See list for abbreviations.

core of HI and M. In the entopallium a low density DCX immunoreactive cells and fibers was observed. At rostral levels of these telencephalic pallial regions, DCX immunoreactive cells and fibers were present in a slightly higher density laterally than medially. Generally there was a rostro-caudal decrease in the amount of DCX immunoreactive material in these pallial regions. The NC and the nidopallium caudolaterale (NCL) exhibited a much higher density of DCX immunoreactive cells and fibers than any other pallial region, particularly in the ventral and lateral aspects. The arcopallium exhibited a very low density of DCX immunoreactive cells and fibers in both parrot species. The HP and APH contained a moderate density of DCX immunoreactive cells and fibers (Figure 5B). In the ventral hippocampus, a moderate density DCX immunoreactive material was observed in the tr and ll while the ml showed a low density (Figure 5D).

### Subpallial Regions

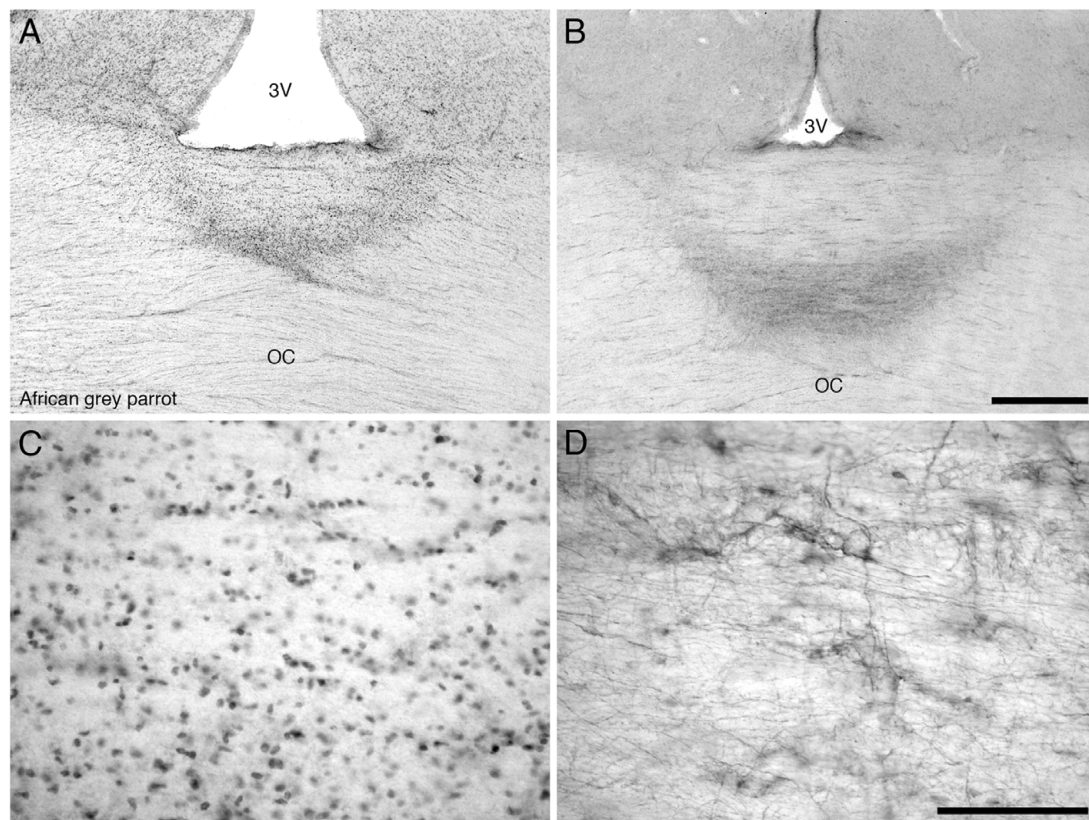
The medial and lateral striatum exhibited a moderate density of DCX immunoreactive cells and fibers at rostral levels, but the density of these cells and fibers decreased

noticeably at levels caudal to the anterior commissure (CA). In the MSt, the DCX immunoreactive cells and fibers were observed in higher density in the medial and ventral aspect than in the dorsal and lateral aspects. A low density of DCX immunoreactive cells and fibers was observed in the SM and SL; however, at caudal levels in both parrots the SL showed a moderate density of DCX immunoreactive cells and fibers. No detectible DCX immunoreactive structures were observed in the regions corresponding to the GP.

