Perineuronal satellite neuroglia in the telencephalon of New Caledonian crows and other Passeriformes: Evidence of satellite glial cells in the central nervous system of healthy birds?

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Clusters of pN-OLG were identified with the use of standard histological procedures in the brains of nine New Caledonian crows, two Japanese jungle crows, two Australian magpies, two Indian mynah and three zebra finches. Heterochromatin masses visible inside the nuclei of these satellite neuroglia indicated that they correspond to dark oligodendrocytes (as described in Mori and Leblond, 1970, and Uranova et al., 2001). Microscopic survey of the brain tissue suggests the largest clusters are located in the hyperpallium densocellulare and mesopallium. No clusters were found in brain sections from one Gruiform (purple swamphen), one Strigiform (barn owl) and one Galliform (chick), suggesting pN-OLG in Aves are brain region- and taxon-specific. Recent studies have linked reduction in number of pN-OLG in the prefrontal cortex with human schizophrenia and other psychiatric disorders (Vostrikov et al., 2007, Kim and Webster, 2010, Kim and Webster, 2011). Therefore, pN-OLG may play a decisive role in the homeostasis and normal activity of the human nervous system. Together, these findings suggest that the presence of pN-OLG in healthy human and avian gray matter is the result of convergent evolution (Vijayan et al., 1993, Kondo et al., 1997, Vostrikov et al., 2007). The discovery of pN-OLG in the zebra finch is of great importance because this species has the potential to

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Short title: Perineuronal oligodendroglia in the telencephala of five passeriform species

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Abstract

We wish to report on the discovery of perineuronal oligodendroglia (pN-OLG) clusters in the telencephala of five healthy Passeriform bird species. The pN-OLG phenotype was recently identified in rats and humans as non-myelinating (Szuchet et al., 2011). In mouse gray matter pN-OLG are thought to support neuronal survival, differentiation and function, and protect against neuronal apoptosis (Takasaki et al., 2010). In humans, they appear to actively retain the potential for myelogenesis by promoting the proliferation of oligodendrocyte progenitor cells and retarding their maturation (Lee et al., 2001, Lelievre et al., 2006, van Landeghem et al., 2007). They also express neuropeptides and region-specific glutamate transporters, which modulate neuronal synaptic strength (Kondo et al., 1997) and participate in the extracellular glutamate balance (van Landeghem et al., 2007).

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Introduction

A neuron-centric view of the function of the nervous system has prevailed since the neuronal doctrine attributed neuroglia with the sole role of maintenance and support of neurons (Vostrikov et al., 2007, Privat et al., 1995). While emerging data on the role of glia (primarily astrocytes) in neuronal homeostasis and function have progressively challenged this view, many functional aspects of neuroglial cells still constitute a mystery for neuroscience (Barres, 2008). Neuroglia have been implicated in the control of the neuronal extracellular space (Simard and Nedergaard, 2004, Haydon et al., 2009), in the development of the nervous system and synapse formation (Barres, 2008), in the regulation of synaptic transmission (Perea et al., 2009), in the plasticity and map formation in the sensory neocortex (Min and Nevian, 2012, Rossi, 2012), and in the development of the nervous system and adult neurogenesis (Malatesta et al., 2000, Miyata et al., 2001, Noctor et al., 2001, Noctor et al., 2002, Alvarez-Buylla and Garcia-Verdugo, 2002, Malatesta et al., 2003, Morest and Silver, 2003, Anthony et al., 2004, Rowitch and Kriegstein, 2010). In the cerebellum neuroglia have been shown to be required for fine motor coordination (Saab et al., 2012). Neuroglia are also able to transmit calcium-wave signals over long distances via gap-junctions (Bennett et al., 2003, Scemes and Giaume, 2006) and, together with microglia, participate in the pathology-induced inflammation and pain responses (Giaume et al., 2007, Milligan and Watkins, 2009).

Satellite glia were first described by Ramón y Cajal (1910, 1899) in healthy peripheral nervous tissue and in the 1930s the term perineuronal satellitosis (PS) was coined to describe neurons closely surrounded by multiple neuroglia (see Vijayan et al., 1993). Today, most neuropathology textbooks teach us to recognise PS in the process of diagnosis of common pathologies that affect the nervous tissue (e.g. grade II astrocytoma, type I neurofibromatosis and anaplastic oligodendroglioma) (Haberland, 2007, Oemichen et al., 2006, Perry and Brat, 2010, Tonn et al., 2010). PS is associated both with normal and pathological nervous tissue depending on the functions performed by the type of perineuronal neuroglia (i.e. NG2-type macroglia, astrocytes, microglia or myelinating and non-myelinating oligodendrocytes) (Ludwin, 1984, Yokota et al., 2008, Faber-Zuschratter et al., 2009, Takasaki et al., 2010, Szuchet et al., 2011).

A potentially different kind of PS has been described in healthy tissue in different regions of the human brain such as the cerebral cortex, hippocampus, basal ganglia and thalamus (Brownson, 1956, Vijayan et al., 1993 , Vostrikov et al., 2007). The satellite neuroglia

present in this type of PS are oligodendroglia (Brownson, 1956, Vijayan et al., 1993, van Landeghem et al., 2007, Vostrikov et al., 2007, Kim and Webster, 2010, Kim and Webster, 2011, Takasaki et al., 2010). Importantly, one recent study has identified that gray matter perineuronal oligodendroglia (pN-OLG) in both rats and humans are of the non-myelinating phenotype (Szuchet et al., 2011). In mice, evidence also suggests that these pN-OLG in the somatosensory cortex support neuronal survival, differentiation, and function, and that they provide protection against neuronal apoptosis but do not play a major role in the plasmalemmal uptake of extracellular glutamate neurotransmitter (Takasaki et al., 2010). In contrast, evidence from one human study (van Landeghem et al., 2007) shows that pN-OLG in the neocortex and hippocampus express specific (PACAP) neuropeptides which are known to (1) both promote proliferation and retard maturation and myelogenesis in oligodendrocyte progenitors (Lee et al., 2001, Lelievre et al., 2006) and (2) modulate neuronal synaptic strength (Kondo et al., 1997). Human pN-OLG also express region-specific glutamate transporters, which suggests they might help clear extracellular glutamate and thus prevent delayed neuronal and glial death in hypoxia-sensitive regions following transient global human ischemia (van Landeghem et al., 2007). Interestingly, recent findings have linked human schizophrenia and other psychiatric disorders with a reduction in number of pN-OLG in the prefrontal cortex (Vostrikov et al., 2007, Kim and Webster, 2010, Kim and Webster, 2011). This suggests that pN-OLG may play an instrumental role in the homeostasis and normal activity of the nervous system involving cognitive functions (see Szuchet et al., 2011).

