RESEARCH PAPER

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Changes in neurogenesis with post-hatching age in the male Japanese quail (*Cortunix japonica*) brain

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Most avian neurogenesis studies have previously focused on the song control system and little attention has been given to non-song birds. The objective of this study was to assess changes in neurogenesis associated with post-hatching age (3-12 weeks) in the Japanese quail brain using proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) immunohistochemistry. PCNA-immunoreactive (ir) cells were observed mainly in the olfactory bulb ventricular zone, telencephalic ventricular zones and cerebellum. Fewer PCNA-ir cells were also observed in the hypothalamus, thalamus and bed nucleus of the stria terminalis. In telencephalic ventricular zones, PCNA-ir cells were concentrated ventrally and dorsally adjacent to the mesopallium and medial striatum, respectively. DCX-ir cells were observed in the olfactory bulb, telencephalon and cerebellum. Furthermore, DCX-ir cells were scattered throughout the pallium except in the entopallium and arcopallium, septal nuclei and striatum. Fewer DCX-ir cells were also observed in the hippocampus and bed nucleus of stria terminalis. The density of PCNA-ir cells and DCX-ir cells in all brain areas declined with post-hatching age. In conclusion, cell proliferation appears to be restricted to the ventricular zones whereas neuronal recruitment is more widespread in the olfactory bulb, telencephalon and cerebellum. Postnatal neuronal incorporation appears to be absent in the diencephalon and mesencephalon.

Key words: neurogenesis, proliferating cell nuclear antigen, doublecortin, post-hatching, Japanese quail brain, neuronal recruitment

INTRODUCTION

Across all vertebrate species, the central nervous system harbours constitutively active neuronal progenitors that are responsible for producing new neurons throughout life (Alunni and Bally-Cuif 2016). The process through which new neurons are formed from these progenitors is termed neurogenesis and involves cell proliferation, differentiation, migration and integration of the newly formed neurons into the existing neuronal circuitry (Barnea and Pravosudov 2011). In mammalian adult brains, neurogenesis is restricted to the subgranular zone of the hippocampus and the subventricular zone of the lateral ventricle (Kaslin et al. 2008, Olaleye and Ihunwo 2014). However, in avian brains new neurons are generated along the walls of the lateral ventricle and migrate radially to be integrated into the different circuits of the telencephalon (Hall et al. 2014, Vellema et al. 2010).

The songbird species such as the canary and zebra finch have been used extensively in post-hatching neurogenesis studies (Brenowitz 2004, Brenowitz and Larson 2015, Nottebohm 1984, 2011). Although neurogenesis in songbird brains was observed to be widespread, the focus of most studies has been almost exclusively on neuronal recruitment and integration into telencephalic circuits involved in song production (Alvarez-Buylla et al. 1992, 1994, Balthazart et al. 2008, Balthazart and Ball 2016, Boseret et al. 2007, Nottebohm 2011). However, songbirds constitute only about 2% of all avian species (Ling et al. 1997). Furthermore, previous studies have revealed that the neurogenesis observed in the telencephalon is not only specific to songbirds but also occurs in other avian species belonging to other orders such as ring doves (Ling et al. 1997, Ling and Cheng

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1995), pigeons (Melleu et al. 2013, 2016, Meskenaite et al. 2016) chicken (Mezey et al. 2012, Nikolakopoulou et al. 2006a) and quails (Balthazart et al. 2010, Bardet et al. 2012, Mouriec and Balthazart 2013, Nikolakopoulou et al. 2006b).

Neurogenesis in the avian brain has been shown to be regulated by several internal and external factors including levels of circulating sex steroids (e.g. testosterone, estradiol), variations in day length, seasonal changes and age (Barker et al. 2014, DeWulf and Bottjer 2002, Guigueno et al. 2016, Meskenaite et al, 2016, Mouriec and Balthazart 2013, Yamamura et al. 2011).

Age-related decline in the production and recruitment of neuronal cells has been reported in the telencephalon and song control nuclei of canaries (Alvarez-Buylla et al. 1994) and zebra finches (DeWulf and Bottjer 2002, Pytte et al. 2007, Wang et al. 2002). In addition, neurogenesis was reported to decline with age in the telencephalic ventricular zones of the ring doves (Ling et al. 1997), the hippocampus and olfactory bulb (OB) of the pigeon (Meskenaite et al. 2016), and the telencephalon of the chicken (Mezey et al. 2012). Interestingly, Mouriec and Balthazart (2013) reported an age-dependent decline in cell proliferation in the medial preoptic nucleus (POM) of the Japanese quail brain during the pre-pubertal stages (postnatal day, PND 1 to 43) which was followed by an increase in cell proliferation at puberty (PND 44 to 56).

The majority of neurogenesis studies in quails focussed on the sex steroid sensitive areas like the POM (Bardet et al. 2012), intermediate medial mesopallium and lateral septal nucleus (Nikolakopoulou et al. 2006b) with little attention given to neurogenesis in other quail brain areas like the telencephalon.

The aim of the present study was therefore to determine the effect of post-hatching age on cell proliferation and neuronal recruitment in the male Japanese quail (*Cortunix japonica*) brain. Japanese quails are precocial birds that belong to the order galliformes. In addition, male quails exhibit crowing, a testosterone dependent behaviour. However, crowing is considered a non-learned vocalisation in contrast to the learned song of songbirds.

To achieve this aim, proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) were used to assess cell proliferation and neuronal recruitment respectively in quail brains.

METHODS

Experimental Animals

Thirty male Japanese quails with post-hatching ages ranging from 3 to 12 weeks obtained from the agricultural research centre (ARC, Irene, South Africa) were used in this study. All animals were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee, which corresponds with those of the National Institute of Health (NIH) for care and use of animals in scientific experimentation. All protocols were approved by the University of the Witwatersrand Animal Ethics Screening Committee (AESC/2012/53/01).

Throughout their life, the birds were maintained on a photoperiod simulating long days (16 : 8 h, light : dark) with access to food and water *ad libitum.* During the first 3 weeks after hatching, chicks were kept in mixed groups in brooder cages at 30°C. Temperature was then progressively reduced to room temperature by the 5th week. From post-hatching age 3 weeks, 3 birds were collected every week up until the 12th week. The quails were allocated to groups according to their sexual maturity as follows: juvenile (sexually immature, 3-5 weeks old, n=9), subadult (sexually matured, 6-8 weeks old, n=9) and adult (sexually active, 9-12 weeks old, n=12).

Tissue collection and processing

All birds were given a dose of heparin (1 ml i.m.) 5 min prior to euthanasia to prevent blood clotting followed by an intraperitoneal injection of Euthapent (0.5–1 ml/kg). Animals were then perfusion-fixed transcardially, initially with 0.9% cold saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) solution. After perfusion, brains were carefully removed from the skull, post-fixed overnight in 4% paraformaldehyde in 0.1 M PB and weighed before being transferred into 30% sucrose in 0.1 M PB until equilibration was reached. The brains were sectioned in a coronal plane at 50 µm using a cryostat (Shandon Cryotome E, Thermo Fischer scientific, UK) at -24°C and repeated series of 5 sections were collected free-floating into 24 multi-well plates containing 0.1 M PB. The first series of sections was mounted on gelatin coated slides before staining with 1% cresyl violet for anatomical orientation, the second series for PCNA and the third for DCX immunohistochemistry. The remaining two series were stored in a cryoprotectant solution at -20°C for future use.