### Diencephalon

The diencephalon exhibited a very low density of DCX immunoreactive cells and fibers. A weak DCX immunoreactivity was observed in the paraventricular nuclei, including POA and POM of the medial hypothalamus and in the VMH of the ventral hypothalamus and in the dorsolateral group of nuclei of the diencephalon, which include the DMA, DLL, DLP, nucleus rotundus (Rt), SPC, and SpL. The OC showed DCX immunoreactive fibers (Figures 6B,D), while no staining was detected in CA.





**FIGURE 6 |** Photomicrographs of PCNA – (A,C) and DCX (B,D) – immunostained coronal sections through the optic chiasm (OC) at its border with the floor of the third ventricle (3V) in the brain of the African grey parrot (*Psittacus erithacus*). (A) Accumulation of PCNA immunoreactive cells in the optic chiasm (OC). (C) A high magnification image of the PCNA immunoreactive cells in the optic chiasm. (B) A concentration of DCX immunoreactive cells and fibers in the optic chiasm. (D) A higher magnification image of DCX immunoreactive cells and fibers showing distinct cells and thick fibers. These images depict a potential neurogenic zone at the dorsal midline of the optic chiasm. In all images dorsal is to the top. Scale bar in (B) = 500  $\mu$ m and applies to (A,B), scale bar in (D) = 100  $\mu$ m and applies to (C,D).

## Mesencephalon, Rhombencephalon, and Cerebellum

A low density of DCX immunoreactive fibers was observed in the layers of the optic tectum (SGF and SGC). Some mesencephalic nuclei and tracts also exhibited weak DCX immunoreactive fibers and vesicles including the GCt, nIV, ICo, and the fasciculus longitudinalis medialis (FLM). In the cerebellum, the Purkinje cell layer was weakly stained, with only fibers being observed (Figures 7B,D). In the pons and medulla oblongata weakly stained fibers and vesicles in very low density were observed in some nuclei including, but not limited to, the LLV, PM, PL, La, raphe nucleus (R), nXIIIts, and nX.