Here we report on the presence of histological structures resembling pN-OLG in the brains of New Caledonian crows (NC crows hereafter) that were found in the process of other anatomical investigations. A survey based on perikaryal stains revealed that similar PS-like structures were present in at least four other passerine species (including zebra finches), but we did not detect them in the other bird taxa that we examined. To our knowledge, this is the first formal description of such structures in non-mammalian brains. Our finding of neuroglial clusters in avian brains resembling those previously described in mammalian cortex provides us with alternative laboratory animal models in which to explore their functionality and possible roles in pathological conditions and cognition.

Materials and Methods

The work involving animals and the transport of material was done in compliance with and with approval from the University of Auckland Animal Ethics Committee (R602, R840 and R469) in accordance with the Animal Welfare Act 1999 (New Zealand) the University of Auckland Code of Ethical Conduct for the Use of Animals for Teaching and Research and the The National Animal Ethics Advisory Committee (NAEAC) Good Practice Guide for the Use of Animals in Research, Testing and Teaching and imported in compliance with New Zealand Ministry of Agriculture and Forestry (New Zealand) regulations, and with approval to collect and export the NC crow brains from by the Customary Authority on Maré, New Caledonia dated 8 July 2008.

New Caledonian crow specimens

Nine New Caledonian crows (*Corvus moneduloides*) (six males and three females) captured on the island of Maré, New Caledonia, in August/October 2007 were used for this study. We captured the birds using a 'whoosh net' (obtained from SpiderTech Bird Nets, Helsinki, Finland). Female and male distinction was based on bill morphology and body weight on the day of capture (Kenward et al., 2004). Crows were housed in a 5-cage outdoor aviary situated in primary forest inland from the coast, for up to a maximum period of five months during which they participated in behavioural experiments (e.g., Taylor et al., 2009, Taylor et al., 2010, Medina et al., 2011). The cages were 3 m high and at least 4 m × 2 m in area. All cages were provided with ample perching space, branches and feeding logs. In addition to getting meat in experimental trials, the crows were fed a main meal in the evening consisting of soaked dog/cat biscuits (40+ mL of dry biscuits soaked in water for 15 min), bread or rice and occasionally raw egg. Papaya and clean drinking and bathing water were available throughout the day.

All crows were euthanised at the end of their captive period. Crows were caught and put into a clean, dark cloth bag and then injected with a lethal dose of pentobarbital (30 mg/kg, intramuscular Ludders, 2008) and held calmly in a dark bag by the experimenter until cardiac arrest. Immediately after cardiac arrest the crow was transcardially-perfused by gravity with 0.5 L of 0.9% saline followed by 0.5 L of 4% formaldehyde solution (obtained from Mesachimie, Nouméa, New Caledonia). The crow's brain was then extracted from the skull and immersed in 4% formaldehyde for importation into New Zealand.

Tissue preparation

Brains were re-immersed and fixed in 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB) upon arrival in New Zealand 10 days after perfusion and kept in cold storage. Brains were finally processed between 2008 and 2011. First, they were sectioned mid-sagitally and both hemispheres were cryoprotected in 30% sucrose in Phosphate Buffered Saline (PBS 0.01M) for 4 to 7 days (until they sank twice in fresh sucrose solution). Brain halves were then placed in a solution of 15% gelatine with 30% sucrose (cryoprotective gelatine solution) at 40° C for one hour. The hemispheres were then placed in a custom-made mould so that fiduciary points could be made in the gelatine for later alignment of tissue sections. The mould consisted of a custom made plastic box with a removable base. The base had small holes drilled in a 3 mm grid pattern, on top of which a perfectly flat 5 mm thick layer of cryoprotective gelatine solution was left to set for 10 min prior to hemisphere placement. The brains were placed on top of this cryoprotective gelatine base with the midline side facing down and left to set for 3 minutes at 6° C. Then, seven to ten sewing pins were inserted into the holes that had been drilled in the base of the mould so that they surrounded the brain hemisphere. A different cryoprotective gelatine solution containing a teaspoon of black fabric dye (to darken the solution) was then poured over the brain. This coloured solution had been previously prepared and kept in liquid state just above room temperature. Once poured, it was left to set first at 6° C, and then at -4° C, for a total time of 15 minutes.

The resulting cryoprotective gelatine block (containing the brain) was then removed from the mould, trimmed and placed, along with the pins, into 4% PFA 30% sucrose overnight. The pins were then removed and the block was sectioned on a sliding freezing microtome at 50 µm thickness in the sagittal plane. Sections were collected in 0.01% sodium azide PBS solution. For each hemisphere every third section was mounted serially onto gelatin chrom-alum coated slides, stained with haematoxylin neutral red, neutral red or cresyl violet (see below), dehydrated and coverslipped with DPX mounting medium (Scharlau) from xylene. The remaining sections were set apart in two series, one for storage and the other for immunocytochemical staining (see below).

Sections and fiduciary points in the surrounding dyed gelatine were imaged using a Leica M205 FA stereomicroscope with a mounted Leica DFC 500 digital camera, and the images subsequently merged and flattened in Adobe Photoshop CS3 Extended and then loaded into Visage Imaging AMIRA 5.2.0 for alignment. The aligned image stacks were then labelled using CorelDRAW X5 for the production of a complete brain atlas for the species. Brain

regions were identified using boundary lines that could be recognised from the histological staining in the image stack. Boundaries were carefully identified with the aid of several avian brain atlases (Karten and Hodos, 1967, Kuenzel and Masson, 1988, Izawa and Watanabe, 2007) and named according to the Avian Consortium Nomenclature (Reiner et al., 2004, Jarvis et al., 2005).

Calcium-binding protein immunocytochemistry

Antibodies for calbindin (mouse monoclonal anti-calbindin D28k; Swant, Cat. no. 300) and parvalbumin (goat anti-parvalbumin; Swant, Cat. no. PVG-214) were used for immunocytochemistry. Detection of the primary antibody was done by incubating the tissue with the appropriate biotinylated secondary antibodies (Jackson Immunoresearch), followed by incubation with and streptavidin-horseradish peroxidase (HRP) conjugate (Invitrogen, Cat. no. S911).

