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Received: 19 October 2023 | Revised: 26 February 2024 | Accepted: 27 February 2024

DOI: 10.1002/cne.25602

RESEARCH ARTICLE

JCN RESEARCH IN SYSTEMS NEUROSCIENCE THE JOURNAL OF COMPARATIVE NEUROLOGY WILEY

Nuclear parcellation and numbers of orexinergic neurons in five species of larger brained birds

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Associate Editor: Kathleen Rockland

Funding information
Carnegie Corporation of New York; Ajman University Research Grant, Grant/Award Number: 2022-IRG-MED-3

Abstract

The orexinergic/hypocretinergic system, while having several roles, appears to be a key link in the balance between arousal and food intake. In birds, to date, this system has only been examined anatomically in four species, all with brains smaller than 3.5 g and of limited phylogenetic range. Here, using orexin-A immunohistochemistry, we describe the distribution, morphology, and nuclear parcellation of orexinergic neurons within the hypothalami of a Congo gray and a Timneh gray parrot, a pied crow, an emu, and a common ostrich. These birds represent a broad phylogeny, with brains ranging in size from 7.85 to 26.5 g. Within the hypothalami of the species studied, the orexinergic neurons were organized in two clusters, and a densely packed paraventricular hypothalamic nucleus cluster located within the medial hypothalamus (Hyp), but not contacting the ventricle, and a more loosely packed lateral hypothalamic cluster in the lateral Hyp. Stereological analysis revealed a strong correlation, using phylogenetic generalized least squares regression analyses, between brain mass and the total number of orexinergic neurons, as well as soma parameters such as volume and area. Orexinergic axonal terminals evinced two types of boutons, larger and the smaller en passant boutons. Unlike the orexinergic system in mammals, which has several variances in cluster organization, that of the birds studied, in the present and previous studies, currently shows organizational invariance, despite the differences in brain and body mass, phylogenetic relationships, and life-histories of the species studied.

KEYWORDS
Aves, hypocretin, immunohistochemistry, orexin, RRID:AB_91545, stereology

Abbreviations: 3n, nervus oculomotorius; 3V, third ventricle; A, arcopallium; ac, anterior commissure; AL, ansa lenticularis; DLL, nucleus dorsolateralis anterior thalami pars lateralis; DLP, nucleus dorsolateralis posterior thalami; DMA, nucleus dorsomedialis anterior thalami; EM, nucleus ectomamillaris; FPL, fasciculus prosencephali lateralis; GLv, nucleus geniculatus lateralis, pars ventralis; GP, globus pallidus; HM, nucleus habenularis medialis; Hyp, hypothalamus; Imc, nucleus isthmi, pars magnocellularis; Inf, infundibulum; Ipc, nucleus isthmi pars parvocellularis; LHyc, lateral hypothalamic orexinergic cluster; LSt, striatum lateral; LV, lateral ventricle; MSt, striatum mediale; OM, tractus occipito-mesencephalicus; Ov, nucleus ovoidalis; PL, nucleus pontis lateralis; PM, nucleus pontis medialis; PMH, nucleus medialis hypothalamic posterioris; POA, nucleus preopticus anterior; PPC, nucleus principalis precommissuralis; PVHc, paraventricular hypothalamic nucleus cluster; PVM, nucleus periventricularis magnocellularis; QF, tractus quintofrontalis; Rt, nucleus rotundus; S, septal nuclear complex; SAC, stratum album centrale; SP, nucleus subpretectalis; SPC, nucleus superficialis parvocellularis; SPM, nucleus spiriformis medialis; STN, nucleus subthalamus; Str, striatum; TeO, tectum opticum; TFM, tractus thalamo-frontalis et frontalis-thalamicus medialis; TrO, tractus opticus; VSt, ventral striatum.

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1 | INTRODUCTION

The orexinergic system plays a role in the regulation of wakefulness/sleep, specifically the maintenance of wakefulness (e.g., Inutsuka & Yamanaka, 2013; Volkhoff, 2012), and appetite, through the stimulation of food intake and the repression of neural circuits controlling satiety (e.g., Kukkonen et al., 2002; Soya & Sakurai, 2020; Zhang et al., 2013), making it a crucial regulatory link balancing the two biological imperatives of obtaining nutrition and sleep, both essential for survival. Orexinergic neurons are typically located within the hypothalamus (Hyp) in vertebrates (e.g., Domínguez et al., 2010; López et al., 2009; Matsuda et al., 2012; Singletary et al., 2006; Nixon & Smale, 2007; Fokidis et al., 2019; Miranda et al., 2013), but the majority of studies have focused upon mammals, with over 50 species having been studied (Oddes et al., 2023), and although there is general similarity in the distribution and nuclear parcellation of the orexinergic neurons in mammals, there are variances.