## DISCUSSION

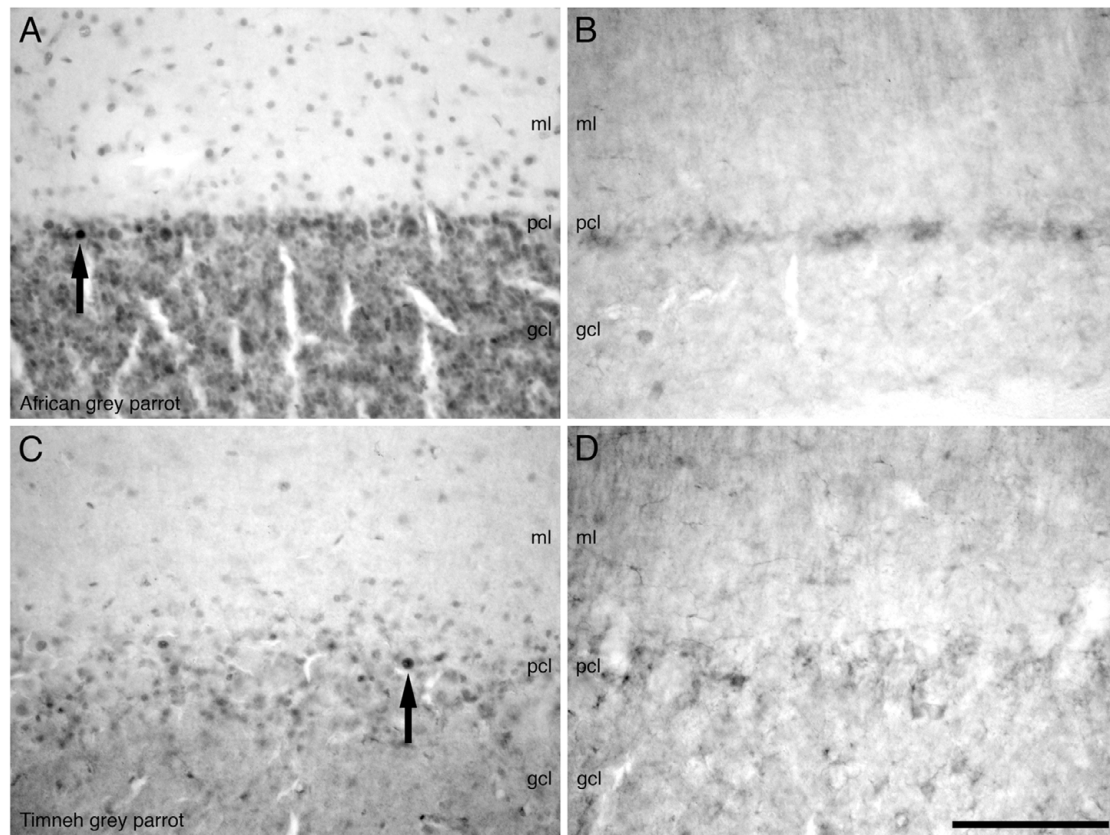
### General Considerations

In the current study we examined putative adult neurogenesis in the brains of the two species of African grey parrots the Congo African grey parrot (*Psittacus erithacus*) and Timneh grey parrot (*Psittacus timneh*) using PCNA and DCX immunohistochemistry. The PCNA immunoreactive cells were distributed heterogeneously in all brain regions including the

olfactory bulb, telencephalon, diencephalon, mesencephalon and rhombencephalon, while DCX immunoreactive structures were dense in the olfactory bulb, telencephalon and some regions of the diencephalon, but far less dense in some parts of the diencephalon, the mesencephalon and rhombencephalon in both African grey parrot species. To a large extent our results conform and are comparable to the wide spread distribution of adult neurogenesis reported in other birds (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014; Mazengenya et al., 2017). Despite the overall similarity in the distribution of PCNA and DCX in both African grey parrot species, region specific differences, particularly associated with the density of PCNA and DCX immunoreactive structures, were observed in the telencephalon. Such region specific differences might highlight the relationship between adult neurogenesis and brain function, particularly in regions responsible for certain behavioral repertoires in different species.

### Validity of PCNA and DCX Antibodies in Determining Adult Neurogenesis in Birds

The PCNA antibody used in the current study recognizes the 36 kDa nuclear protein subunit of the DNA polymerase that



**FIGURE 7 |** Photomicrographs of PCNA – (A,C) and DCX (B,D) – immunostained coronal sections through the cerebellar cortex in the brains of the two parrot species – (A,B) – African grey parrot (*Psittacus erithacus*), (C,D) – Timneh grey parrot (*Psittacus timneh*). A clear similarity in the distribution of PCNA immunoreactive cells in the layers of the cerebellar cortex in the African grey parrot (A) and Timneh grey parrot (C) is observed. A similar distribution and staining intensity of DCX immunoreactive cells and fibers in the layers of the cerebellar cortex in the African grey parrot (*Psittacus erithacus*) (B) and Timneh grey parrot (*Psittacus timneh*) (D) is also observed. Note large and deeply stained PCNA immunoreactive cells (arrows) in the Purkinje cell layer (pcl) in both parrots. In all images dorsal is to the top and medial to the right. Scale bar in (D) = 100  $\mu$ m and applies to all. See list for abbreviations.