All steps were separated by three 5 min washes in PBS. Floating brain sections left in 0.01% sodium azide PBS solution were collected and washed thrice in PBS before being placed in bleaching solution (50% methanol 1% H_2O_2 in distilled water) for 10 min. Sections were then incubated for 24 h in primary antibody (1:5000 in a 2% normal horse serum 0.4% Triton X-100 PBS solution) on a shaker at room temperature. They were then incubated in the secondary antibody for 1 h (1:1000 in a 2% normal horse serum 0.4% Triton X-100 PBS solution), followed by 1 h in streptavidin-HRP (1:1,000). Sections were rinsed in PBS and the incubation with the secondary antibody and streptavidin-HRP were repeated prior to the chormatogenic reaction with DAB. Sections were finally washed, then incubated in DAB chromagen solution (0.025% 3,3′-diamino-benzidine [DAB], 0.005% H2O2 and 0.015% $CoCl_2$ in PBS) for 90 s, washed again in PBS, mounted on gelatine chrom-alum coated slides, left to dry and finally dehydrated and coverslipped with DPX mounting medium (Scharlau) from xylene.

Immunocytochemistry was performed as a tool to aid in the description and identification of brain boundaries and to gain insight into neuronal morphology. No claims are made about the biochemical specificity of the antibodies since we were not able to perform western blots to examine antibody specificity. The presence of immunocytochemical staining is therefore referred to as “-like immunureactivity” (-LI) and no conclusions are drawn with regards to the biochemical signature of the tissue under study.

Cell morphology and classification

We classified the NC crow brain cell classes according to standard cell morphology descriptions found in textbooks (e.g. Nieuwenhuys et al., 2008, Nieuwenhuys, 1994, Fortune and Margoliash, 1992, Tombol et al., 2000, Srivastava et al., 2009).

Classical Histology

Brain sections were mounted on gelatine subbed glass slides and subjected to different classical histological methods for histological analysis.

Neutral red nuclear staining method. Sections were first dehydrated then re-hydrated and quickly rinsed in tap water. They were then placed in neutral red solution (5 mg neutral red in 500 mL distilled water and 20 mL acetate buffer 0.037M, pH 4.8) for 2-4 minutes, briefly rinsed with tap water and finally differentiated in 95% ethanol, dehydrated and coverslipped from xylene.

Carazzi's modified haematoxylin staining method. Sections were first dehydrated then rehydrated and quickly rinsed in tap water. They were then left in haematoxylin solution (5 g haematoxylin and 25 g aluminium potassium sulfate, 100 mg potassium sodium iodate, in 100 mL glycerol, 400 mL distilled water and either 20mL/L glacial acetic acid or 5mL/L HCl) for 30-40 minutes, blued in running warm tap water for 5 minutes, and finally differentiated in 1% HCl in 70% ethanol for 2-5 seconds, dehydrated and coverslipped from xylene.

Haematoxylin neutral red nuclear staining method (with Carazzi's haematoxylin). Sections were dehydrated then rehydrated and quickly rinsed in tap water. They were then left in haematoxylin solution for 30-40 minutes before being blued in running warm tap water for 5 minutes and differentiated in 95% ethanol. The sections were next left in neutral red solution for 2 minutes, rinsed in warm tap water and finally differentiated in 95% ethanol, dehydrated and coverslipped from xylene.

Fast cresyl violet staining method. Sections were dehydrated then rehydrated and quickly rinsed in tap water. They were then left in cresyl violet solution (60 mL of 10 g/L cresyl violet in 540 mL distilled water and 5 mL 10% acetic acid) for 2 minutes, briefly rinsed in warm tap water, and finally differentiated in 95% ethanol, dehydrated and coverslipped from xylene.

Other specimens

Stained sagittal brain sections from two Indian mynahs (*Acridotheres tristis*), two Australian magpies (*Gymnorhina tibicen*), one pukeko (*Porphyrio porphyrio*) and two zebra

finches (*Taeniopygia guttata*) were also examined. The mynahs, Australian magpies and pukeko were perfused post-mortem after being collected in the wild between 2005 and 2008 in the North Island of New Zealand. The brains of these three species were sectioned and stained with cresyl violet by Dr. Jeremy Corfield at the University of Auckland using the same methods described above. The sagittal brain sections of two zebra finches (35 µm thick, cresyl violet staining) were provided by Dr. Priscilla Logerot at the University of Auckland. High-resolution images of sagittal brain sections (50 µm thick, cresyl violet staining) of two Japanese jungle crows (*Corvus macrorhyncos*) were provided by Dr. Ei-Ichi Izawa (Keio University, Tokyo, Japan).

Tissue analysis

Microphotographs of the tissue were taken from different regions of the telencephalon (labelled as in Fig. 1) using a Nikon Digital Sight DS- 5MC camera attached to an Eclipse 80i Nikon microscope. The microphotographs were then loaded into Adobe Photoshop CS3 for cropping and figure production.

Results and Discussion

Our microscopy survey based on antibodies for calcium-binding proteins revealed that the NC crow telencephalon has the typical neuronal classes described previously in other avian species (Fortune and Margoliash, 1992, Tombol et al., 2000, Srivastava et al., 2009). In contrast, our survey based on perikaryal stains in five passerine species (including NC crows) revealed the presence of cell clusters that had not been previously formally described in the avian brain literature. These clusters resemble a specific subtype of perineuronal satellitosis described previously in humans and rodents (see Introduction). These clusters were not reactive to calbindin or parvalbumin immunocytochemistry.

General description of the cellular morphology in the NC crow

We identified several types of neurons showing calcium binding protein-LI in the telencephalon of the NC crow brain (Fig. 2). In most cases, the calbindin antibody (CB-LI) produced a more complete view of the neuronal processes than parvalbumin (PV-LI), which resulted in a higher level of neuropil staining. We identified the following cell classes: fusiform bipolar neurons, pyramidal-like neurons, and round, ovoid and angular multipolar neurons. A few fusiform bipolar neurons were found in the dorsal portion of the hyperpallium

densocellulare with processes running parallel to the boundaries of the nucleus (Fig. 2a), and in the striatum mediale with processes aligned in the horizontal direction (rostro-caudal axis) (not shown). Pyramidal-like neurons were found in the hyperpallium apicale, hyperpallium densocellulare, and mesopallium (Fig. 2b). Multipolar neurons were much more ubiquitous. Round multipolar cells were found only in the hyperpallium apicale (Fig. 2c), whereas ovoid multipolar cells were located in the nidopallium and area corticoidea dorsolateralis (Fig. 2d,f). Both round and ovoid types of multipolar neurons were found in the striatum mediale. Angular multipolar neurons were located in hyperpallium apicale, hyperpallium densocellulare, mesopallium, nidopallium caudale, striatum mediale and arcopallium (Fig. 2e).