Some of the reported differences in the distribution and parcellation of orexinergic neurons in mammals appear to be associated with high volume diets, such as the presence of the parvocellular orexinergic cluster in the medial Hyp (Davimes et al., 2017; Dell et al., 2012; Dell, Karlsson, et al., 2016; Dell, Patzke, et al., 2016; Ettrup et al., 2010; Iqbal et al., 2001; Malungo et al., 2020; Maseko et al., 2013; Oddes et al., 2023), while others, such as the presence of orexinergic neurons in the ventral aspect of the medial supraoptic region of the Hyp (Oddes et al., 2023; Williams et al., 2022), have uncertain function. In addition, it should be noted that these variations in the parcellation of orexinergic neurons in mammals appear to be broadly associated with brain size, such that larger brained mammals appear to have more orexinergic clusters than smaller brain mammals; for example, smaller brained carnivores (compare ferret and nine-banded mongoose, Pillay et al., 2017, with African and Asian lion and cheetah, Oddes et al., 2023), and smaller brained primates (compare prosimians/macaque monkeys with apes, Williams et al., 2022; Oddes et al., 2023) have fewer distinct orexinergic clusters than the larger brained representatives within these lineages.

Studies of the orexinergic neuronal distribution in birds have, to our knowledge, been undertaken in only four species of bird, the Japanese quail (Phillips-Singh et al., 2003), the house finch (Singletary et al., 2006), the domestic chicken (Miranda et al., 2013), and the song sparrow (Fokidis et al., 2019). These reports indicate that the orexinergic neurons of the avian Hyp form a moderately dense cluster centered on the paraventricular hypothalamic nucleus, with a low density of orexinergic neurons spreading into the lateral hypothalamic regions. Thus, the nuclear parcellation of the orexinergic neurons in avian species appears to be less varied than seen in mammals, but this lack of variance may be related to three factors: (1) Only smaller brained avian species have been investigated; (2) the limited phylogenetic diversity of avian species explored; or (3) the presence of both orexin 1 and 2 receptors in mammals that do not appear in nonmammalian vertebrates, including birds (Soya & Sakurai, 2020).

Here, we present both qualitative and quantitative results of immunohistochemical staining for an orexin-A (OxA) antibody in the hypothalami of five birds, a Congo gray parrot, a Timneh gray parrot, a pied crow, an emu, and a common ostrich. These birds represent species that have, in avian terms, large brains (all five species). These birds also represent lineages in which this system has not been previously investigated and are species with vastly different life histories to those that have been studied. The results of this investigation allow us to begin to address in more detail the potential variation, or potential lack thereof, in the organization of the orexinergic system in birds.

2 | MATERIALS AND METHODS

2.1 | Specimens

Brains were obtained from adult males from one Congo gray parrot (*Psittacus erithacus*), one Timneh gray parrot (*Psittacus timneh*), one pied crow (*Corvus albus*), one emu (*Dromaius novaehollandiae*), and one common ostrich (*Struthio camelus*) for use in the current study. The live birds were sourced from a local breeder in South Africa. All birds showed no behavioral problems or stereotypies indicative of any neurological impairments. The birds used in this study were treated according to the guidelines of the University of Witwatersrand Animal Ethics Committee (Clearance number 2013/05/02B), which correspond with those of the National Institutes of Health (NIH) for care and use of animals in scientific experimentation. Five minutes prior to being euthanized, the parrots and the crow were given an intramuscular dose of heparin (2500 units in 0.5 mL) to prevent blood clotting. The parrots and the crow were then injected with an intraperitoneal dose of Euthapent (1 mL/kg). Following euthanasia, these birds were transcardially perfusion-fixed, initially with a rise of 0.9% saline solution at a temperature of 4°C followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C. The emu and common ostrich were given an intramuscular dose of heparin (5000 units in 1 mL) and after 5 min were euthanized with an intraperitoneal injection of Euthapent (1 mL/kg, body masses: emu ~50 kg, common ostrich ~120 kg). Following euthanasia, the carotid arteries of the emu and ostrich were cannulated, and the heads were perfused with the same solutions as described for the parrots and crow. The brains, which showed no overt signs of neuropathology, were removed from the skull, weighed, and postfixed in 4% paraformaldehyde in 0.1 M PB (24 h at 4°C) and allowed to equilibrate in 30% sucrose in 0.1 M PB before being stored in an antifreeze solution at −20°C until use (Manger et al., 2009).

2.2 | Sectioning and immunohistochemical staining

Blocks containing the Hyp were dissected from these brains, allowed to equilibrate in 30% sucrose in 0.1 M PB, and then frozen in crushed dry ice. The frozen brain blocks were mounted to an aluminum stage, and coronal sections of 50 μm thickness were cut using a sliding microtome. Brain blocks from the Congo gray parrot, Timneh gray parrot and pied crow were sectioned in a coronal plane into 1 in 10 series, while those

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appears to be limited to two clusters that may have homologues in both mammals and reptiles, but these suggested homologues are made tentatively and require further investigation. It is possible that with the investigation of further avian species, varying in size, phylogenetic relationships, and life histories that more than the two orexinergic clusters consistently reported to date will be observed.