is essential for DNA replication and is expressed selectively in proliferating cells (Hall et al., 1990; Balthazart and Ball, 2014). The PCNA antibody is present during the various stages of the cell cycle except the G<sub>0</sub> stage (Kurki et al., 1988; Hall et al., 1990; Balthazart and Ball, 2014). In birds, the PCNA antibody has been characterized in developing quail, parakeets, zebra finches (Charvet and Striedter, 2008, 2009) and chickens (Hannan et al., 1999; Capes-Davis et al., 2005). The regions where PCNA immunoreactivity was observed in the African grey parrots correspond with those reported in quails after BrdU injection (Charvet and Striedter, 2008). Apart from labeling proliferating cells, the PCNA antibody also labels cells repairing their DNA (Hall et al., 1990; Shivji et al., 1992; Essers et al., 2005) and remains detectable several days after cells exit the cell cycle (Balthazart and Ball, 2014). Such characteristics can affect the reliability of the PCNA antibody as a marker of adult neurogenesis in birds since they lead to an overestimation of proliferating cells.

Doublecortin is a microtubule associated protein that plays a key role in the migration of neurons during development and in post mitotic neurons undergoing migration, remodeling of their

dendritic processes, and synaptogenesis in adulthood (Francis et al., 1999; Gleeson et al., 1999; Nacher et al., 2001; Brown et al., 2003; Yang et al., 2004; Capes-Davis et al., 2005; Couillard-Despres et al., 2005). DCX expression levels have been found to decline with age in mammals (Brown et al., 2003; Couillard-Despres et al., 2005) and in birds (Ling et al., 1997; Hannan et al., 1999; Capes-Davis et al., 2005; Kim et al., 2006). In rats DCX expression is first observed 1 day after cell division and last for about 2–3 weeks until the neurons begin to express markers of mature neurons such as NeuN (Brown et al., 2003). In birds, DCX expression has been found to last for up to 30 days post mitosis (Balthazart et al., 2008). The DCX antibody was recommended for adult neurogenesis (Rao and Shetty, 2004; Couillard-Despres et al., 2005) and its expression has been consistent in areas known to recruit new neurons in mammals (Brown et al., 2003). In contrast, DCX expression in birds has been found in locations not associated with adult neurogenesis. In adult canaries BrdU immunoreactive cells were found to co-express the DCX antibody in the subtelencephalic regions (Vellema et al., 2014). The DCX antibody used in the current study has been characterized in the following birds: canaries



(Boseret et al., 2007; Balthazart et al., 2008; Yamamura et al., 2011; Vellema et al., 2014), chickadees (Fox et al., 2010), zebra finches (Kim et al., 2006), sparrows (LaDage et al., 2010), starlings (Hall and MacDougall-Shackleton, 2012), pigeons (Melleu et al., 2013, 2015; Mazenganya et al., 2017) Japanese quail (Balthazart et al., 2010) and chickens (Hannan et al., 1999; Capes-Davis et al., 2005; Mezey et al., 2012).