Our tissue preparation prevented us from carrying out a detailed, rigorous classification of neurons based on their dendritic trees and axons, or on the number and orientation of processes projecting from the soma, as these parameters depend on the thickness of the slice and the orientation of the neuron within the plane of section (always a single section thickness on sagittal in our study).

Description of a novel histological structure in the telencephalon of NC crows

When inspecting the NC crow tissue stained for perikarya with classical histology we found conspicuous structures, first identified in the mesopallium consisting of one or two larger central cells with long projections (classified as neurons) surrounded by a tight and compact cluster of four or more cells with no visible or very short projections (we classified these cells as satellite neuroglia) (Fig. 3). We refer to these multi-cell structures consisting of a neuron and associated satellite neuroglia as Perineuronal Satellitosis (PS). Initially, we saw satellite neuroglia in cresyl violet stained tissue, and then confirmed their presence with three other stains (haematoxylin, neutral red and haematoxylin-neutral red) in multiple sections from different individuals. This allowed us to eliminate the possibility that the identified PS were the result of a histological artifact. Notably, these structures were not visible in the immunocytochemical preparations.

A well differentiated haematoxylin stain followed by a rapid neutral red stain provided the clearest delineation of the different cell types and their distribution within the cluster. Based on our perikarya stains, neurons were distinguished from neuroglia by the presence of dark, coarsely stained Nissl substance, a large pale nucleus with a distinct central nucleolus, and lightly stained proximal segments of dendritic processes (Fig. 3). In contrast, glial cells contained much less visibly stained endoplasmic reticulum, giving them a compact round or

oval form with darker stained nuclei and often with multiple spots of condensed chromatin. Similar criteria have been previously used to distinguish neuroglia from neurons (e.g. Sherwood et al., 2006).

Upon closer inspection of the NC crow tissue, the visible chromatin masses of our satellite cell nuclei suggest that the cells correspond to dark oligodendrocytes initially described by Mori & Leblond (1970) (see also Mori and Hama, 1971, Ling et al., 1973, Uranova et al., 2001, Faber-Zuschratter et al., 2009). An independent neuropathologist also identified these cells as oligodendrocyte-like after inspecting our microphotographs (Dr. Arie Perry, University of California, San Francisco, personal communications). This indicates that the observed PS has a composition similar to that of pN-OLG.

Gross distribution of cell clusters in the telencephalon of Passeriformes

Once we had successful criteria to identify pN-OLG in the mesopallium of NC crows we carried out a careful microscopy survey in the other telencephalic areas in this and four other passerine species (Japanese jungle crow, Australian magpie, Indian mynah and zebra finch), as well as in a non-passerine (the pukeko, order Gruiformes) using cresyl violet stained tissue (Figs. 4, 5). Far from having a simple, homogeneous pattern of distribution (i.e. presence or absence) across the whole telencephalon, we found that the number of pN-OLG clusters and their size varied according to the brain region under study.

In the striatum mediale the high cell density prevented unambiguous identification of pN-OLG clusters in most species, although we were able to identify a few pN-OLG clusters in both *Corvus* species. With the exception of the nidopallium caudale in the NC crows, pN-OLGs clusters in the pallial subdivisions of the passerine telencephala were easily recognised because in these regions cells are more sparsely distributed. The pN-OLG clusters were most conspicuous in the mesopallium (Fig. 4) and hyperpallium densocellulare (Fig. 5) of the five passerines, where their number appeared to be higher.

Clusters of cells were more difficult to identify in the pukeko tissue due to its poor quality. Nevertheless we found some clusters, but these appeared to be mainly composed of large neurons. Neuron clusters (as distinct from PS) were also detected in the NC crow nidopallium caudale and have been described elsewhere (Fortune and Margoliash, 1992). Finally, cell clusters appeared absent in the hippocampus, area parahippocampalis, area corticoidea dorsolateralis, arcopallium and entopallium.

In the two *Corvus* species, the Australian magpie, Indian mynah and zebra finch the largest pN-OLG clusters appeared to be present in the densocellular subregion of the hyperpallium and the mesopallium (Figs. 4, 5). We could not determine unambiguously whether pN-OLG were present in the pukeko brain tissue. Also, when closely inspecting whole high-resolution sagittal images of perikarya stained brains of the barn owl (*Tyto alba*, Strigiformes) and the chick (*Gallus gallus*, Galliformes) (available at http://brainmaps.org) we failed to find evidence of pN-OLG. Together, our findings indicate that avian pN-OLG clusters are both region- and taxon-specific, suggesting that their presence in healthy passerines and humans (as shown by Brownson, 1956, Vijayan et al., 1993, van Landeghem et al., 2007, Vostrikov et al., 2007) is the result of convergent evolution.

It is surprising that the presence of pN-OLG clusters has not been previously reported in avian species, perhaps because neuropathology textbooks focus on pN-OLG in relation to mammalian brain disease (Haberland, 2007, Oemichen et al., 2006, Perry and Brat, 2010, Tonn et al., 2010). For example, previous observations of such clusters in zebra finch brains were interpreted as pathological (Wild, unpublished observations). It seems highly unlikely that the selective presence of pN-OLG-type clusters in the telencephala of the brains that we studied was associated with developed neuropathologies. This is because the brains that we studied came from birds in different geographical locations that showed no obvious signs of illness before being perfused. Rather, our comparative studies suggest that pN-OLG structures are present in non-pathological avian brains, consistent with evidence of PS in both ill and healthty rodents (for evidence of PS in both ill and healthy rodents, see Ludwin, 1984, Krinke et al., 2000, Szuchet et al., 2011).

Conclusions

The view that animal behaviour depends solely on the activity of neurons may not be reasonably sustained despite our limited understanding of the mechanisms responsible for the functioning of the neuronal-glial circuitry (Verkhratsky, 2006). Recent findings linking human schizophrenia and other psychiatric disorders with a reduction in the number of pN-OLG in the prefrontal cortex (Vostrikov et al., 2007, Kim and Webster, 2010, Kim and Webster, 2011), suggest that pN-OLG may contribute to the homeostasis and normal activity of the nervous system and, directly or indirectly, affect cognitive function (Szuchet et al., 2011). Such findings

underscore the need to gain a better understanding of the role(s) of different neuroglia types in neuronal function and how these interactions are related to normal and pathological conditions.