Immunohistochemistry

Brain sections were pre-treated for 30 min at room temperature under gentle shaking with an endogenous peroxidase inhibitor (0.1 M PB, 100% meth-

anol and 30% H_2O_2 in the ratio 49.2 : 49.2 : 1.66). Following three 10 min rinses in 0.1 M PB, the sections were subsequently pre-incubated in a blocking buffer solution (3% normal horse serum or 3% normal rabbit serum, 2% bovine serum albumin, 0.25% Triton X-100 in 0.1 M PB) for 2 h under gentle shaking at room temperature to prevent non-specific binding. Sections were then transferred into a primary antibody solution 1 : 500, mouse anti-PCNA, clone PC 10; NCL-L-PCNA, Novocastra, UK or 1 : 300, goat anti-DCX, C-18 : SC-8066, Santa Cruz Biotechnology, Inc, USA, in the blocking buffer solution, and were incubated for 48 h at 4°C under gentle shaking. Following incubation, sections were subjected to three 10 min rinses in 0.1 M PB before being incubated in secondary antibody solution 1 : 1000 biotinylated anti-mouse IgG, Novocastra, UK or 1 : 1000 dilution of biotinylated anti-goat IgG, BA-5000; Vector Laboratories, USA in blocking buffer solution, for 2 h at room temperature under gentle shaking. Following three 10 min rinses in 0.1 M PB, the sections were incubated in an avidin-biotin solution 1 : 125, Vector Laboratories in 0.1 M PB for 1 h. The sections were then transferred into three 10 min 0.1 M PB rinses before being placed in a solution containing 0.05% 3, 3 Di-amino-benzidine tetrachloride (DAB) in 0.1 M PB for 5 min. To each 1 ml of this solution, 3.3 µl of 30% H_2O_2 was added, and chromatic precipitation was visually monitored under a low power stereomicroscope.

Development was subsequently stopped by placing the sections in 0.1 M PB, followed by a final 10 min rinse in 0.1 M PB. Sections were mounted on 0.5% gelatinised slides, left to dry overnight, dehydrated in a graded series of alcohols, cleared in xylene and cover slipped with Depex. To ensure that non-specific staining did not affect the results, control sections taken at random were processed in the same manner, but either the primary or the secondary antibody was omitted. No labeled cells were observed in either case.

Data Analysis

Sections were analysed qualitatively with both low and high power microscopy to yield a comparative description of the distribution of PCNA and DCX-ir neurons. Brain structures were identified based on the atlas of the quail or chicken brain (Baylé et al. 1974, Kuenzel and Masson 1988, Puelles 2007) and we adopted the nomenclature by the Avian Brain Forum (Reiner et al., 2004). High power microscopic observations were used to determine the relative densities of stained structures throughout the different regions of the brain. The relative densities of immunostained structures were visually compared and recorded on a scale ranging from absent (-) to low (+) to moderate (++) to high (+++). A second observer was used to eliminate observer bias.

High resolution microscopic images were digitally captured using an AxioCam HRC digital camera on a Zeiss Axioplan 2 compound microscope and a Zeiss Discovery SteREO v20 Stereomicroscope connected to a PC running Axiovision software. Composite images were prepared with the Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA). No pixilation adjustment or manipulation of the captured images was undertaken, except for the adjustment of contrast and brightness levels.

RESULTS

We report here changes in PCNA-ir and DCX-ir from post-hatching age 3 weeks to 12 weeks.

Age related changes in PCNA expression in the Japanese quail brain

Olfactory bulb

In juvenile quails, a high density of PCNA-ir cells was observed in the ependymal and subependymal lining of the olfactory ventricles (Fig. 1A, B). In addition, a few PCNA-ir cells were observed extending dorsally into the hyperpallium densocellulare (HD) (Fig. 1C). The distribution of PCNA-ir cells in the OB of subadult and adult quails was similar to that observed in juvenile quails except that no PCNA-ir cells were observed extending into the HD in adult quails older than 9 weeks (Fig. 3A-C, Table I).

Telencephalon

In the juvenile telencephalon, a high density of PCNA-ir cells was observed throughout the rostrocaudal extent of the lateral ventricles (Fig. 1D-L). In the ventricular zones, PCNA-ir cells were mostly abundant ventrally adjacent to the medial striatum (Mst) and dorsally adjacent to the mesopallium (M) (Fig. 1D, E). In addition, a few PCNA-ir cells were observed in the M (arrows in Fig. 1E) of quails aged 3 and 4 weeks. However, no PCNA-ir cells were observed in the rest of the pallial areas. In the subpallium, a low density of PCNA-ir cells was observed in the Mst adjacent to the lateral ventricles (Fig. 1H, I). Fewer cells were also observed in the bed nucleus of stria terminalis (bed nucleus of the stria terminalis, medial division, BSTM and bed nucleus of the stria terminalis, lateral division, BSTL) (arrows in Fig. 1L).

The distribution of PCNA-ir cells in the telencephalon of subadult and adult quails was similar to that observed in the juvenile group although the density of PCNA-ir cells was lowest in the adult group (Figs 3D-I). However, no PCNA-ir cells were observed in the pallial areas of the subadult and adult quails. In addition, PCNA-ir cells were absent in the BSTM and BSTL of adults older than 9 weeks or in the Mst of adult quails (Table I).