## Adult Neurogenesis in African Grey Parrots Compared to Other Birds

Adult neurogenesis has been observed in budgerigars (*Melopsittacus undulatus*), which are small parrots (Nottebohm, 2011), but the current study is the first to report the presence of adult neurogenesis in the brains of the larger African grey parrots. There are remarkable similarities and as well as some variations in the pattern of adult neurogenesis across the avian species studied to date. In the avian brain, new neurons are generated in the SVZ of the lateral ventricle, with the largest density of proliferating cells reported in its dorsal and ventral poles. These cells migrate to different target regions of the brain (Alvarez-Buylla et al., 1994). In the present study proliferating cells were observed in high density in the SVZ of the lateral ventricle and throughout its rostrocaudal extent. The Congo African grey parrot exhibited an accumulation of proliferating cells in the dorsal and ventral poles of the lateral ventricle, at the level of CA, similar to the proliferative “hot spots” described by Alvarez-Buylla (1990). These aggregates of proliferating cells were only observed in the dorsal pole of the lateral ventricle in the Timneh grey parrot. The proliferative hot spots phenomenon was also reported in other adult birds, including pigeons (Melleu et al., 2013), canaries (Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1998; Mazenganya et al., 2017), chickens (Mezey et al., 2012), and marsh tits (*Poecile palustris*) (Patel et al., 1997); however, they were absent in the adult ring dove (*Streptopelia risoria*) (Ling et al., 1997). The identity of the proliferating cells in these germinal zones remains contentious in both mammals and non-mammals. In the song bird, *Serinus canaria* the proliferative hot spots were associated with large accumulations of radial glial cells (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1988), suggesting that radial glial cells were the primary cells undergoing mitosis in the germinal zones (Alvarez-Buylla et al., 1990; Goldman, 1990; Vellema et al., 2010). In contrast, cell proliferation, including in the present study, was observed in the brain parenchyma (Alvarez-Buylla et al., 1988; Vellema et al., 2010; Mazenganya et al., 2017), the SVZ of the cerebral aqueduct, third, fourth, and tectal ventricles, albeit in low density, and in the midbrain, hindbrain and cerebellum (Vellema et al., 2010). This localized proliferative activity suggests that certain neurons may be produced and integrate locally without the need for cell migration, and or thus proliferating cells can be glia or endothelial cells (Vellema et al., 2010).

The recruitment of new neurons is widespread in the telencephalon of adult birds and affected by various factors including age, environmental complexity, seasonal variation, hormones, stress and social complexity (for review, see Barnea and Pravosudov, 2011). In the African grey parrots, the distribution of DCX immunoreactive cells and fibers exhibited

a similar pattern to that observed in the adult canaries (Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014), starlings (Absil et al., 2003) and zebra finches (Kim et al., 2006); however, DCX immunoreactivity was reported to be restricted to the telencephalon in other studies on adult canaries (Alvarez-Buylla and Nottebohm, 1988; Kirn et al., 1994; Vellema et al., 2010), pigeons (Melleu et al., 2013), ring doves (Ling et al., 1997), and chickens (Mezey et al., 2012).

Neuronal recruitment exhibited regional differences in the telencephalic subdivisions of adult canaries, zebra finches, domestic pigeons, and rock pigeons (Kim et al., 2006; Boseret et al., 2007; Balthazart et al., 2008; Melleu et al., 2013; Mazenganya et al., 2017). In the adult canaries and zebra finches, the highest densities of DCX immunoreactive cells were observed in the M, NC, and striatum. Intermediate DCX expression was observed in the hyperpallium and rostral nidopallium, while a low density of DCX immunopositive cells was noted in the hippocampus, arcopallium and subtelencephalic regions (Kim et al., 2006; Boseret et al., 2007; Vellema et al., 2014). We observed similar trends in the adult African grey parrots, but the hippocampus and parahippocampal regions exhibited moderate to high density of DCX immunoreactive cells and fibers. In addition, DCX immunoreactive neurons and fibers showed some area specific distribution in certain regions of the telencephalon and the brain stem nuclei in the two species of African grey parrots. For example, rostral nidopallium exhibited a moderate density DCX immunoreactive cells and fibers, whereas the caudal nidopallium exhibited the densest DCX immunoreactive cells and fibers. In addition, the distribution of DCX immunoreactive cells was not uniform in most telencephalic regions, where DCX immunoreactive cells and fibers were found in higher density in the medial and lateral margins compared to the core regions. The area of specific distribution of DCX immunoreactive cells and fibers observed in these parrot species was also reported in adult canaries (Vellema et al., 2010, 2014) and might highlight brain areas where plastic changes are necessary for the maintenance of behavioral repertoires in different species. In both song and non-song birds, the arcopallium and the entopallium exhibited absent to low densities of DCX immunoreactive cells (Goldman and Nottebohm, 1983; Vellema et al., 2010; Melleu et al., 2013; Mazenganya et al., 2017). Similarly, the two species of African grey parrots showed a low density of DCX immunoreactive cells in the entopallium and the arcopallium. In the arcopallium, the DCX immunoreactive cells and fibers were distributed only in the ventrocaudal margins encompassing the nucleus taenia (Tn).

