Received: 11 September 2023 | Revised: 28 November 2023 | Accepted: 9 January 2024

DOI: 10.1002/cne.25587

JCN RESEARCH IN SYSTEMS NEUROSCIENCE THE JOURNAL OF COMPARATIVE NEUROLOGY

WILEY

RESEARCH ARTICLE

Apparent absence of hypothalamic cholinergic neurons in the common ostrich and emu: Implications for global brain states during sleep

Pedzisai Mazengenya[1,2,3] | **John A. Lesku**[4] | **Niels C. Rattenborg**[5] | **Paul R. Manger**[3]

[1] College of Medicine, Ajman University, Ajman, United Arab Emirates

[2] Center of Medical and Bio-allied Health Sciences Research, Ajman University, Ajman, United Arab Emirates

[3] School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, Parktown, Johannesburg, Republic of South Africa

[4] Sleep Ecophysiology Group, School of Agriculture, Biomedicine and Environment, La Trobe University, Melbourne, Australia

[5] Avian Sleep Group, Max Planck Institute for Biological Intelligence, Seewiesen, Germany

Correspondence
Pedzisai Mazengenya, College of Medicine, Basic Medical Sciences Department, Ajman University, Ajman, United Arab Emirates.
Email: p.mazengenya@ajman.ac.ae

Associate Editor: Kathleen Rockland.

Funding information
Ajman University Research Grant, Grant/Award Number: 2022-IRG-MED-3

Abstract

We examined the presence/absence and parcellation of cholinergic neurons in the hypothalami of five birds: a Congo grey parrot (*Psittacus erithacus*), a Timneh grey parrot (*P. timneh*), a pied crow (*Corvus albus*), a common ostrich (*Struthio camelus*), and an emu (*Dromaius novaehollandiae*). Using immunohistochemistry to an antibody raised against the enzyme choline acetyltransferase, hypothalamic cholinergic neurons were observed in six distinct clusters in the medial, lateral, and ventral hypothalamus in the parrots and crow, similar to prior observations made in the pigeon. The expression of cholinergic nuclei was most prominent in the Congo grey parrot, both in the medial and lateral hypothalamus. In contrast, no evidence of cholinergic neurons in the hypothalami of either the ostrich or emu was found. It is known that the expression of sleep states in the ostrich is unusual and resembles that observed in the monotremes that also lack hypothalamic cholinergic neurons. It has been proposed that the cholinergic system acts globally to produce and maintain brain states, such as those of arousal and rapid-eye-movement sleep. The hiatus in the cholinergic system of the ostrich, due to the lack of hypothalamic cholinergic neurons, may explain, in part, the unusual expression of sleep states in this species. These comparative anatomical and sleep studies provide supportive evidence for global cholinergic actions and may provide an important framework for our understanding of one broad function of the cholinergic system and possible dysfunctions associated with global cholinergic neural activity.

KEYWORDS
Aves, choline acetyltransferase, cholinergic, immunohistochemistry, RRID:AB_2079751

Abbreviations: 3V, third ventricle; ac, anterior commissure; AL, ansa lenticularis; CoS, nucleus commissurae septi; DMH, dorsomedial hypothalamic cholinergic cluster; DMP, nucleus dorsomedialis posterior thalami; EM, nucleus ectomamillaris; FLM, fasciculus longitudinalis medialis; FPLfasciculus prosencephali lateralis, fasciculus prosencephali lateralis; Hyp, hypothalamus; Ipc, nucleus isthmi pars parvocellularis; LHyd, nucleus lateralis hypothalamus, dorsal cholinergic cluster; LHyv, nucleus lateralis hypothalamus, ventral cholinergic cluster; LSt, striatum laterale; ME, median eminence cholinergic cluster; MSt, striatum mediale; OM, tractus occipitomesencephalicus; Ov, nucleus ovoidalis; PaP, nucleus papilloformis; PM, nucleus pontis medialis; PMH, medialis hypothalamic posterioris cholinergic cluster; Rt, nucleus rotundus; S, septal nuclear complex; SL, lateral septal nucleus; SNc, substantia nigra pars compacta; SPC, nucleus superficialis parvocellularis; SpM, nucleus spiriformis medialis; TrO, tractus opticus; TSM, tractus septopallio-mesencephalicus; Tu, tuberal region cholinergic cluster.

J Comp Neurol. 2024;532:e25587.
https://doi.org/10.1002/cne.25587

1 | INTRODUCTION

The cholinergic neurons that form the discrete cholinergic nuclei, nuclear groups, and system of the brain are involved in a vast array of functions from overt movements to consciousness, arousal, perception, selective attention, and memory (Woolf & Butcher, 2011). This system is also intricately involved in the regulation of emotion and motivation, and the production and modulation of features of the sleep/wake cycle, including arousal and rapid-eye-movement (REM) sleep (e.g., Platt & Reidel, 2011; Mu & Huang, 2019). There are several cholinergic cell groups/nuclei, found from the very rostral aspect of the forebrain through to the spinal cord (e.g., Woolf, 1991; Medina & Reiner, 1994); however, while these cell groups are discrete morphological units and have discrete functions, the cholinergic system also appears to form a global system, using dendritic connectivity to make the cholinergic system an interconnected, continuous aggregation of neurons spanning the rostral forebrain through to the spinal cord, capable of coordinating large-scale neural activity, such as the variations seen in the sleep–wake cycle, across the whole brain (Woolf, 1991).