That the presence of pN-OLG is brain region- and taxon-specific in birds invites speculation about their general function. Verkhratsky (2010 p. 1) stated that the neuronal web is embedded into a glial syncytium and gives rise to the sophisticated neuronal–glial network in which "*... both types of neural cells [work] in concert, ensuring amplification of brain computational power*". Further, perineuronal oligodendrocytes constitute a recently identified lineage of oligodendrocytes, one with a phenotype that "*...blur[s] the boundary between a neuron and a glial cell...*" (Szuchet et al., 2011). As pN-OLG clusters appear to be characteristic of passerine brains where they commonly occur in associative forebrain regions, it is tempting to hypothesise that they may play an important role in the flexible behaviour and problem-solving abilities seen in the highly successful, larger-brained passerine group (Emery and Clayton, 2005, Taylor et al., 2009). It will be therefore informative to determine their presence or absence in parrots, which, like passerines, are endowed with larger brains and are known for their higher cognitive abilities.

Our findings add to the increasing evidence suggesting that neuroglia play a major role in normal brain functioning. Future work, aimed at characterising in detail the biochemical and genetic signature of avian satellite neuroglia herein described, will be necessary. Most importantly, our discovery opens the way for comparative animal experimentation, which will help determine how pN-OLG may influence normal neuronal function. The zebra finch provides a highly suitable animal model for comparative and functional studies to investigate the physiological function and possible role of pN-OLG in behaviour. Future biochemical studies will determine the degree of phenotypic similarity between avian and mammalian pN-OLG, and comparative and functional studies in Passeriformes may elucidate whether they have evolved in association with the emergence of complex tool behaviour and/or advanced problem-solving skills in this group.

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Author Contributions

This work is part of Felipe S. Medina's (FSM) PhD Thesis, School of Psychology, University of Auckland. Experiments were conceived by all authors. Experimental work was performed by FSM, with input from all other authors. Data were analysed by FSM with assistance from MFK and JMW. The manuscript was written by FMS with contribution from all authors.

Abbreviation list

A	Arcopallium
B	Nucleus basalis
Cb	Cerebellum
CDL	Area corticoidea dorsolateralis
CIO	Capsula interna occipitalis
DLA	Nucleus dorsolateralis anterior thalami
DLP	Nucleus dorsolateralis posterior thalami
FPL	Fasciculus prosencephali lateralis
FRL	Formatio reticularis mesencephali pars lateralis
GP	Globus pallidus
HA	Hyperpallium apicale
HD	Hyperpallium densocellulare
HVC	High vocal centre
ICo	Nucleus intercollicularis
Imc	Nucleus isthmi pars magnocellularis
Imp	Nucleus isthmi pars parvocellularis
LM	Nucleus lentiformis mesencephali
M	Mesopallium
MAN	Nucleus magnocellularis nidopallii anterioris
MLd	Nucleus mesencephalicus lateralis pars dorsalis
MSt	Striatum mediale
N	Nidopallium
NC	Nidopallium caudale
SAC	Stratum album centrale
TeO	Tectum opticum
TnA	Nucleus taeniae amygdalae
VeM	Nucleus vestibularis medialis
X	Area X

FIGURE LEGENDS

Figure 1 Medial sagittal section through the right brain of an adult male NC crow stained with Cresyl Violet (top) and schematic of the same section showing regional boundaries (bottom). Rostral is left, ventral is down. Scalebar: 2 mm.

Figure 2 Morphology of neurons (identified with a green arrowhead) in the major telencephalic nuclei of the NC crow brain revealed by calcium-binding protein immunocytochemistry (a-e: CB-LI cells; f: PV-LI cells). **a:** fusiform bipolar neuron in the hyperpallium densocellulare; **b:** pyramidal-like neuron in the mesopallium; **c:** round multipolar neuron in the hyperpallium apicale; **d:** ovoid multipolar neuron in the nidopallium; **e:** angular multipolar neuron in the arcopallium; **f:** ovoid multipolar neuron in the area corticoidea dorsolateralis. Scalebar: 20 μm.

Figure 3 Microphotographs of pN-OLG clusters in the NC crow telencephalon. Orange and green arrowheads indicate neurons within a pN-OLG cluster and green arrows indicate neurons devoid of a perineuronal glial cluster. White arrowheads show unclustered oligodendrocytes, and the asterisks indicate presence of blood vessels or blood cells. **a:** Medium-sized pN-OLG cluster in the hyperpallium (light haematoxylin stain). Red arrowheads point to neurons surrounded by a cluster or pN-OLG, white arrows indicate unclustered glia and green arrowhead indicates unclustered neuron. Inset shows a large neuron (N) with its dendritic projections (visible contour indicated by dashed black line) showing a round nucleus (solid black circle) with a darkly stained nucleolus (n) in its centre, surrounded by nine pN-OLG (white dashed lines) **b:** Large pN-OLG cluster in the mesopallium (haematoxylin neutral-red stain). Inset showing the visible contour of the central neuron (black dashed line) and 14 surrounding neuroglia (white dashed line), neurons devoid of a glial cluster is also seen (green arrowhead); **c:** Neuron in the hyperpallium apicale devoid of perineuronal cluster (green arrowhead) and isolate oligodendrocytes (white arrowhead). **d:** Neurons and neuroglia in the area parahippocampalis (dark haematoxylin stain) with no indication of perineuronal clustering. **e:** Neurons (green arrowhead) and neuroglia (white arrowhead) showing no clustered arrangement found in area corticoidea dorsolateralis (cresyl violet stain); **f:** Unclustered neurons and neuroglia in the arcopallium (haematoxylin neutral-red stain); **g:** Unclustered neurons and neuroglia in the entopallium (neutral-red stain).

Figure 4 Microphotographs of the mesopallium of six avian species. White arrowheads indicate neurons with pN-OLG (perineuronal oligodendroglia) and green arrowhead indicate

neurons devoid of perineuronal clusters. **a:** NC crow; **b:** Japanese jungle crow; **c:** Australian magpie; **d:** Indian mynah; **e:** zebra finch; **f:** pukeko. Scalebar: 100 µm.