The morphology of DCX immunoreactive cells varied in the different regions of the brain in the two species of African grey parrots. In regions closer to the lateral ventricle particularly the ventral striatum the DCX immunoreactive cells exhibited elongated somata with unipolar or bipolar morphology. In other regions of the telencephalon elongated DCX immunoreactive cells were mixed with cells with round and multipolar morphology, although the former were more abundant. Elongated unipolar or bipolar cells represent migrating young neurons, usually emanating from the SVZ, whereas the round and multipolar neurons characterize mature neurons (Alvarez-Buylla, 1990). Migration of neurons in the telencephalon of

birds follows two defined modes including tangential and radial migration (Alvarez-Buylla et al., 1988; Alvarez-Buylla, 1990). Vellema et al. (2010) further described the random migration of neuroblasts, whereby migrating immature neurons follow undefined routes within the telencephalon. In birds, the most pronounced mode of migration is radial migration, whereby migrating neuroblasts are guided on cellular processes of radial glial cells (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990; Vellema et al., 2010). Under this mode of migration, young neurons migrate from the SVZ of the lateral ventricle preferentially toward the lateral and ventrocaudal aspects of the telencephalon (Alvarez-Buylla, 1990; Vellema et al., 2010). In birds including in the current study, proliferative hot spots, both dorsal and ventral (PCNA immunoreactive as in the present study) coincide with accumulations of immature and migrating neurons (DCX immunoreactive cells and fibers) particularly in the hyperpallium and the striatum, respectively (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990). According to Alvarez-Buylla (1990), radial glia processes extend laterally for about 2 mm into the parenchyma of the adult avian telencephalon and these radial cells are concentrated in proliferative hot spots suggesting that migration through scaffolds of the radial glia processes is only possible for very short distances. In the current study, the Congo African grey parrot showed accumulation of a higher density of DCX immunoreactive cells and fibers in regions of the telencephalon corresponding to the dorsal and ventral hot spots whereas in the Timneh grey parrot a higher density of DCX immunoreactive structures was observed in the dorsal regions of the telencephalon.

This preferred direction of migration may explain the presence of the high densities of DCX immunoreactive cells and fibers in the lateral and caudal regions of the telencephalon when compared to the medial and rostral regions. Migration of immature neurons furthest from the extends of the radial glia processes becomes random and neuroblasts migrate and differentiate in regions that are topographically disjointed from the proliferative hot spot indicating that there is no SVZ specification for the final destination and position where new neurons differentiate and integrate (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990). This may also help to explain the variations in the topography of the proliferative hot spots observed in the current study and in other birds examined to date. According to Melleu et al. (2013), the majority of round and multipolar cells expressing the microtubule marker DCX do not co-express with the adult neuronal marker NeuN, suggesting that they are immature neurons even though they show the morphology of mature neurons.

Recruitment of new neurons in the diencephalon, mesencephalon and rhombencephalon of adult birds has been reported in experimental animals (Cao et al., 2002; Chen et al., 2006), but no studies support adult neuronal recruitment under normal physiological conditions. Despite this, reports in adult canaries (Boseret et al., 2007; Balthazart et al., 2008; Vellema et al., 2014) and zebra finches (Kim et al., 2006) indicate the presence of DCX immunoreactive cells in these regions. In the species of the African grey parrots studied herein, similar, but low density, distributions of DCX immunoreactive cells

and fibers were observed. In agreement with Kim et al. (2006) and Boseret et al. (2007) the low density expression of DCX immunoreactivity in the brain stem may not signify adult recruitment of new neurons, but represent cellular plasticity of adult neurons (Nacher et al., 2001; Brown et al., 2003).