One of the discrete groups of cholinergic nuclei is located within the hypothalamus, where, in most mammals, three cholinergic clusters are generally reported (e.g., Woolf, 1991; Dell et al., 2010). To our knowledge, the only avian species in which these hypothalamic cholinergic clusters have been fully defined is the pigeon (*Columba livia*) (Medina & Reiner, 1994), although hypothalamic cholinergic neurons have been noted in the budgerigar (*Melopsittacus undulatus*) (Roberts et al., 2002) and chicken (*Gallus domesticus*) (González-Cabrera et al., 2015). In the pigeon, cholinergic neurons were found in the nucleus preopticus medialis (POM), the nucleus medialis hypothalamic posterioris (PMH), the infundibular region and nucleus tuberis, dispersed neurons throughout the nucleus lateralis hypothalamic (LHy), the stratum cellulare externum (SCE), and the dorsal aspect of the stratum cellular internum (SCI) (Medina & Reiner, 1994). Thus, the complement of cholinergic hypothalamic clusters appears to be more complex in the pigeon when compared to mammals.

While the complement of cholinergic nuclei in the mammalian hypothalamus is consistent across most species (e.g., Dell et al., 2010), in the monotremes (platypus [*Ornithorhynchus anatinus*] and short-beaked echidna [*Tachyglossus aculeatus*]) these hypothalamic cholinergic neurons were not observed (Manger et al., 2002). The absence of hypothalamic cholinergic neurons in the monotreme hypothalamus creates a hiatus in the potential global cholinergic system (Woolf, 1991). Interestingly, this absence coincides with the unusual sleep phenomenology of the monotremes, where the forebrain exhibits the cortical electrophysiological activity of non-REM sleep, while brainstem and behavioral measures are consistent with REM sleep (Siegel et al., 1996, 1998, 1999). Thus, this hypothalamic cholinergic hiatus may explain the unusual combination of sleep elements observed in the monotremes (Manger et al., 2002).

While most birds show globally synchronized sleep states (e.g., Lesku & Rattenborg, 2014; Tisdale et al., 2017), one exception, the common ostrich (*Struthio camelus*), has been noted (Lesku et al., 2011). In the ostrich, during episodes of REM sleep characterized by eye movements under closed eyelids, reduced neck muscle tone, and a wobbling head and neck, forebrain activity would flip between activation (typical of REM sleep) and slow waves (typical of non-REM sleep) (Lesku et al., 2011), showing instances of similarity to monotreme sleep. In addition, the amount of REM sleep in the ostrich was greater than that observed in most other avian species (Lesku et al., 2011; please note (n.b.) ostriches spend 26.3 ± 1.3% of total sleep time in REM, while a recent report in the budgerigar indicates that they spend 26.5 ± 8.97% of total sleep time in REM, Canavan & Margoliash, 2020, an amount marginally higher than the ostrich), a second characteristic in common with sleep in the monotremes compared to other mammals (e.g., Siegel et al., 1999). This then raises the question of whether, like the monotremes, ostriches and related birds lack hypothalamic cholinergic neurons. To investigate this question, we employed choline acetyltransferase (ChAT) immunohistochemistry on brain sections through the hypothalamus from an ostrich and the closely related emu (*Dromaius novaehollandiae*), and for methodological validation and comparison to non-related avian species, a Congo grey parrot (*Psittacus erithacus*), a Timneh grey parrot (*P. timneh*), and a pied crow (*Corvus albus*).

2 | MATERIALS AND METHODS

2.1 | Specimens

Brains from adult (animals of breeding age, but not senescent) males of one Congo grey parrot (*P. erithacus*), one Timneh grey parrot (*P. timneh*), one pied crow (*Corvus albus*), one emu (*D. novaehollandiae*) and one common ostrich (*S. camelus*) were used in this 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 NIH for the 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, active ingredient pentobarbital). Following euthanasia, these birds were transcardially perfusion-fixed, initially with a rinse of 0.9% saline solution at a temperature of 4°C followed by 4% formaldehyde in 0.1 M phosphate buffer (PB) at 4°C (mixed from paraformaldehyde powder). The emu and 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 and post-fixed in 4% paraformaldehyde in 0.1 M PB (for 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

The brains were allowed to equilibrate in 30% sucrose in 0.1 M PB and then frozen in crushed dry ice. The frozen brains were mounted to an aluminum stage and coronal sections of 50 μm thickness were cut using a sliding microtome. Brains from the Congo grey parrot, Timneh grey parrot, and pied crow were sectioned in a coronal plane into 1 in 10 series, while those of the emu and ostrich were sectioned in a coronal plane into 1 in 20 series, of which two series were used in the current study. The remaining 8/18 series were placed in an antifreeze solution and stored at −20°C for future use. The two series used in this study were stained for Nissl and immunostained for ChAT (see below). Sections used for Nissl staining were mounted on 0.5% gelatine-coated glass slides, and cleared in a solution of 1:1 chloroform and 100% alcohol overnight, after which the sections were stained with 1% cresyl violet. The Nissl-stained sections were used to define the architecture of the hypothalamus and surrounding structures.

The series of sections used for ChAT immunohistochemistry were initially treated for 30 min with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1 M PB: 1.6% of 30% H_2O_2), followed by three 10-min rinses in 0.1 M PB. The sections were then preincubated at room temperature for 3 h in a blocking buffer solution comprised of 3% normal goat rabbit, 2% bovine serum albumin (BSA, Sigma), and 0.25% Triton X-100 (Merck) in 0.1 M PB. The sections were then placed in a primary antibody solution (blocking buffer with appropriately diluted primary antibody) and incubated at 4°C for 48 h under gentle shaking. To reveal neurons that produce acetylcholine as a neurotransmitter, we employed the AB144P anti-ChAT goat polyclonal antibody (AB144P, Merck-Millipore; RRID AB_2079751) at a dilution of 1:3000. This antibody is known to reliably identify cholinergic neurons in all regions of the brain in a range of vertebrate species (e.g., Kaiser et al., 2011; Laux et al., 2012; Imam et al., 2018).