Figure 5 Microphotographs of the hyperpallium densocellulare of six avian species. White arrowheads indicate neurons with pN-OLG (perineuronal oligodendroglia) and green arrowhead indicate neurons devoid of perineuronal clusters. **a:** NC crow; **b:** Japanese jungle crow; **c:** Australian magpie; **d:** Indian mynah; **e:** zebra finch; **f:** pukeko. Scalebar: 100 µm.

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Figure 1

Figure 1

Medial sagittal section through the right brain of an adult male NC crow stained with Cresyl Violet (top) and schematic of the same section showing regional boundaries (bottom). Rostral is left, ventral is down. Scalebar: 2 mm.

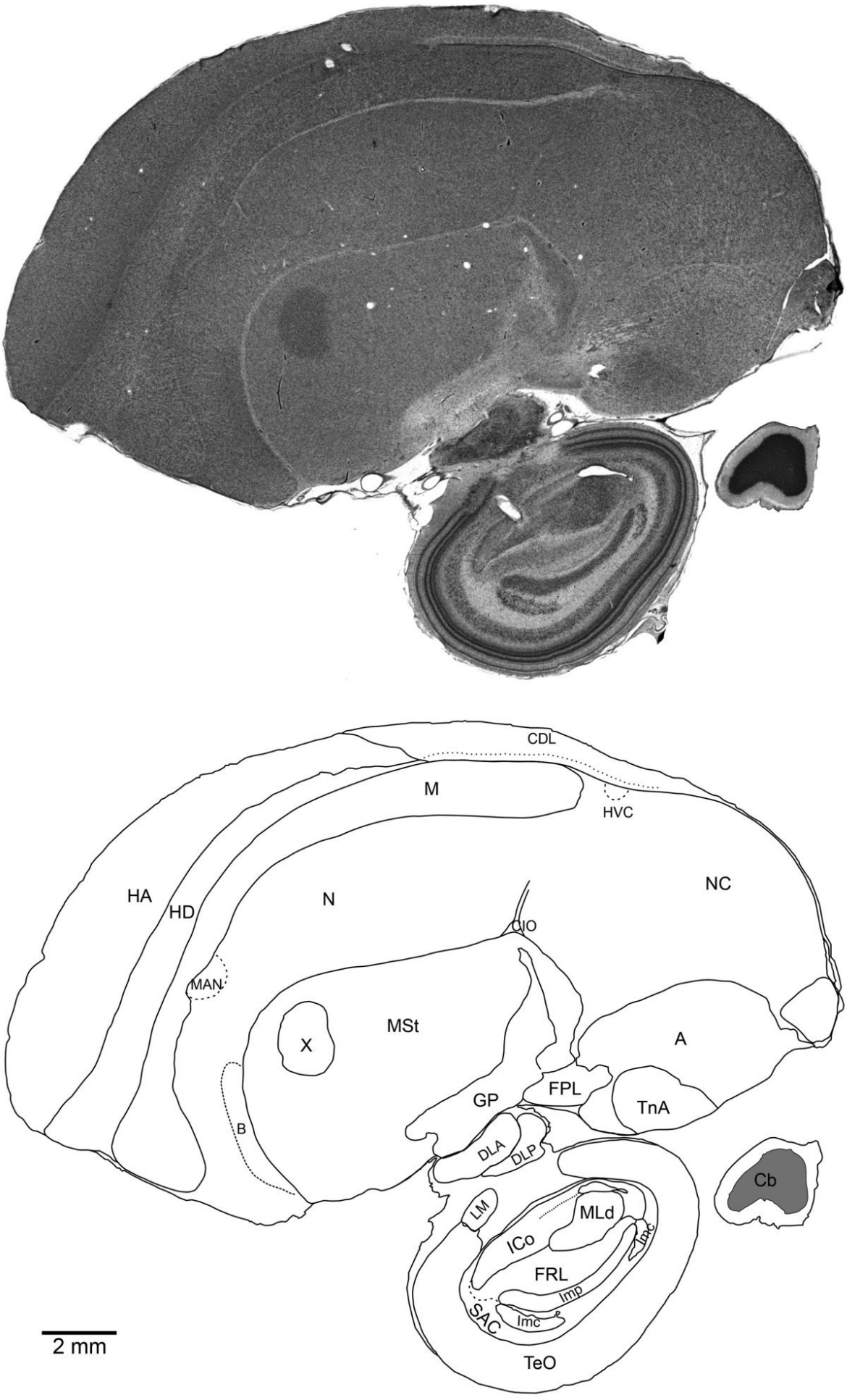


Figure 2

Figure 2

Morphology of neurons (identified with a green arrowhead) in the major telencephalic nuclei of the NC crow brain revealed by calcium-binding protein immunocytochemistry (a-e: CB-LI cells; f: PV-LI cells). **a:** fusiform bipolar neuron in the hyperpallium densocellulare; **b:** pyramidal-like neuron in the mesopallium; **c:** round multipolar neuron in the hyperpallium apicale; **d:** ovoid multipolar neuron in the nidopallium; **e:** angular multipolar neuron in the arcopallium; **f:** ovoid multipolar neuron in the area corticoidea dorsolateralis. Scalebar: 20 µm. al bou