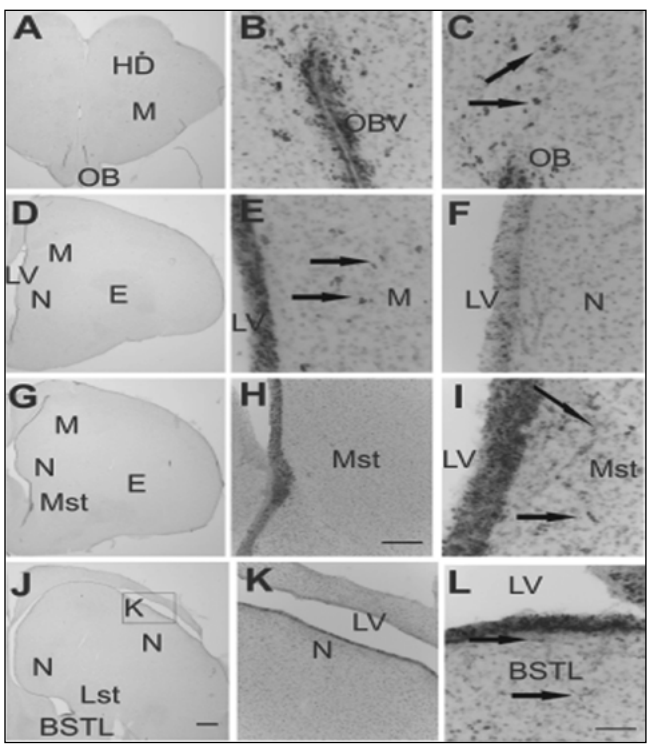

Fig. 1. Photomicrographs showing distribution of PCNA-ir cells in the olfactory bulb (OB) and telencephalon of the juvenile Japanese quail brain. (A) Low power photomicrograph showing distribution of PCNA-ir cells in the OB. (B) High power photomicrograph showing distribution of PCNA-ir cells along the OB ventricular lining. (C) High power photomicrograph showing PCNA-ir cells (black arrows) extending from the OB to the hyperpallium densocellulare (HD). (D) Low power image showing distribution of PCNA-ir cells long the lining of the lateral ventricle (LV) of the rostral telencephalon. (E) High power image showing distribution PCNA-ir cells along the lining of the LV adjacent to the mesopallium (M) (black arrows showing PCNA-ir cells in the M). (F) High power image showing distribution PCNA-ir cells in the lining of the lateral ventricle adjacent to the nidopallium (N). (G) Low power image showing distribution of PCNA-ir cells long the lining of the lateral ventricle of the mid telencephalon. (H) Photomicrograph showing an increase in PCNA-ir cells on the ventral reach of the lateral ventricular zones adjacent to medial striatum (Mst). (I) High power image showing PCNA-ir cells in the Mst (black arrows) adjacent to the lateral ventricle. (J) Low power photomicrograph showing distribution of PCNA-ir cells long the lining of the lateral ventricle of the caudal telencephalon (insert shows part of the lateral ventricle magnified in figure K). (K) High power image showing distribution of PCNA-ir cells along the lateral ventricular lining of the caudal telencephalon. (L) High power photomicrograph showing the distribution of PCNA-ir cells in the bed nucleus of the stria terminalis lateral division (BSTL) (black arrows). E, entopallium; OBV, olfactory bulb ventricle. Scale bars: J=500 μm and applies to A, D, G; L=50 μm and applies to B, C, E, F, I, K and H=200 μm.

Diencephalon

In the hypothalamus of juvenile quails, medium densities of PCNA-ir cells were observed in the POM (Figs 2A, 3J), nucleus paraventricularis (PVN), nucleus ventromedialis hypothalami (VMN) and nucleus tuberis (Tu). In the thalamus, low densities of PCNA-ir cells were observed in the nucleus rotundus (Rt), nucleus ovoidalis (OV), nucleus dorsolateralis anterior thalami (DLA), nucleus dorsomedialis anterior thalami (DMA) and nucleus geniculatus lateralis pars ventralis (Glv) (Fig. 2B). A low density of PCNA-ir cells was also observed lining the ventricular zones of the 3rd ventricle of the diencephalon. The pattern of expression of PCNA in the diencephalon of subadult and adult quails was identical to that observed in juvenile quails although the density of PCNA-ir cells appeared to decline with age (Table I).

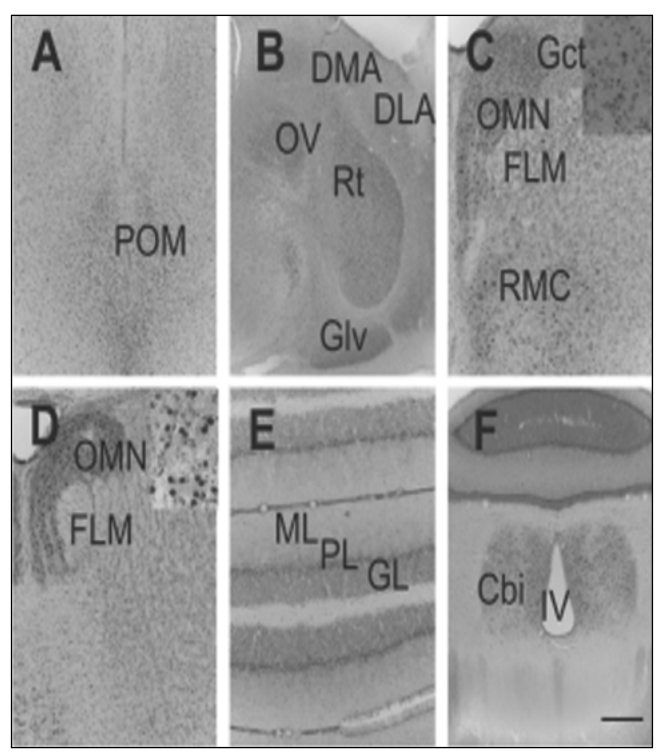

Fig. 2. Photomicrographs showing distribution of PCNA-ir cells in the nucleus preopticus medialis (POM) (A), thalamic nuclei (B), mesencephalic nuclei (C, D) nuclei, cerebellar cortex (E) and the nucleus cerebellum internum (Cbi) (F) of the juvenile Japanese quail brain. Inserts in figures C and D represent high magnifications of the griseum centrale (Gct) and oculomotor nucleus (OMN) respectively. DLA, nucleus dorsolateralis anterior thalami; DMA, nucleus dorsomedialis anterior thalami; FLM, fasciculus longitudinalis medialis; GL, granule cell layer; Glv, nucleus geniculatus lateralis, pars ventralis; ML, molecular cell layer; OV, nucleus ovoidalis; PL, Purkinje cells; Rt, nucleus rotundus; RMC, red nucleus magnocellular part; IV, fourth ventricle. Scale bars: F=200 μm and applies to images A-F; inserts in C and D=20 μm.

Mesencephalon and optic tectum

In the juvenile mesencephalon, a high density of strongly stained PCNA-ir cells was observed in the oculomotor nucleus (OMN) and red nucleus magnocellular part (RMC) located ventral to the OMN (Figs 2C-D, 3M). In addition, a high density of small weakly stained PCNA-ir cells was also observed in the griseum centrale (Gct) (Fig. 2C). Furthermore, a low density of PCNA-ir cells was observed in the lining of the mesencephalic aqueduct. The distribution of PCNA-ir cells in the mesencephalon of subadult and adult groups was similar to that of the juvenile groups. However, the density of PCNA-ir cells was lowest in the adult group. In the optic tectum, no PCNA-ir cells were observed in all age-groups (Table I).

Cerebellum

In the cerebellum of juvenile quails, a high density of PCNA-ir cells was observed in the Purkinje and granule cell layers of the cerebellum (Figs 2E, 3P). In addition to this, fewer PCNA-ir cells were found in the nucleus cerebellum internum (Cbi) situated on either side of the cerebellar recess of the fourth ventricle (Fig. 2F). A similar pattern of PCNA expression was observed in subadult quails although the number of PCNA-ir cells decreased with age. In the adult quails, fewer PCNA-ir cells were observed in the cerebellar layers whereas no PCNA-ir cells were observed in the Cbi (Table I).

Age-related changes in DCX expression in the Japanese quail brain

Two main types of DCX-ir cells were observed in the Japanese quail brain. Type 1 cells were round multipolar cells that displayed spherical or triangular shapes with cytoplasm filled with DCX and pale nuclei devoid of DCX staining. Long DCX-ir fibres extending from the cell bodies were also observed (Fig. 4A). The type 1 pattern of staining was mostly observed in the pallial (towards the external surface) and striatal parts of the telencephalon. In addition, smaller type 1 cells were observed in the cerebellum.