4.2 | Scaling of orexinergic neuronal numbers and soma parameters in birds

The consistency noted above in the parcellation, or clustering, of the avian orexinergic neurons is also seen in the scaling of both total numbers and soma sizes with brain mass in the birds studied. Both OLS and PGLS regression analyses revealed statistically significant correlations between brain mass and total orexinergic neuronal numbers in the five birds studied. In contrast, in mammals, the numbers of orexinergic neurons and brain mass do not show a clear correlation with brain mass, perhaps due to the variations observed in mammals, although larger brained mammals tend to have more orexinergic neurons than smaller brain mammals (e.g., Dell et al., 2012, Dell, Karlsson, et al., 2016; Dell, Patzke, et al., 2016; Williams et al., 2022). Total numbers of orexinergic neurons have not been reported in reptiles. OLS analysis revealed significant correlations between brain mass and soma volume and area in the birds studied, but these correlations were weaker, but still statistically supported, with PGLS analysis. Such correlations do not appear to be present in the mammalian data (e.g., Dell et al., 2012; Dell, Karlsson, et al., 2016; Dell, Patzke, et al., 2016; Williams et al., 2022) and have not been examined in reptiles.

4.3 | Orexinergic boutons and communication channels

Orexin is known to produce a postsynaptic excitatory effect on the neurons where orexinergic receptors are found (Song et al., 2006; Yan et al., 2012). It is possible that the excitatory effects on the recipient neurons could occur through either chemical synaptic or volume transmission communication channels, or indeed both (Fuxe et al., 2010). Orexin has been shown to be able to communicate through either of these channels, with the volume transmission channel producing a longer sustained activation than that initiated by chemical synaptic transmission (Del Cid-Pellitero & Garzón, 2011). In the birds studied herein, as well as in mammals previously studied (Dell et al., 2015), we noted two different types of orexinergic boutons, distinguished by size, and these different boutons may be involved in the different communication channels. It has been noted in regions of the brain processing sensory information that different bouton types, based on size, are involved in different functions, with larger boutons considered to be "drivers," while smaller en passant boutons are considered "modulators" (Sherman & Guillery, 1998). Thus, it is possible that the larger OxA+ boutons are involved in chemical synaptic communication having excitatory effects on specific postsynaptic neurons, while the smaller en passant boutons may be involved in volume transmission communication and may modulate excitation levels across neuronal populations. The current observations indicate that, whatever communication channels are used by the orexinergic system, these types of communication channels are likely to be similar in both birds and mammals.

4.4 | An unusually consistent orexinergic system in birds?

Taken together, the consistency in parcellation, significant correlations between brain mass and total numbers/soma parameters, and bouton morphology in birds, especially in comparison to the variations observed in mammals and reptiles, indicate that in birds, the orexinergic system is possibly not as evolutionarily plastic as it is in other amniotes. The birds in which the orexinergic system has now been investigated represent a broad phylogenetic spectrum, brains that range in size from under 1 g to over 25 g, and birds that have very distinct life histories, including flightlessness and unusual sleep phenomenology (e.g., Lesku et al., 2011). It is possible that the absence of orexin 1 receptors in birds (Soya & Sakurai, 2020) might limit the evolutionary plasticity of the orexinergic system, but this does not seem to have affected plasticity in this system in reptiles (Domínguez et al., 2010) where the orexin 1 receptors are also absent (Soya & Sakurai, 2020). At present, we cannot suggest why the avian orexinergic system appears so consistent across species. It is possible that with the study of further avian species that certain examples of variations in the orexinergic system, in terms of parcellation, distribution, scaling relationships, soma parameters, and bouton types may be observed.

AUTHOR CONTRIBUTIONS

Pedzisai Mazengenya and Paul R. Manger conceptualized the study. Pedzisai Mazengenya and Paul R. Manger obtained and prepared the brains used in this study. Pedzisai Mazengenya, Muhammad A. Spocter, and Paul R. Manger performed the staining and analysis. Pedzisai Mazengenya and Paul R. Manger wrote the initial draft of the manuscript, and Muhammad A. Spocter contributed to the editing and improvement of drafts of the manuscript. All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

ACKNOWLEDGMENTS

No acknowledgements to declare.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data have not been shared due to this study being based on histological sections.

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PEER REVIEW

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How to cite this article: Mazengenya, P., Spocter, M. A., & Manger, P. R. (2024). Nuclear parcellation and numbers of orexinergic neurons in five species of larger brained birds. *Journal of Comparative Neurology*, *532*, e25602. https://doi.org/10.1002/cne.25602

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