The incubation in the primary antibody solution was followed by three 10 min rinses in 0.1 M PB, after which the sections were incubated in a secondary antibody solution, comprised of a 1:1000 dilution of biotinylated anti-goat IgG (BA-5000, Vector Labs) in a solution containing 3% normal rabbit serum and 2% BSA in 0.1 M PB, for 2 h at room temperature. This was followed by three 10 min rinses in 0.1 M PB after which the sections were incubated in AB solution (Vector Labs) for 1 h. After three further 10 min rinses in 0.1 M PB, the sections were placed in a solution of 0.05% diaminobenzidine in 0.1 M PB for 5 min, followed by the addition of 3 μL of 30% H_2O_2 to each 1 mL of solution in which each section was immersed. Chromatic precipitation of the sections was monitored visually under a low-power stereomicroscope. This process was allowed to continue until the background staining of the sections was appropriate for architectonic analysis without obscuring any immunopositive structures. The precipitation process was stopped by immersing the sections in 0.1 M PB and then rinsing them twice more in 0.1 M PB. To check for nonspecific staining from the immunohistochemistry protocol, we omitted the primary antibody and the secondary antibody in selected sections, which produced no evident staining. The immunohistochemically stained sections were mounted on 0.5% gelatine-coated slides and left to dry overnight. The sections were then dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped with Depex.

2.3 | Anatomical reconstruction, microphotography, and nomenclature

A low-power stereomicroscope was used to examine the Nissl-stained sections and camera lucida drawings outlining architectural borders were made. The associated ChAT-immunostained sections were matched to these drawings and the stained neurons were marked. The drawings were then scanned and redrawn using the Canvas Draw 6 drawing program (Canvas GFX, Inc., FL, USA). Digital photomicrographs were captured using an Axiocam 208 color camera mounted to a Zeiss Axioskop microscope (with Zeiss A-Plan 5X/012, Zeiss Plan-NeoFluar 10×/0.30 and Zeiss Plan-NeoFluar 40×/0.75 objectives). No pixelation adjustments or manipulation of the captured images were undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop. The neuroanatomical demarcations and nomenclature used in the current study were derived from Kuenzel and van Tienhoven (1982), Medina and Reiner (1994), Reiner et al. (2004), Izawa and Watanabe (2007), and Brauth et al. (2011).

3 | RESULTS

Employing an antibody raised against ChAT, we were able to reveal cholinergic neurons (ChAT+) in all the birds studied. In the Congo grey parrot, the Timneh grey parrot, and the pied crow, cholinergic neurons were readily identified in the hypothalamus and were readily parcellated into six distinct clusters (that may or may not coincide with previously defined avian hypothalamic nuclei). In contrast, while ChAT+ neurons were observable in many other parts of the brain, no cholinergic neurons were observed in the hypothalami of either the ostrich or emu.

3.1 | Cholinergic neuronal clusters in the hypothalami of the grey parrots and pied crow

Within the hypothalami of the Congo grey parrot, the Timneh grey parrot, and the pied crow, we could identify six clusters of cholinergic neurons (Figure 1). The location and appearance of these clusters were essentially similar in each species; thus, the following description applies to all three species except where noted. The most distinct cholinergic nucleus observed was located in the dorsal medial aspect of the hypothalamus, in close proximity to but not in contact with the