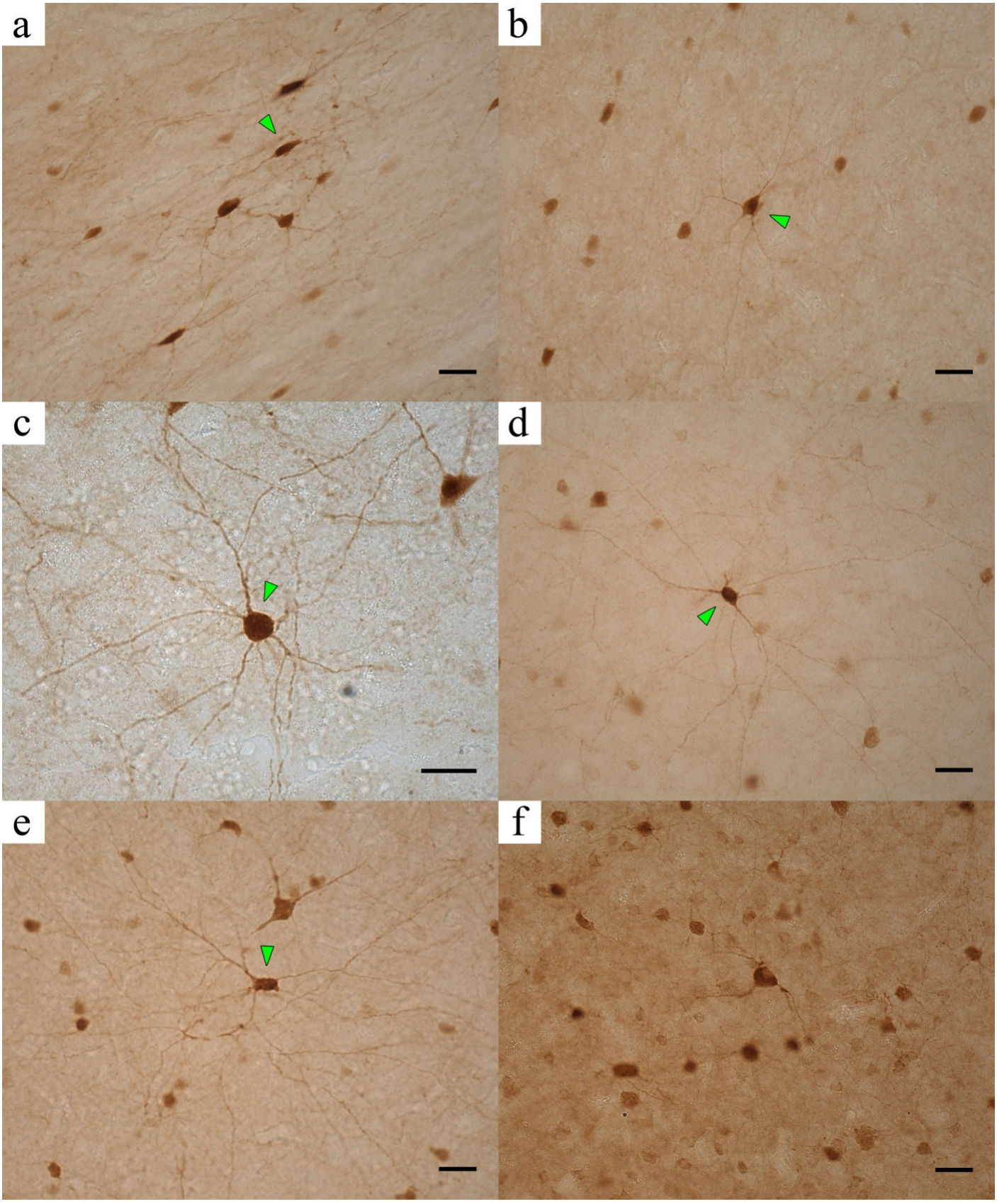


Figure 3

Figure 3

Microphotographs of pN-OLG clusters in the NC crow telencephalon. Orange and green arrowheads indicate neurons within a pN-OLG cluster and green arrows indicate neurons devoid of a perineuronal glial cluster. White arrowheads show unclustered oligodendrocytes, and the asterisks indicate presence of blood vessels or blood cells. **a:** Medium-sized pN-OLG cluster in the hyperpallium (light haematoxylin stain). Red arrowheads point to neurons surrounded by a cluster or pN-OLG, white arrows indicate unclustered glia and green arrowhead indicates unclustered neuron. Inset shows a large neuron (N) with its dendritic projections (visible contour indicated by dashed black line) showing a round nucleus (solid black circle) with a darkly stained nucleolus (n) in its centre, surrounded by nine pN-OLG (white dashed lines) **b:** Large pN-OLG cluster in the mesopallium (haematoxylin neutral-red stain). Inset showing the visible contour of the central neuron (black dashed line) and 14 surrounding neuroglia (white dashed line), neurons devoid of a glial cluster is also seen (green arrowhead); **c:** Neuron in the hyperpallium apicale devoid of perineuronal cluster (green arrowhead) and isolate oligodendrocytes (white arrowhead). **d:** Neurons and neuroglia in the area parahippocampalis (dark haematoxylin stain) with no indication of perineuronal clustering. **e:** Neurons (green arrowhead) and neuroglia (white arrowhead) showing no clustered arrangement found in area corticoidea dorsolateralis (cresyl violet stain); **f:** Unclustered neurons and neuroglia in the arcopallium (haematoxylin neutral-red stain); **g:** Unclustered neurons and neuroglia in the entopallium (neutral-red stain).