Type 2 cells were either unipolar or bipolar cells that displayed elongated DCX-ir cell bodies with long

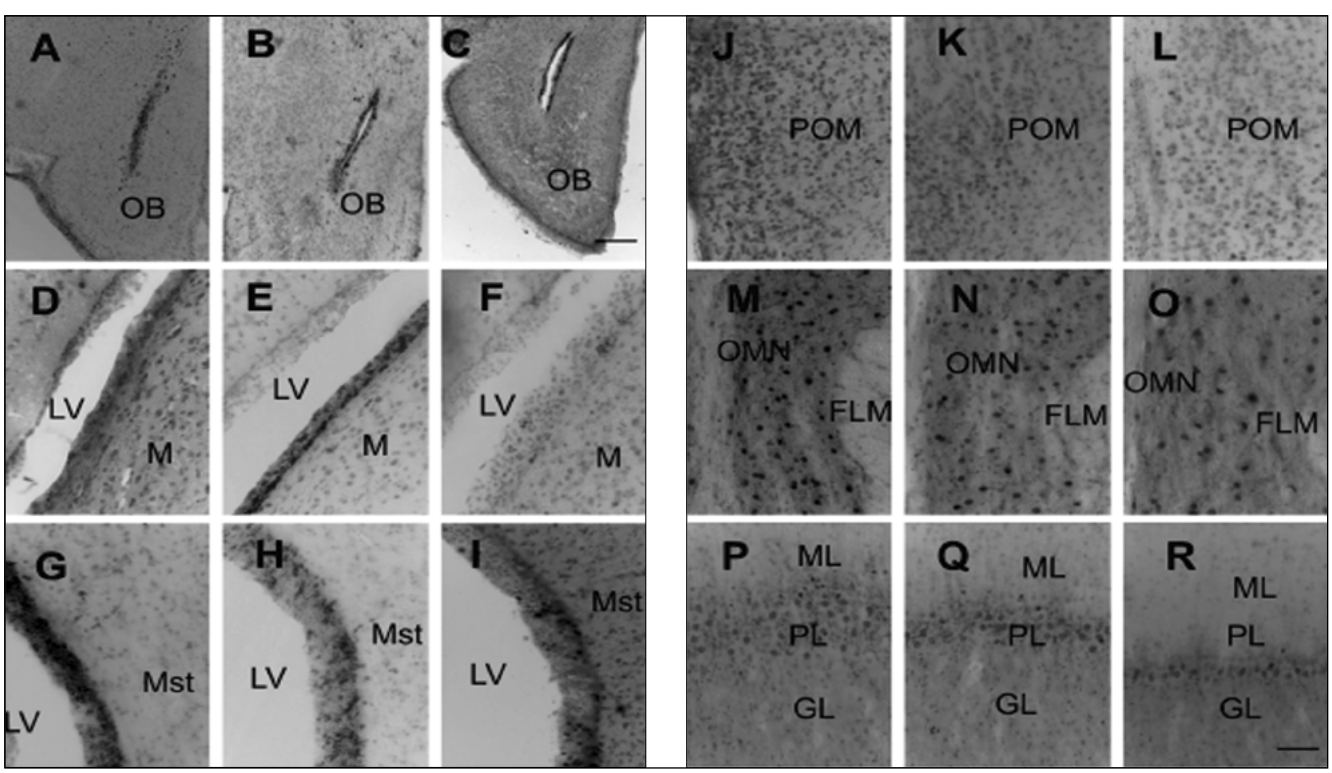

Fig. 3. Photomicrographs showing the distribution of PCNA-ir cells in the different parts of the brain of juvenile (A, D, G, J, M and P), subadult (B, E, H, K, N and Q) and adult (C, F, I, L, O and R) Japanese quails. Ventricular lining of olfactory bulb (OB) (A-C), ventricular lining adjacent to mesopallium (M) (D-F), ventricular lining adjacent to medial striatum (Mst) (G-I), nucleus preopticus medialis (POM) (J-L), oculomotor nucleus (OMN) (M-O) and cerebellum (P-R). FLM, fasciculus longitudinalis medialis; GL, granule cell layer; LV, lateral ventricle; ML, molecular cell layer; PL, Purkinje cell layer. Scale bars: C=200 μm and applies to A and C; R=50 μm and applies to D-Q.

fibres originating from one or both ends. Furthermore, the cell bodies of type 2 cells displayed elongated pale staining nuclei (Fig. 4B). The type 2 staining pattern was mostly observed in the pallial areas adjacent to the lateral ventricles, striatum, septum, and cerebellar cortex.

Olfactory bulb

In juvenile quails, the OB was intensely stained with a compact plexus of DCX-ir fibres interspersed with small round or fusiform (types 1 and 2) weakly stained cell bodies in the ependymal and subependymal linings of the olfactory ventricles (Figs 5A-B, 7A). In addition, a dense network of DCX-ir fibres extended dorsally into the HD (Fig. 5C). The pattern of DCX staining in the OB of subadult and adult animals was identical to that observed in juvenile animals, although there was a marked decline in the density of DCX-ir cells and fibres with age (Fig. 7A-C). In contrast to juvenile and subadult quails, no DCX-ir fibres were observed extending into the HD in adult quails.

Telencephalon

In juvenile quails, the highest density of DCX-ir cells and fibres were observed in the pallial part (hyperpallium apicale, HA, HD, M and nidopallium, N) of the telencephalon, although the density of positive cells varied across different pallial areas (Fig. 5D-N). Type 1 cells were mostly observed near the external surfaces of the pallium, whereas type 2 cells were observed near the lateral ventricles. The HA, HD, M and N were densely populated by types 1 and 2 DCX-ir cells. A much higher density of type 1 and 2 DCX-ir cells was observed on the ventral aspect of the nidopallium caudale (NC). In addition, a high density of DCX-ir fibres was observed in the pallial areas adjacent to the lateral ventricles with the highest densities observed in the M and Mst. The hippocampus (HP) had the lowest density of DCX-ir cells in the pallium (Fig. 5G, H). However, DCX-ir elements (cells or fibres) were absent in the entopallium (Fig. 5D, K) and arcopallium.

In the subpallium, a high density of type 1 and 2 cells was observed in the Mst (Fig. 5D, G, I) and lateral striatum (Lst) (Fig. 5G, J, K). A low density of type 2 cell bodies was also observed in the septum (lateral septum, SL and medial septum, SM) (Fig. 5J) and in the bed nucleus of stria terminalis (BSTM and BSTL) (Fig. 5N). However, no DCX-ir cells or fibres were observed in the globus pallidus (Fig. 5G).

Throughout the entire telencephalon, the lateral ventricular walls were lined with a high density of DCX-ir elements. The distribution of DCX-ir cells in the pallium of subadult and adult quails was similar to that observed in juvenile quails except for a reduction in the density of DCX-ir elements with age (Figs 7D-I, Table II). Similarly, the density of DCX-ir elements declined with age in the subpallium of subadult and adult quails (Figs 7J-O, Table II). However, no DCX-ir elements were observed in the BSTM and BSTL of adult quails older than 9 weeks (Table II).

Diencephalon, mesencephalon and optic tectum

At all ages studied, no type 1 or type 2 DCX-ir cells were observed in the ventricular or parenchymal areas of the diencephalon, mesencephalon and optic tectum (Fig. 6A-C).

Cerebellum

A high density of small bipolar DCX-ir cells (type 2) was observed in the granular and Purkinje layers of the juvenile quail cerebellar cortex (Fig. 6D, F, G). In addition, a few type 2 cells with long fibres were also observed in the molecular layer of the cerebellum (Fig. 6D, E).