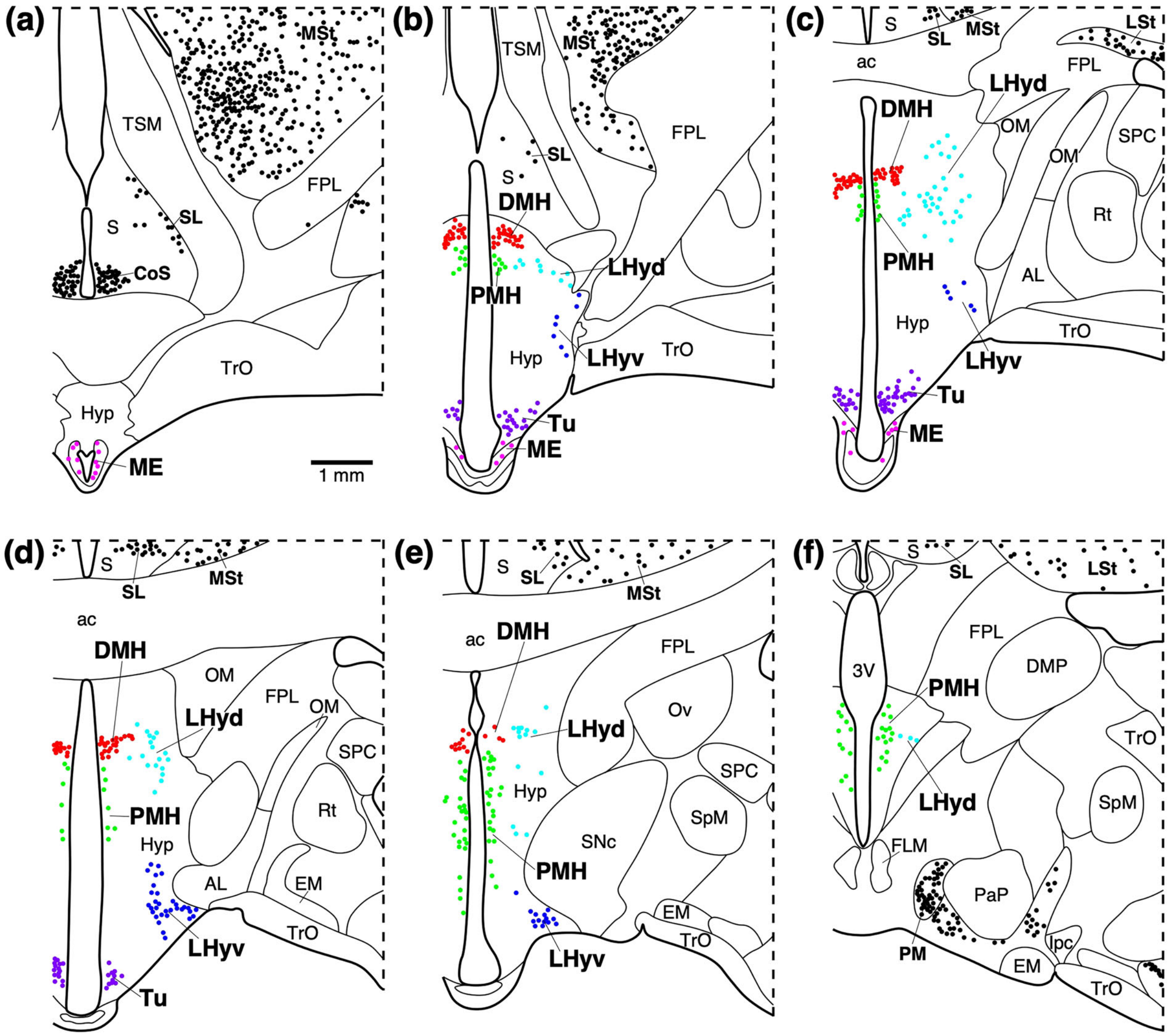

FIGURE 1 Serial drawings of coronal sections through one-half of the Congo grey parrot hypothalamus showing the distribution of ChAT immunopositive neurons (ChAT+). (a) is the most rostral section, (f) the most caudal. The outlines of the architectonic regions were drawn from Nissl-stained sections, with ChAT+ neurons marked on these drawings. Solid red circles represent cholinergic neurons of the dorsomedial hypothalamic cholinergic cluster (DMH); solid green circles represent cholinergic neurons of the nucleus medialis hypothalamic posterioris cholinergic cluster (PMH); solid light blue circles represent cholinergic neurons of the nucleus lateralis hypothalamus, dorsal cholinergic cluster (LHyd); solid dark blue circles represent cholinergic neurons of the nucleus lateralis hypothalamus, ventral cholinergic cluster (LHyv); solid purple circles represent cholinergic neurons of the tuberal region, or arcuate hypothalamic cholinergic cluster (Tu); solid pink circles represent cholinergic neurons of the median eminence cholinergic cluster (ME); solid black circles represent cholinergic neurons located outside of the hypothalamus. Each circle represents an individual neuron. The drawings are approximately 1000 μm apart, and in each drawing dorsal is to the top and the medial to the left. (See the list for abbreviations.)

wall of the third ventricle (Figure 1b–e). We term this group the dorsomedial hypothalamic cholinergic cluster (DMH), based on location and neurochemistry. The ChAT+ neurons of the DMH reveal the highest relative density of cholinergic neurons in the avian hypothalami studied, with extensive local dendritic arbors forming a distinct, intensely stained neuropil (Figures 2a, 3a, 4a). This appearance of the DMH was most marked in the two species of parrot (Figures 2a, 3a), with the DMH of the pied crow exhibiting similar neuronal densities, but a less intense neuropil stain (Figure 4a). The ChAT+ neurons of the DMH were a mixture of multipolar and bipolar types and did not evince any specific preferred dendritic orientation (Figures 2a, 3a, 4a).

A relatively moderate-to-low density of ChAT+ neurons was observed to extend laterally from the DMH within the dorsal aspect of the lateral hypothalamus (Figure 1b–f), which we term the nucleus lateralis hypothalamus, dorsal cholinergic cluster (LHyd). The neurons comprising the LHyd were primarily bipolar in type, with dendrites showing a preferential mediolateral orientation (Figures 2b, 3c, 4b). The relative density and overall number, based on our qualitative impression, of these LHyd ChAT+ neurons were highest in the Congo grey parrot, becoming lesser in number and density in the Timneh grey parrot and the pied crow (Figures 2b, 3c, 4b). A similar cluster of ChAT+ neurons was observed in the ventrolateral aspect of the hypothalamus