## Functional Implications of Adult Neurogenesis Related to Certain Behaviors of the African Grey Parrots

The role of adult neurogenesis in brain function and behavior in various species is still contentious. In mammals adult hippocampal neurogenesis is generally associated with acquisition of new and clearance of old memories (Kempermann et al., 2004; Zhao et al., 2008); however in birds, neuronal recruitment is wide spread in the telencephalon, making it difficult to understand the precise functional implications of this widespread characteristic. In addition, birds live comparably longer than mammals of similar body mass, and adult neurogenesis in avian species may be adaptive, promoting continuous learning by updating and renewing memories (Nottebohm, 1991; Gahr et al., 2002). The effect of learning on adult neurogenesis is bidirectional. Learning has been found to increase the survival, maturation and the response to stimuli of adult born neurons (Kee et al., 2007; Tronel et al., 2010; Lemaire et al., 2012). In contrast, adult neurogenesis facilitates learning in new conditions, which may include new habitats, new members of social groups, or new sources of food. In avian species adult neurogenesis is coupled with cell death (Kirn and Nottebohm, 1993; Rasika et al., 1994; Yamamura et al., 2011) and has been found to be task-dependant in various species (Barker et al., 2011). According to Nottebohm et al. (1986) increased singing in canaries leads to increased neuronal recruitment in the high vocal centre (HVC). In food storing species increased hippocampal neurogenesis was observed during the peak periods of food caching in autumn, whereas in non-storing species no addition of hippocampal neurons was recorded during the same period (Barnea and Nottebohm, 1996; Sherry and Hoshoooley, 2009). Moreover in migratory species, which rely on spatial memory, species with larger home ranges exhibited increased hippocampal neurogenesis compared to related species that inhabit smaller territories (Cristol et al., 2003; Hoshoooley and Sherry, 2007; LaDage et al., 2009). The olfactory bulbs recruit new neurons in adults of almost all studied species, except for a few studies of canaries and zebra finches (Barker et al., 2011; Melleu et al., 2013). In the current study we observed a high density of PCNA and DCX immunoreactive structures in the inner layers of the olfactory bulbs in both African grey parrots. The functional implications of olfactory neurogenesis remain elusive, but the olfactory system is involved in reproduction, the monitoring of environmental changes, the maintenance of survival by detecting presence of predators, the identification of clan members and the location of food (Jones and Roper, 1997; Patzke et al., 2010; Barker et al., 2011).

Apart from adult hippocampal and olfactory neurogenesis, neuronal recruitment in the NC has been shown to increase under various states of environmental enrichment. In a study



conducted on zebra finches, adult neurogenesis in the NC was found to increase in adults introduced to communal living compared to individuals living in pairs (Lipkind et al., 2002). The reason associated with this increase is the possibility that individuals introduced to communal living need to identify new members and establish new social relationships (Lipkind et al., 2002; Adar et al., 2008). Similar changes in neuronal recruitment in the NC were identified during the reproductive cycle, when adult individuals of zebra finches were feeding their young (Barkan et al., 2007). In the present study, the NC and the NCL exhibited the densest DCX immunoreactivity in both species of African grey parrots. The NC is associated with reproductive behavior (Melleu et al., 2013), whereas the NCL, a proposed analog of the mammalian prefrontal cortex, participates in higher order cognitive functions, such as decision making, speech and planning of behavior (Güntürkün, 2005).

African grey parrots live in large colonies of approximately 10,000 individuals (Parr and Juniper, 2010). Although few studies have examined the behavior of African grey parrots in the wild, results show that their social ecology is comparable to that of primates (Emery et al., 2007). The African grey parrots lead a complex social life coupled with long

term monogamous relationships (Seibert, 2006; Emery et al., 2007). Shultz and Dunbar (2010) postulated that species in monogamous relationships develop larger brain to body mass ratios to preserve the stable pair bonded relationships. The African grey parrots may be useful models to assess the impact of changing social environments on adult neurogenesis.

## AUTHOR CONTRIBUTIONS

AI and PRM designed the study, analyzed the data, and reviewed the final manuscript for submission. PM and AB collected and processed tissue and carried out the initial analysis of data. PM prepared the initial manuscript draft.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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