In the subadult and adult quail cerebellar cortex, the pattern of DCX-ir cells was similar to that observed

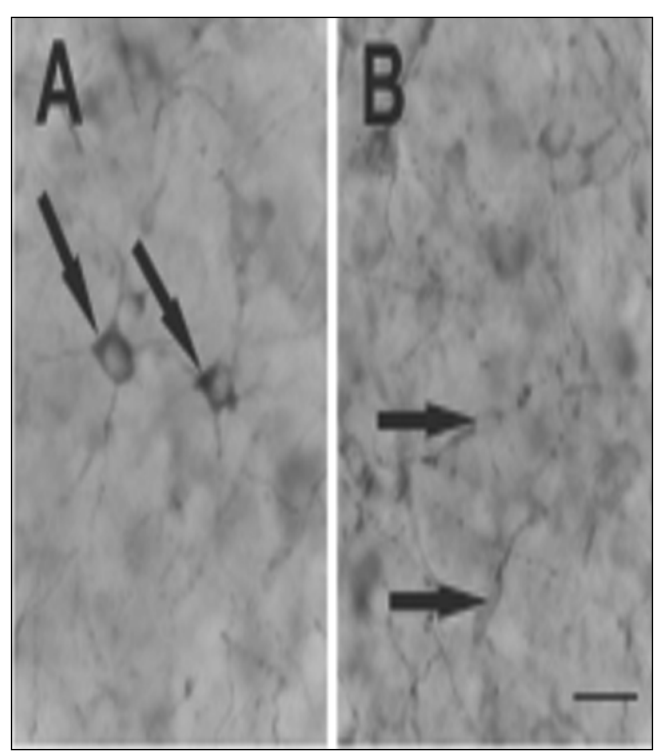

Fig. 4. Photomicrographs showing type 1 (A) and type 2 (B) DCX-ir cells in the Japanese quail brain (black arrows). Scale bar: B=20 μm and also applies to A.

in juveniles although the densities declined with age (Fig. 7P-R, Table II). However, no type 1 or type 2 DCX-ir cells were observed in the Cbi at all ages studied (Fig. 6H).

Table I. Age-related changes in PCNA immunoreactivity in the Japanese quail brain.

			Post hatching age									
			3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks
Olfactory Bulb	OBV		+++	+++	+++	++	++	++	++	+	+	+
Telencephalon	LV		+++	+++	+++	+++	+++	+++	++	++	++	++
	Pallium	HA	–	–	–	–	–	–	–	–	–	–
		HD	–	–	–	–	–	–	–	–	–	–
		M	–*	–*	–	–	–	–	–	–	–	–
		N	–	–	–	–	–	–	–	–	–	–
		E	–	–	–	–	–	–	–	–	–	–
		A	–	–	–	–	–	–	–	–	–	–
		HP	–	–	–	–	–	–	–	–	–	–
	Subpallium	Mst	–**	–**	–	–	–	–	–	–	–	–
		Lst	–	–	–	–	–	–	–	–	–	–
		GP	–	–	–	–	–	–	–	–	–	–
		SL	–	–	–	–	–	–	–	–	–	–
		SM	–	–	–	–	–	–	–	–	–	–
		BSTM	+	+	+	+	+	+	+	–	–	–
		BSTL	+	+	+	+	+	+	+	–	–	–
Diencephalon	Hypothalamus	POM	++	++	++	+	+	+	+	+	+	+
		PVN	+	+	+	+	+	+	+	+	+	+
		VMN	+	+	+	+	+	+	+	+	+	+
		Tu	+	+	+	+	+	+	+	+	+	+
	Thalamus	Rt	+++	+++	+++	++	++	++	++	+	+	+
		OV	+++	+++	+++	++	++	++	++	+	+	+
		DLA	++	++	++	+	+	+	+	+	+	+
		DMA	++	++	++	+	+	+	+	+	+	+
		Glv	+++	+++	+++	++	++	++	++	+	+	+
Mesencephalon		Gct	+++	+++	+++	++	++	++	++	++	+	+
		OMN	+++	+++	+++	++	++	++	++	++	+	+
		RMC	++	++	++	+	+	+	+	+	+	+
Optic Tectum			–	–	–	–	–	–	–	–	–	–
Cerebellum		CB	+++	+++	+++	++	++	++	+	+	+	+
		Cbi	+++	+++	+++	++	++	++	+	+	+	+

+++– high density, ++– medium density, +– low density. PCNA–ir cells in the mesopallium (*) and medial striatum (**). – No positive PCNA–ir.

DISCUSSION

Our study used PCNA and DCX to investigate cell proliferation and neuronal incorporation in the Japanese quail brain to determine how these processes change with post-hatching age.

Methodological considerations

Proliferating cell nuclear antigen, also known as cyclin, is a protein associated with catalytic activity of DNA polymerase A and is critical to the extension of the DNA strand during replication (Balthazart and Ball 2014b). PCNA is therefore expressed during the G1 and S phases of the cell cycle and not in G0 or the resting phase. In addition, PCNA expression is highly conserved among eukaryotes (Charvet and Striedter 2008) and has been employed in the current study as a marker of cell proliferation in the Japanese quail brain. PCNA immunolabelling was used previously in the avian brain to compare cell proliferation during ontogeny in birds that possess large (Parakeets) *vs.* small (Bobwhite quails) brains in adulthood (Charvet and Striedter 2008). Additionally, Bardet et al. (2012) and Mouriec and Balthazart (2013) also used PCNA to demonstrate age dependent changes in cell proliferation in the POM of Japanese quail brains. In the quail