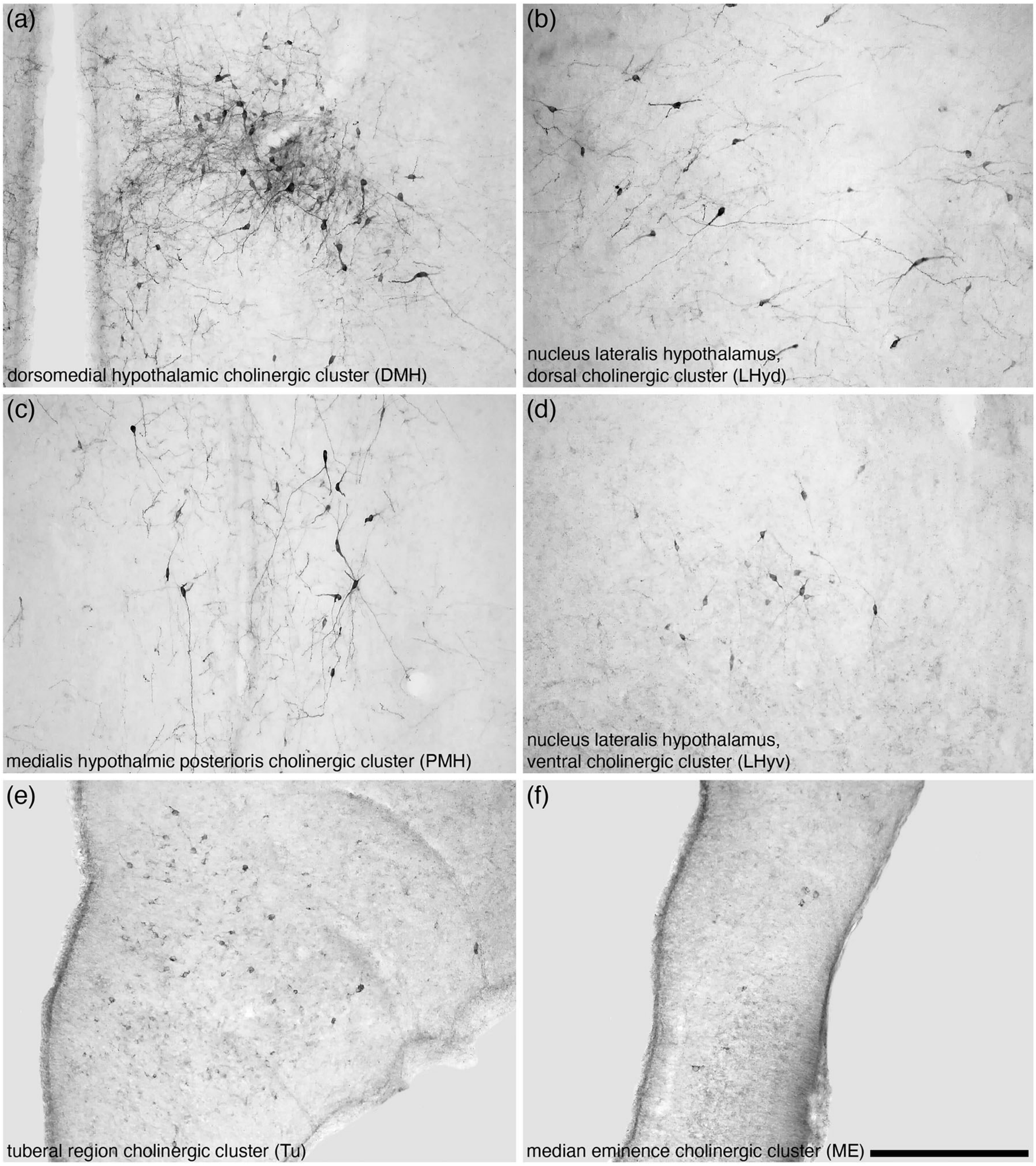

FIGURE 2 Photomicrographs of neurons in the hypothalamus of the Congo grey parrot that were immunoreactive for the ChAT antibody used in the current study. Cholinergic neurons were observed in six clusters including: (a) the dorsomedial hypothalamic cholinergic cluster (DMH); (b) the nucleus lateralis hypothalamic, dorsal cholinergic cluster (LHyd); (c) the nucleus medialis hypothalamic posterioris cholinergic cluster (PMH); (d) the nucleus lateralis hypothalamic, ventral cholinergic cluster (LHyv); (e) the tuberal region, or arcuate hypothalamic cholinergic cluster (Tu); and (f) the median eminence cholinergic cluster (ME). In all images, the dorsal is to the top and medial to the left, apart from (c) where the midline is found in the center of the image. Scale bar in (f) = 250 μm and applies to all.

(Figure 1b-e), which we term the nucleus lateralis hypothalamus, ventral cholinergic cluster (LHyv). Again, the relative density and number of these ChAT+ neurons were highest in the Congo grey parrot, being lesser in number and density in the Timneh grey parrot and the pied crow (Figures 2d, 4d). The ChAT+ neurons of the LHyv were primarily bipolar in type, with a preferred dorsomedial to the ventrolateral orientation of the dendrites (Figures 2d, 4d). While the neurons forming the LHyd and LHyv are similar, there appears to be a topographical discontinuity between these two cholinergic clusters.

ChAT+ neurons extending ventrally from the DMH, formed a loosely arranged, relatively low-density column aligned parallel to the wall of the third ventricle. These were assigned to the nucleus medialis hypothalamic posterioris cholinergic cluster (PMH) (Figure 1b–f). The ChAT+ neurons of the PMH were primarily bipolar in type, but