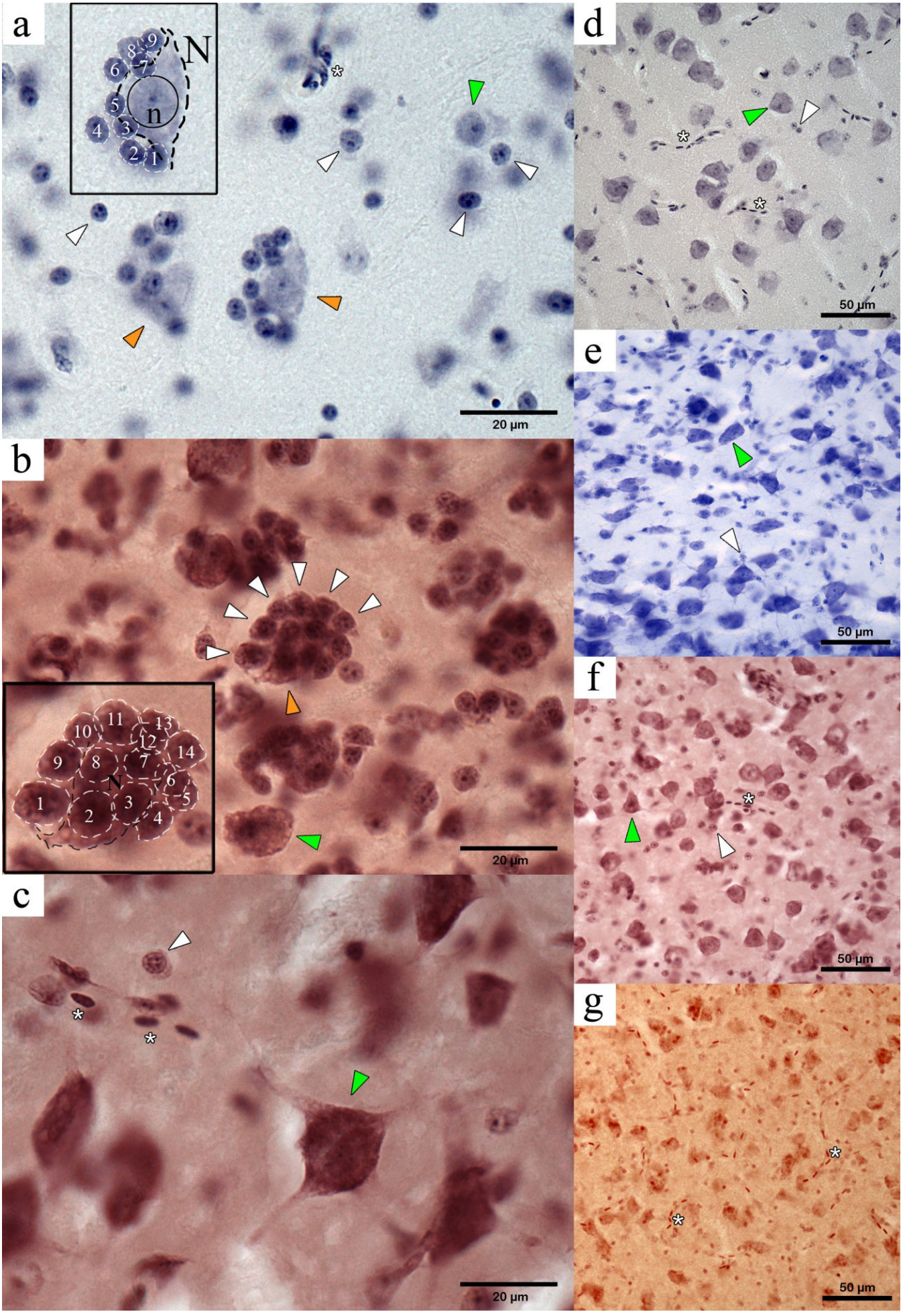
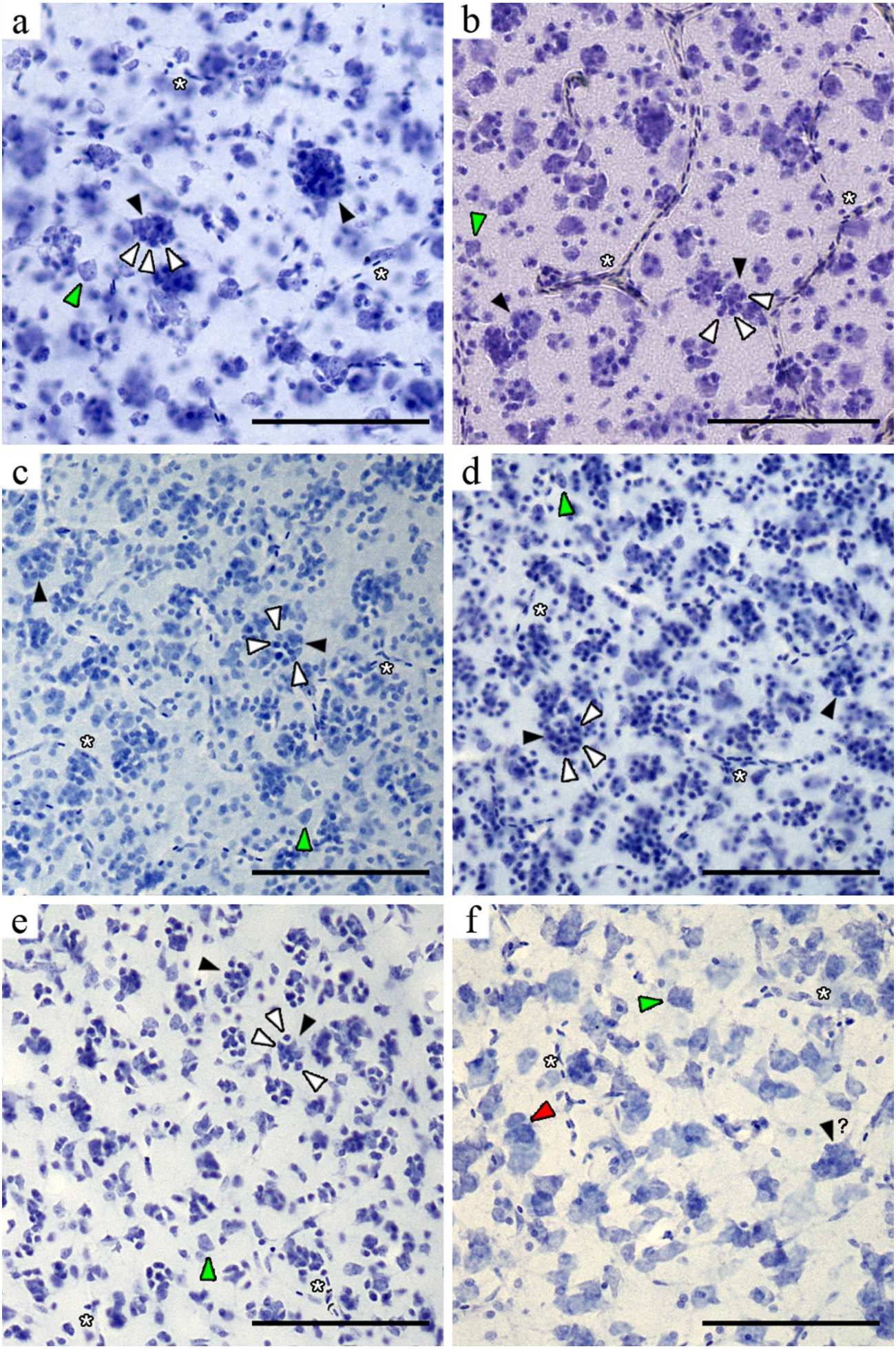


Figure 4

Figure 4

Microphotographs of the mesopallium of six avian species. White arrowheads indicate neurons with pN-OLG (perineuronal oligodendroglia) and green arrowhead indicate neurons devoid of perineuronal clusters. **a:** NC crow; **b:** Japanese jungle crow; **c:** Australian magpie; **d:** Indian mynah; **e:** zebra finch; **f:** pukeko. Scalebar: 100 µm. r neuro



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Microphotographs of the hyperpallium densocellulare of six avian species. White arrowheads indicate neurons with pN-OLG (perineuronal oligodendroglia) and green arrowhead indicate neurons devoid of perineuronal clusters. **a:** NC crow; **b:** Japanese jungle crow; **c:** Australian magpie; **d:** Indian mynah; **e:** zebra finch; **f:** pukeko. Scalebar: 100 µm.