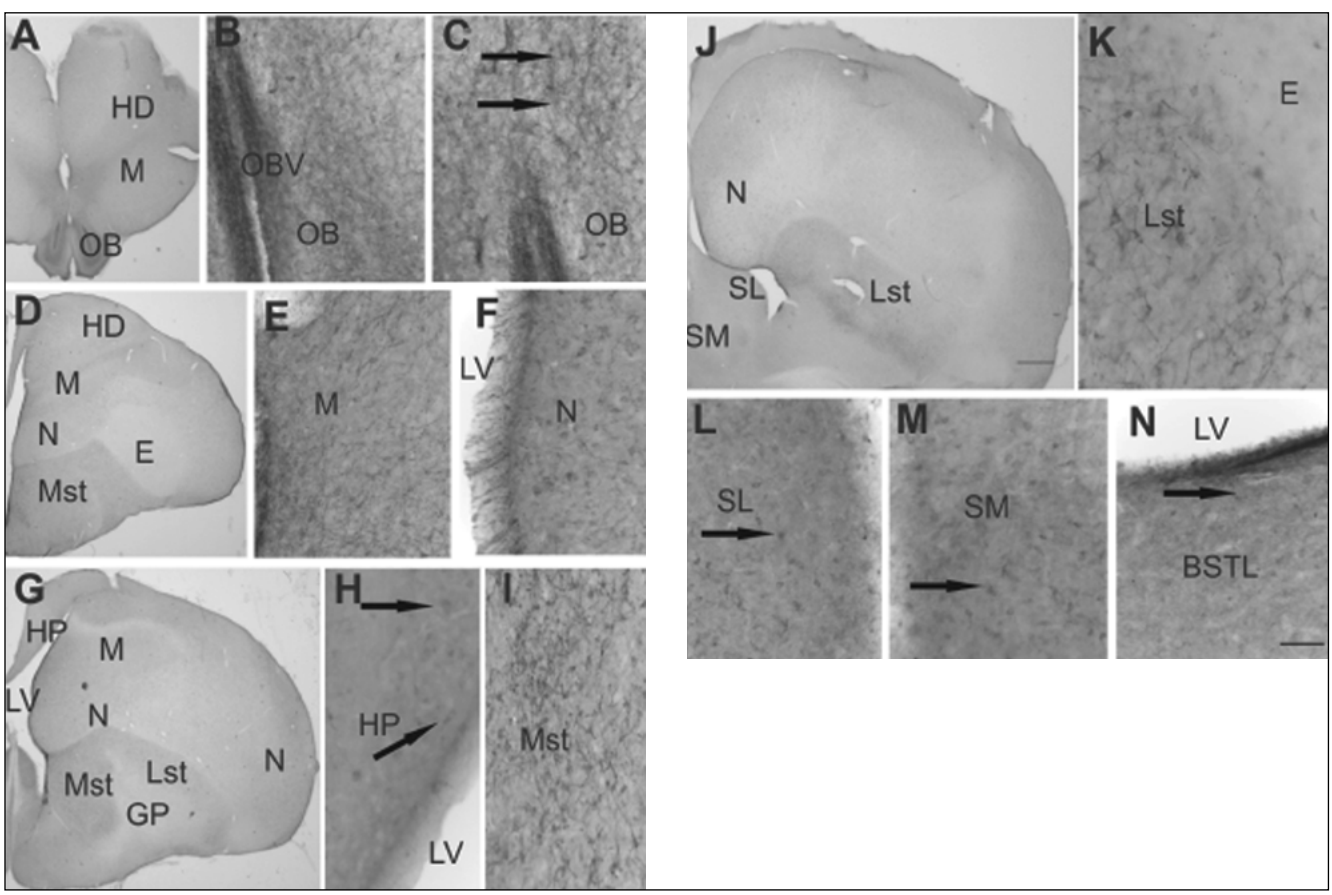

Fig. 5. Photomicrographs showing distribution of DCX-ir cells in the olfactory bulb and telencephalon of the Japanese quail brain. (A) Low power photomicrograph showing distribution of DCX-ir cells in the olfactory bulb (OB). (B) High power photomicrograph showing distribution of DCX-ir cells along the OB. (C) High power photomicrograph showing DCX-ir cells (black arrows) extending from the OB to the hyperpallium densocellulare (HD). (D) Low power image showing distribution of DCX-ir cells in the rostral telencephalon, E-high power image showing distribution DCX-ir cells in the mesopallium (M). (F) High power image showing distribution DCX-ir cells in the nidopallium (N). (G) Low power image showing distribution of DCX cells in the mid telencephalon. (H) Photomicrograph showing DCX-ir cells in the hippocampus (HP) (black arrows). (I) High power image showing DCX-ir cells in the medial striatum (Mst). (J) Low power photomicrograph showing distribution of DCX-ir cells in the caudal telencephalon. (K) High power image showing distribution of DCX-ir cells in the Lst near the lateral striatal- entopallial junction. (L) High power photomicrograph showing the distribution of DCX-ir cells in the lateral septum (SL) (black arrow). (M) High power photomicrograph showing the distribution of DCX-ir cells in the medial septum (SM) (black arrow). (N) High power photomicrograph showing the distribution of DCX-ir cells in the bed nucleus of the stria terminalis lateral division (BSTL) (black arrow). GP, globus pallidus; Lst, lateral striatum; LV, lateral ventricle; OBV, olfactory bulb ventricle. Scale bars: J=500 μm and applies to A, D, G; N=50 μm and applies to B, C, E, F, H, I, K, L, M.

specifically, the pattern of PCNA staining was reported to coincide with bromodeoxyuridine (BrdU) labelling observed after injections with short survival times (Bardet et al. 2012, Striedter and Charvet 2008) and this therefore justifies its use as a marker of cell proliferation in the current study.

Table II. Age-related changes in DCX immunoreactivity in the Japanese quail brain.

			Post hatching age									
			3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks
Olfactory Bulb	OB		+++	+++	+++	+++	++	++	++	+	+	+
Telencephalon	LV		+++	+++	+++	+++	+++	+++	++	++	+	+
	Pallium	HA	+++	+++	+++	++	++	++	+	+	+	+
		HD	+++	+++	+++	+++	+++	+++	++	++	++	++
		M	+++	+++	+++	+++	+++	+++	++	++	++	++
		N	+++	+++	+++	+++	+++	+++	++	++	++	++
		E	–	–	–	–	–	–	–	–	–	–
		A	–	–	–	–	–	–	–	–	–	–
		HP	++	++	+	+	+	+	+	+	+	+
	Subpallium	Mst	+++	+++	+++	+++	+++	+++	++	++	+	+
		Lst	+++	+++	+++	++	++	++	+	+	+	+
		GP	–	–	–	–	–	–	–	–	–	–
		SL	++	++	++	+	+	+	+	+	+	+
		SM	+	+	+	+	+	+	+	+	+	+
		BSTM	+	+	+	+	+	+	+	–	–	–
		BSTL	+	+	+	+	+	+	+	–	–	–
Diencephalon	Hypothalamus	POM	–	–	–	–	–	–	–	–	–	–
		PVN	–	–	–	–	–	–	–	–	–	–
		VMN	–	–	–	–	–	–	–	–	–	–
		Tu	–	–	–	–	–	–	–	–	–	–
	Thalamus	Rt	–	–	–	–	–	–	–	–	–	–
		OV	–	–	–	–	–	–	–	–	–	–
		DLA	–	–	–	–	–	–	–	–	–	–
		DMA	–	–	–	–	–	–	–	–	–	–
		Glv	–	–	–	–	–	–	–	–	–	–
Mesencephalon		Gct	–	–	–	–	–	–	–	–	–	–
		OMN	–	–	–	–	–	–	–	–	–	–
		RMC	–	–	–	–	–	–	–	–	–	–
Optic tectum			–	–	–	–	–	–	–	–	–	–
Cerebellum		CB	+++	+++	+++	++	++	++	+	+	+	+
		Cbi	–	–	–	–	–	–	–	–	–	–

+++– high density, ++– medium density, +– low density of DCX–ir type 1 and 2 cells only. – No positive DCX–ir.

DCX is a microtubule-associated protein that binds to microtubules, increasing their bundling and stabilization (Horesh et al. 1999). DCX controls the polymerization of the leading process and stabilization of the cytoskeleton during neuronal migration (Balthazart and Ball 2014a, 2014b) thus playing a critical role in the positioning of new born neurons. However, mammalian studies have also shown that DCX is not only a marker of neurogenesis but is also related to the extensive neuronal plasticity present in the adult brain (Brown et al. 2003, Klempin et al. 2011, Kremer et al. 2013, Nacher et al. 2001). Given the role of DCX in microtubule reorganization, DCX also supports the reorganization of the dendritic arbour, neurite outgrowth and synaptogenesis in plastic neurons.

The DCX antibody has been validated for both mammals and birds and it has been established that it is specific to the DCX protein (Balthazart et al. 2008, Balthazart and Ball 2014b). In galliformes, DCX has been identified in the developing or post- hatching brain of the chicken (Capes-Davis et al. 2005, Mezey et al. 2012) and Japanese quail (Balthazart et al. 2010). In this study, DCX immunolabelling was also used to investigate neuronal incorporation in the Japanese quail brain.

Two main types of DCX-ir elements were observed in this study. Type 1 cells were observed mostly near the external surfaces of the brain and are presumably differentiating neurons that have reached their destination following migration since DCX expression begins in late mitotic neuronal precursors and extends to early post mitotic neurons (Balthazart et al. 2008, Brown et al. 2003). Type 2 cells were mostly observed in the telencephalic areas adjacent to the lateral ventricles and resemble migrating neurons with leading and trailing processes that will presumably transform into multipolar morphology on reaching their target area (Boseret et al. 2007, Mezey et al. 2012).