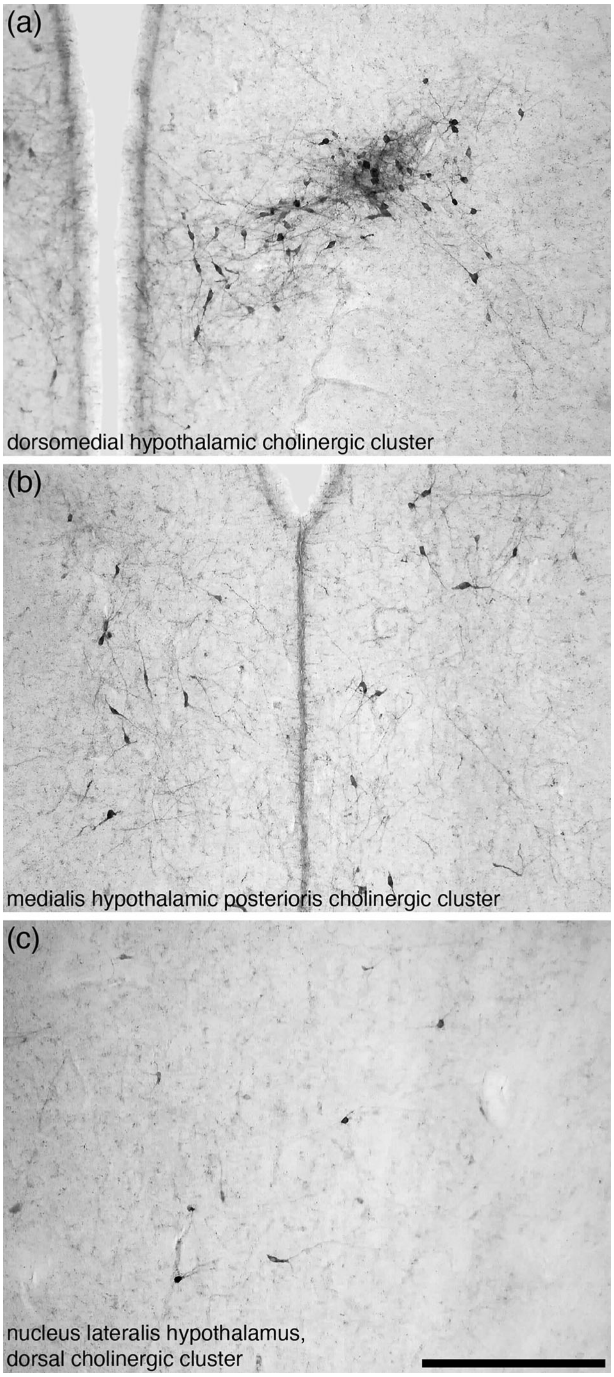

FIGURE 3 Photomicrographs of neurons in the hypothalamus of the Timneh grey parrot that were immunoreactive for the ChAT antibody used in the current study. Cholinergic neurons were observed in six clusters, of which we depict: (a) the dorsomedial hypothalamic cholinergic cluster (DMH); (b) the nucleus medialis hypothalamic posterioris cholinergic cluster (PMH); and (c) the nucleus lateralis hypothalamic, dorsal cholinergic cluster (LHyd). In all images, the dorsal is to the top and medial to the left, apart from (b) where the midline is found in the center of the image. Scale bar in (c) = 250 μm and applies to all.

occasional multipolar neurons were observed (Figures 2c, 3b, 4c). The dendrites of these PMH neurons were primarily oriented in a dorsoventral plane (Figures 2c, 3b, 4c). The appearance of PMH did not appear to vary markedly between the species. Additional ChAT+ neurons were observed within the ventromedial aspect of the hypothalamus, in the tuberal (or arcuate) region, and in the median eminence that we term the tuberal/arcuate hypothalamic cholinergic cluster (Tu) and the cholinergic cluster of the median eminence (ME) (Figure 1a–d). The ChAT+ neurons of both the Tu and ME were less intensely immunostained than those described above, with distinct dendrites emanating from the soma not being clearly stained (Figure 2e,f). The ChAT+ neurons assigned to the Tu formed a distinct, relatively high-density, cluster extending from the wall of the third ventricle across the hypothalamus to the medial aspect of the ventrolateral edge of the hypothalamus (Figure 2e). Within the ME, the ChAT+ neurons were observed in a very low relative density (Figure 2f). The appearance of the Tu and ME was similar across the three species studied.

3.2 | Absence of cholinergic neurons in the hypothalami of the common ostrich and emu

In contrast to our findings in the parrots and pied crow, no ChAT+ neurons were observed in the hypothalami of the ostrich or emu (Figure 5). Despite this lack of ChAT+ hypothalamic neurons in these two birds, many ChAT+ neurons were observed in other regions of the brain, for example, the striatum mediale (Figures 5, 6a, 6b) and the oculomotor nucleus (Figure 6c, 6d), with the antibody labeling the axonal efferents of the neurons of the oculomotor nucleus (Figure 6c).

4 | DISCUSSION

Using immunohistochemical techniques with a ChAT antibody, we were able to reveal the location and clustered organization of cholinergic neurons in five avian species of which this system has not been investigated previously. The current study was specifically focused on the question of whether cholinergic neurons were present in the hypothalamus of the ostrich. This focus is due to the unusual expression of sleep in the ostrich (Lesku et al., 2011), which has several similarities to that observed in monotremes (Siegel et al., 1996, 1998, 1999) that lack cholinergic neurons in the hypothalamus (Manger et al., 2002). While in three species—the Congo grey and Timneh grey parrots, and the pied crow—hypothalamic cholinergic neurons were clearly present, no convincing evidence for the presence of these hypothalamic cholinergic neurons was observed in either the ostrich or the closely related emu. It should be noted here that we generalize the results from one individual to the entire species, thus we temper our discussion with this potential source of error in mind.