Ontogenetic considerations

Japanese quails are precocial birds and therefore require minimal parental care after hatching. For precocial birds it is vital to have a highly developed brain by the time of hatching especially subtelencephalic areas that are important for homeostatic processes and simple behavioural tasks (Mezey et al. 2012). Neurogenesis in the quail brain is therefore supposed to be complete before

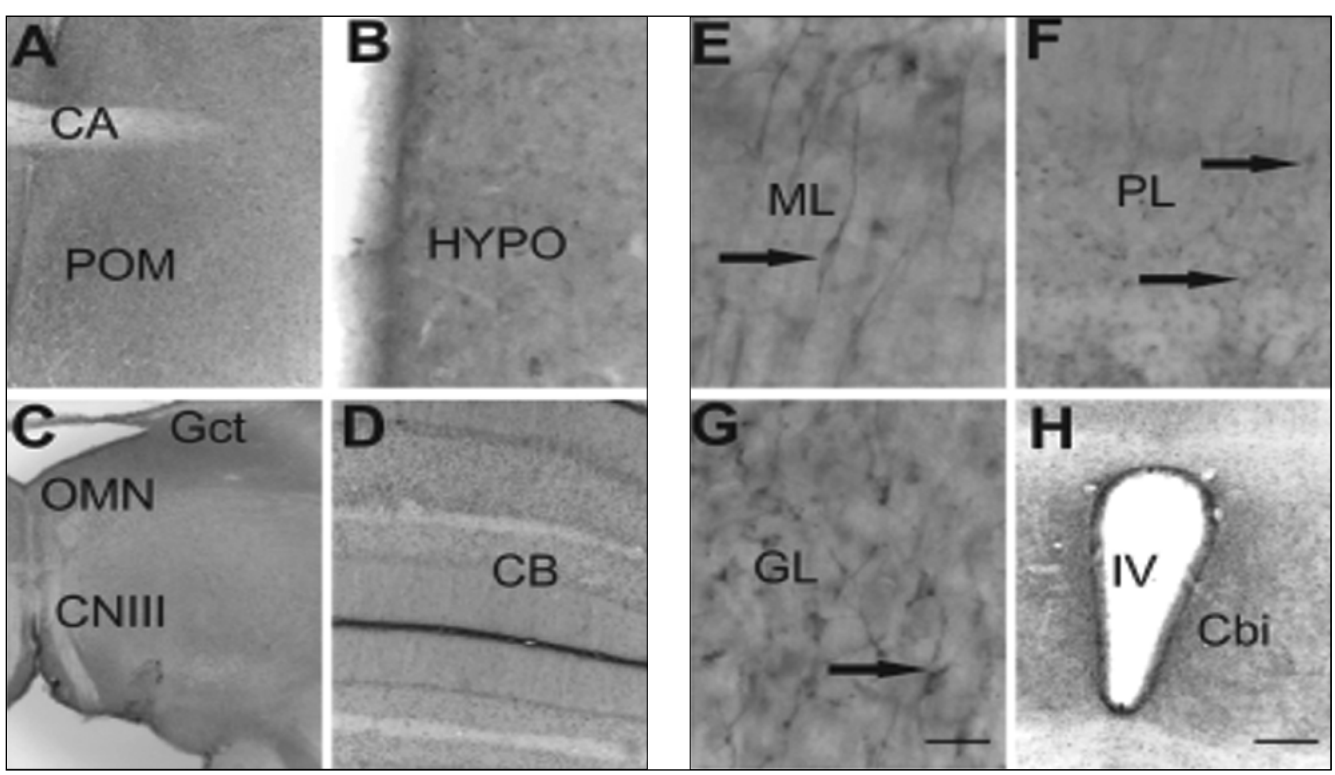

Fig. 6. Photomicrographs showing Japanese brain sections through the nucleus preopticus medialis (POM), hypothalamus (HYPO), mesencephalon and cerebellum. DCX-ir cells were absent in the POM (A), hypothalamus (B), mesencephalon (C) and nucleus cerebellum internum (Cbi) (H). DCX-ir cells (black arrows) were observed in all the layers (molecular cell layer (ML), Purkinje cell layer (PL) and granule cell layer (GL) of the cerebellar cortex (D, E, F and G). IV, fourth ventricle, CA, anterior commissure; CB, cerebellum; Gct, griseum centrale; OMN, oculomotor nucleus. Scale bars: G=20 μm and applies to E and F; H=200 μm and applies to A, B, C and D.

hatching (Tsai et al. 1981). In galliformes such as quails and chicks, neurogenesis starts at about 16-30% of hatching time and is presumed to be complete for the medulla by 30%, the tectum by 50% and the telencephalon by 50% of hatching time (Mezey et al. 2012). However Bardet et al. (2012) reported that neurons in most brain areas in the quail except for the telencephalon and the cerebellum are born and become post-mitotic before embryonic day 6 (E6). Even if neurogenesis persists throughout life in the telencephalon of adult birds, the bulk of neurons in this part of the brain are produced before E6 in quails (Bardet et al. 2012) and E7-E9 in chicks (Tsai et al. 1981). Our results reveal that the post hatch development of higher order brain areas such as the telencephalon and cerebellum is protracted in precocial birds in a similar way as observed in other birds and mammals.

Age-related changes in PCNA and DCX expression in different brain regions

PCNA-ir and DCX-ir elements were observed in the OB of juvenile, subadult and adult quails. Our findings suggest that the lining of the olfactory ventricle is the birth site of neurons that are incorporated in the OB. This is in contrast with the situation in mammals where neurons are born in the lateral ventricular zones and migrate through the rostral migratory stream to reach the OB (Maheu et al. 2015). A qualitative analysis of the results revealed an age-dependent decline in PCNA-ir and DCX-ir cells in the OB especially in the subadult and adult groups. A similar age-dependent decline in DCX expression was also reported by Mezey et al. (2012) in chicks and Meskenaite et al. (2016) in pigeons. DCX-ir cells and fibres running between the OB and the HD have been previously observed in the adult chick (Mezey et al. 2012) and in the pigeon (Melleu et al. 2013). These fibres may represent an extensive network of newly generated neurons being continuously exchanged along the rostrocaudal extension of the olfactory pathways (Melleu et al. 2013). The presence of neuronal production and incorporation in the OB of the quail is not surprising as this bird has been reported to have a keen sense of smell and is known to be able to detect the presence of certain pesticides, as well as avoid food containing a toxic chemical called lectin, using only the sense of smell (Mills et al. 1997).

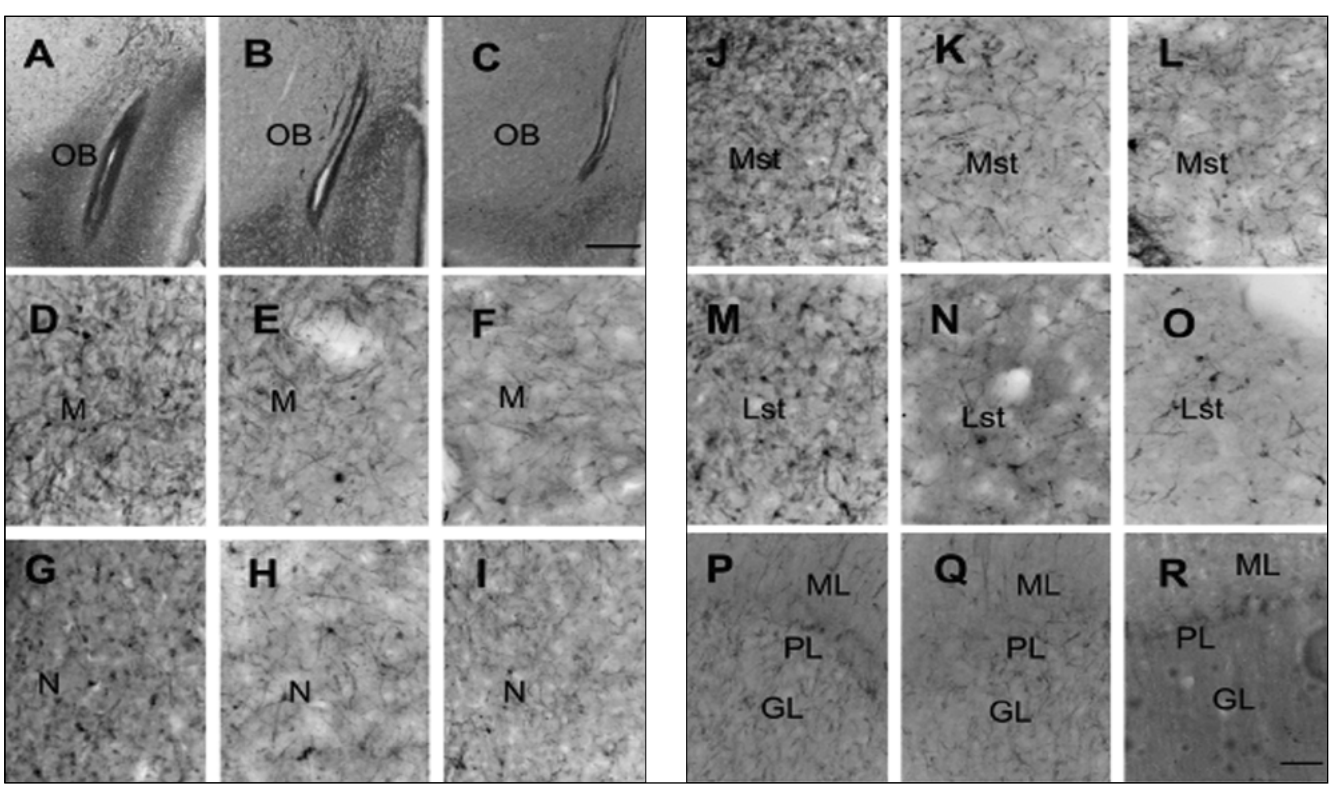

Fig. 7. Photomicrographs showing the distribution of DCX-ir cells the different parts of the brain of juvenile (A, D, G, J, M and P), subadult (B, E, H, K, N and Q) and adult (C, F, I, L, O and R) Japanese quails. Olfactory bulb (OB) (A-C), mesopallium (M) (D-F), nidopallium (N) (G-I), medial striatum (Mst) (J-L), lateral striatum (Lst) (M-O) and cerebellum (P-R). GL, granule cell layer; ML, molecular cell layer; PL, Purkinje cell layer. Scale bars: C=200 μm and applies to A-C; R=50 μm and applies to D-Q.

In the pallium, the majority of PCNA-ir cells observed throughout the rostrocaudal extent of the lateral ventricles appeared to decline with post-hatching age. PCNA-ir cells were mostly found to be abundant ventrally and dorsally in the ventricular zones adjacent to the medial striatum and mesopallium, respectively, corresponding to the proliferative "hotspots" described by Alvarez-Buylla et al. (1990). In juvenile quails, very few PCNA-ir cells were observed in the mesopallium and caudal part of the nidopallium adjacent to the lateral ventricles. Similarly, Bardet et al. (2012) reported the presence of a few PCNA and BrdU labelled cells in the quail pallium and concluded that these could be slow cycling progenitors. Alternatively, the presence of PCNA-ir cells in the parenchyma may not be related to cell proliferation but to DNA repair in mature neurons as proposed by Essers et al. (2005). Apart from those few cells in the pallial parenchyma of juvenile quails, cell proliferation in the subadult and adult Japanese quails appears to be restricted to the lateral ventricular zones.

DCX-ir cells were observed throughout the pallium except for the entopallium and the arcopallium. DCX expression in the pallium suggests that the Japanese quail, like other birds, continues to recruit neurons into the pallium throughout life although at a slower rate in adult as compared to juvenile and subadult quails. The very low density of DCX-ir cells in the quail hippocampus throughout juvenile, subadult and adult stages is not surprising given that spatial memory is not as major a factor in the life of quails as it is for food storing birds (Mezey et al. 2012) or homing pigeons (Meskenaite et al. 2016).

In the subpallium, PCNA-ir cells declined with post hatching age eventually disappearing in the medial striatum and in the bed nucleus of stria terminalis (BSTM and BSTL) in adult quails older than 9 and 10 weeks respectively. DCX expression ceased in the BSTL and BSTM at 11 weeks suggesting that these brain structures reach full maturation at 10 weeks after which no new neurons are incorporated. However the striatum continues to express DCX throughout the juvenile, subadult and adult ages.

The observation that DCX is expressed in the septum and the striatum, areas where PCNA is not expressed, implies that there could be another source for neurons that are added to these brain areas, presumably the adjacent lateral ventricles. Furthermore, our findings suggest that the septum continues to recruit neurons throughout the ages studied. This is contrary to the findings by Nikolakopoulou et al. (2006b) who reported an early maturation of the SL in the quail at post-hatching day 20 (P20) using BrdU staining. They reported that about 50% of BrdU-ir cells in the SL were of an unknown phenotype as they neither co-expressed with neuronal or glial markers. Since the SL is a steroid sensitive brain area, this population of BrdU-ir cells could be slow cyclin progenitors that are quiescent at P20 but may be reactivated at puberty when the level of circulating steroids increases as observed by Mouriec and Balthazart (2013).

Given the fact that no type 1 or type 2 DCX-ir cells were observed in the diencephalon, the PCNA immunoreactivity observed in this region is presumably related to glial cell production. Additionally, Balthazart et al. (2010) also reported a lack of DCX immunoreactivity in the adult quail POM. In support of our observation, Bardet et al. (2012) reported that neuronal production in this region of the quail brain is complete by embryonic day 6 and that further cell proliferation after this date is related to production of glial cells. However, previous attempts to test this hypothesis through double labelling experiments in birds proved to be technically challenging because many antibodies available to identify glial cells in mammals (S100, GFAP or CD11) either do not label any cell in birds or only label subpopulations of glial cells in specific brain regions (Castagna et al. 2003, Bardet et al. 2012).

As was the case with the diencephalon, no type 1 or type 2 DCX-ir cells were observed in these two regions of the quail brain. As a result, it can be concluded that the intense PCNA immunoreactivity observed in the oculomotor nucleus, griseum centrale and the red nucleus of the mesencephalon may not have related to neurogenesis but possibly related to glial cell production. No PCNA-ir or DCX-ir cells were observed in the optic tectum in any of the quails suggesting that this structure reaches full maturity before hatching.

Our findings suggest that neuronal birth and incorporation continue to take place in the cerebellum of juvenile, subadult and adult quails just like other birds species (Bardet et al. 2012, Stamatakis et al. 2004,). The PCNA immunoreactivity observed in the Cbi of juvenile and subadult quails may be related to glial cell production since no type 1 or type 2 DCX-ir cells were observed in that nucleus.

In conclusion, post-hatch neuronal production appears to be restricted to olfactory and telencephalic ventricles whereas neuronal incorporation is restricted to the telencephalon and cerebellum. In addition, both processes continue into adulthood and appear to decline with post-hatching age beginning from the 6^{th} week. Furthermore, subtelencephalic regions such as the diencephalon, mesencephalon and optic tectum appear to reach full maturity prior to hatching and post-hatching proliferation observed in these regions is most likely related to glial cell production.

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