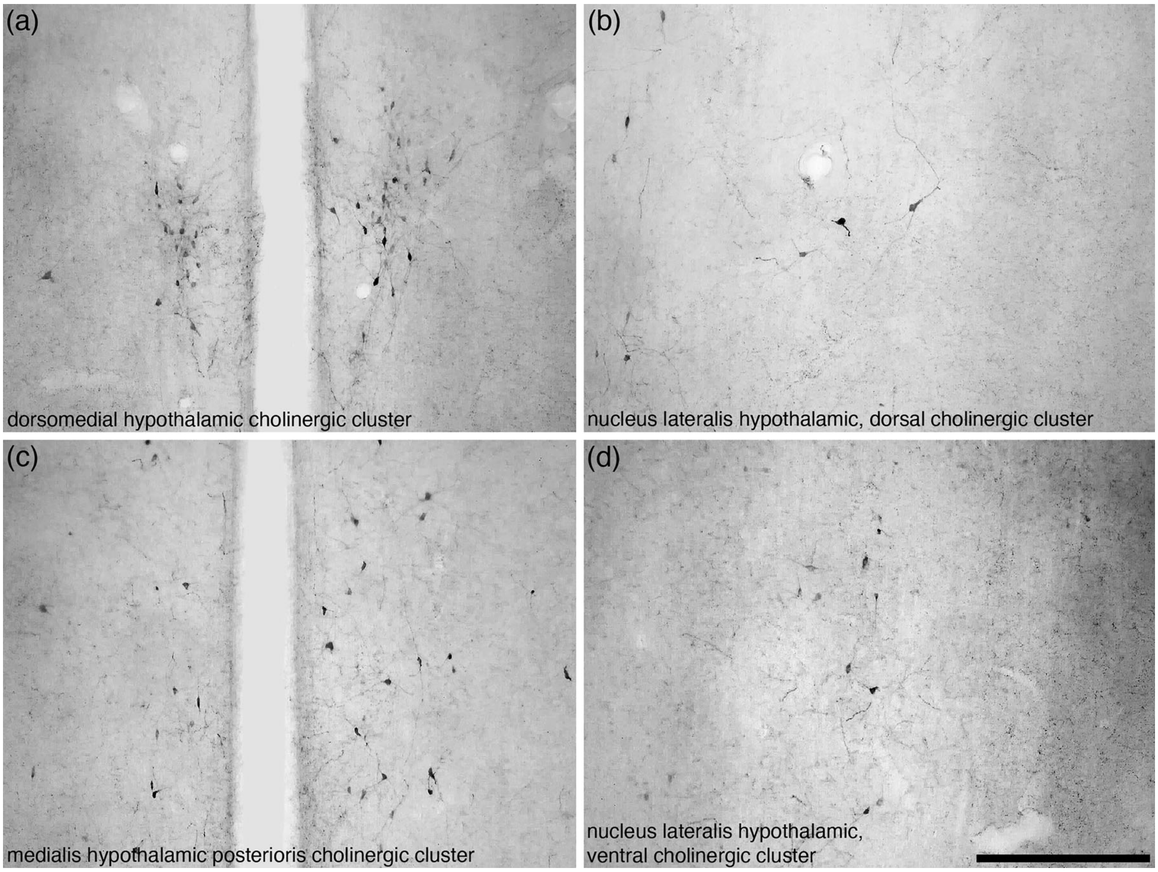

FIGURE 4 Photomicrographs of neurons in the hypothalamus of the pied crow that were immunoreactive for the ChAT antibody used in the current study. Cholinergic neurons were observed in six clusters, of which we depict: (a) the dorsomedial hypothalamic cholinergic cluster (DMH); (b) the nucleus lateralis hypothalamic, dorsal cholinergic cluster (LHyd); (c) the nucleus medialis hypothalamic posterioris cholinergic cluster (PMH); and (d) the nucleus lateralis hypothalamic, ventral cholinergic cluster (LHyv). In (a) and (b), the midline is found in the center of the image; in (c) and (d), the dorsal is to the top and medial to the left. Scale bar in (d) = 250 μm and applies to all.

4.1 | Comparison of hypothalamic cholinergic nuclei in birds

Although hypothalamic cholinergic neurons have been noted previously in pigeons (Medina & Reiner, 1994), budgerigar (Roberts et al., 2002), and chicken (González-Cabrera et al., 2015), these have only been fully mapped and parcellated in the pigeon. The current study adds data regarding the presence and parcellation of hypothalamic cholinergic neurons from five further avian species. Hypothalamic cholinergic neurons appear to be absent from the ostrich and emu, and these species are discussed further below. The description of the hypothalamic cholinergic clusters in the parrots and crow in the current study, despite some variations, is similar to that previously reported in the pigeon (Medina & Reiner, 1994). The PMH cluster appears to be a consistent feature of the avian species studied, based on location and preferential dendritic orientation. The DMH cluster observed in the parrots and crow in the current study, which is differentiated from the PMH cluster based on the location, lack of a predominant dendritic orientation, and extensive cholinergic neuropil staining, was not noted in the pigeon (Medina & Reiner, 1994). Thus, this cholinergic cluster may be a novel feature of the parrots and crow; alternatively, it may not be as strongly "expressed" in the pigeon compared to the parrots and crow (this nucleus is most distinct in the Congo grey parrot) and was thus not differentiated from the PMH by Medina and Reiner (1994).

In the pigeon, parrots, and crow, cholinergic neurons were observed in the lateral hypothalamus, although it appears that the overall number of these neurons in the pigeon was appreciably lower than observed in the parrots and crow, with the Congo grey parrot appearing to have the greatest number of cholinergic neurons in the lateral hypothalamus of the species studied to date. In addition, in the parrots and crow, we could parcellate the cholinergic neurons of the lateral hypothalamus into dorsal and ventral cholinergic clusters, based on location and predominant dendritic orientation, that was not noted in the pigeon (Medina & Reiner, 1994). However, in the pigeon it was noted that cholinergic neurons were observed in the SCI and the SCE, which we did not specifically define in the current study of the parrots and crow as these cholinergic neurons appear to be a caudal continuation of the lateral hypothalamic cholinergic neurons.

The last hypothalamic region noted in the current study to have cholinergic neurons was the tuberal/arcuate region and the occasional neuron in the median eminence, which appear to correspond

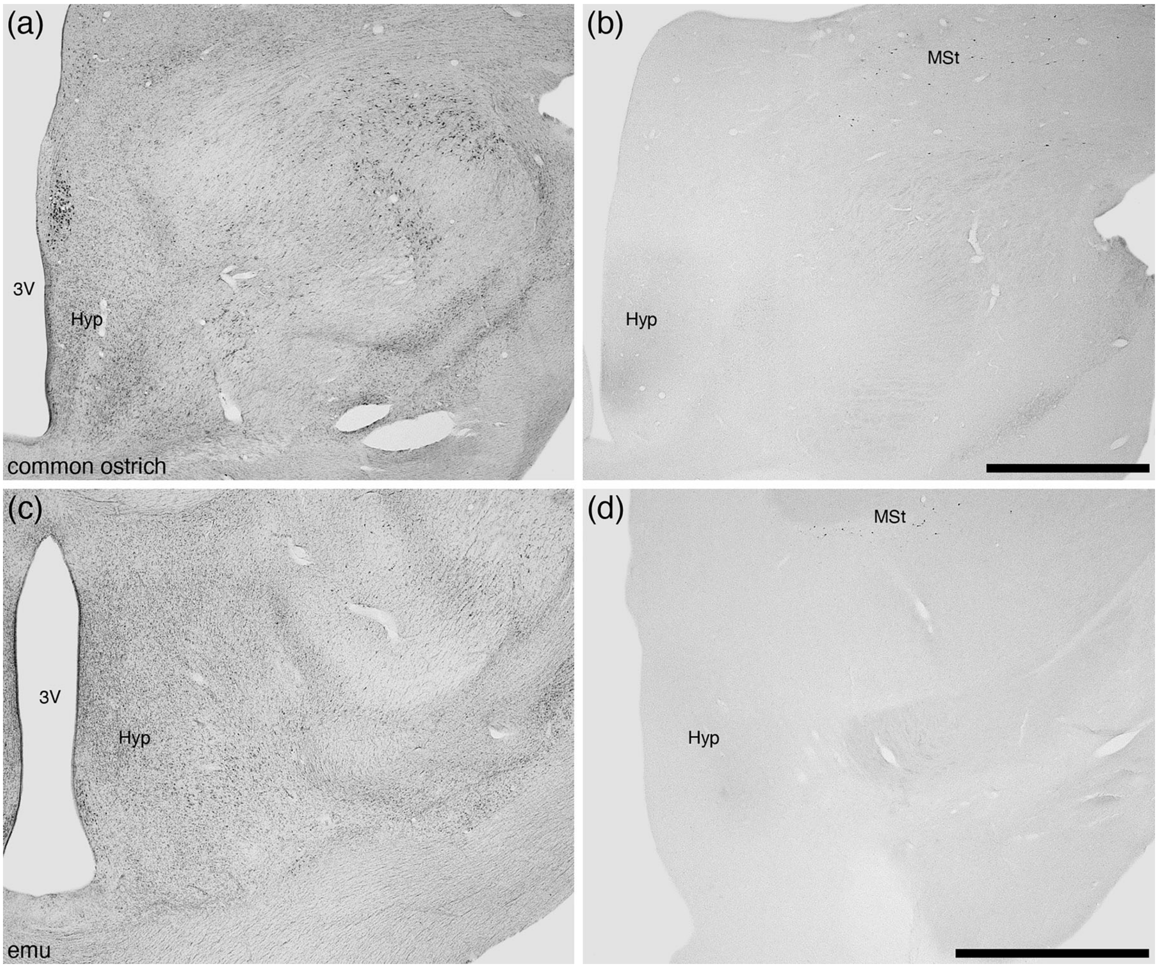

FIGURE 5 Low magnification photomicrographs of Nissl (a, c) and ChAT (b, d) stained sections through the hypothalamus (Hyp) of the common ostrich (a, b) and emu (c, d). Despite the absence of ChAT immunoreactive neurons in the hypothalamus of both species, ChAT+ neurons were observed in the adjacent striatum mediale (MSt). In all images, the dorsal is to the top and medial to the left. Scale bar in (b) = 2 mm and applies to (a) and (b). Scale bar in (d) = 2 mm and applies to (c) and (d). third ventricle (3 V).

directly with that observed in the nucleus preoptic medialis (POM) and infundibular/tuberal region of the pigeon (Medina & Reiner, 1994). Thus, broadly speaking, the organization of the hypothalamic cholinergic system is quite similar across the avian species studied, but there does appear to be a differential expression of the number of cholinergic neurons in the lateral hypothalamus and at the dorsal portion of the PMH (that we term the DMH cholinergic cluster), especially so in the Congo grey parrot.

4.2 | Absence of hypothalamic cholinergic neurons in ostrich and emu: Methodological issues

In the current study, we used the AB144P anti-ChAT goat polyclonal antibody (Merck-Millipore; RRID AB_2079751). It is possible that the target antigen of this antibody may not be detectable using immunohistochemical techniques in the hypothalamus of the ostrich and emu, as the transferase enzyme housing the antigen may be folded in such a way as to physically prevent the antibody from binding to the antigen site. Alternatively, it is possible that the AB144P antibody lacks specificity for the intended purpose. Despite these possibilities, several lines of evidence support the conclusion that the antibody, and methodology used, support the observation of a lack of cholinergic neurons in the hypothalami of the ostrich and emu.

The first line of evidence supporting this conclusion is that the cholinergic neurons in the rest of the brain of the ostrich and emu were labeled by this antibody, often occurring in the same sections as the hypothalamus. Second, omission of the primary antibody led to no staining of cholinergic neurons in any region of the ostrich or emu brain (as well as in the parrots and pied crow). Third, the possible folding of the transferase enzyme to prevent binding of the antibody to the antigen site would need to only occur within the cholinergic neurons of the hypothalamus and not have occurred in other regions of the ostrich and emu brain, which appears unlikely. Fourth, the antibody clearly revealed cholinergic neurons in the hypothalami of the two parrots and pied crows studied herein with exactly the same techniques and solutions. Fifth, this antibody has revealed hypothalamic cholinergic neurons in a broad range of mammals (e.g., Dell et al., 2010) as

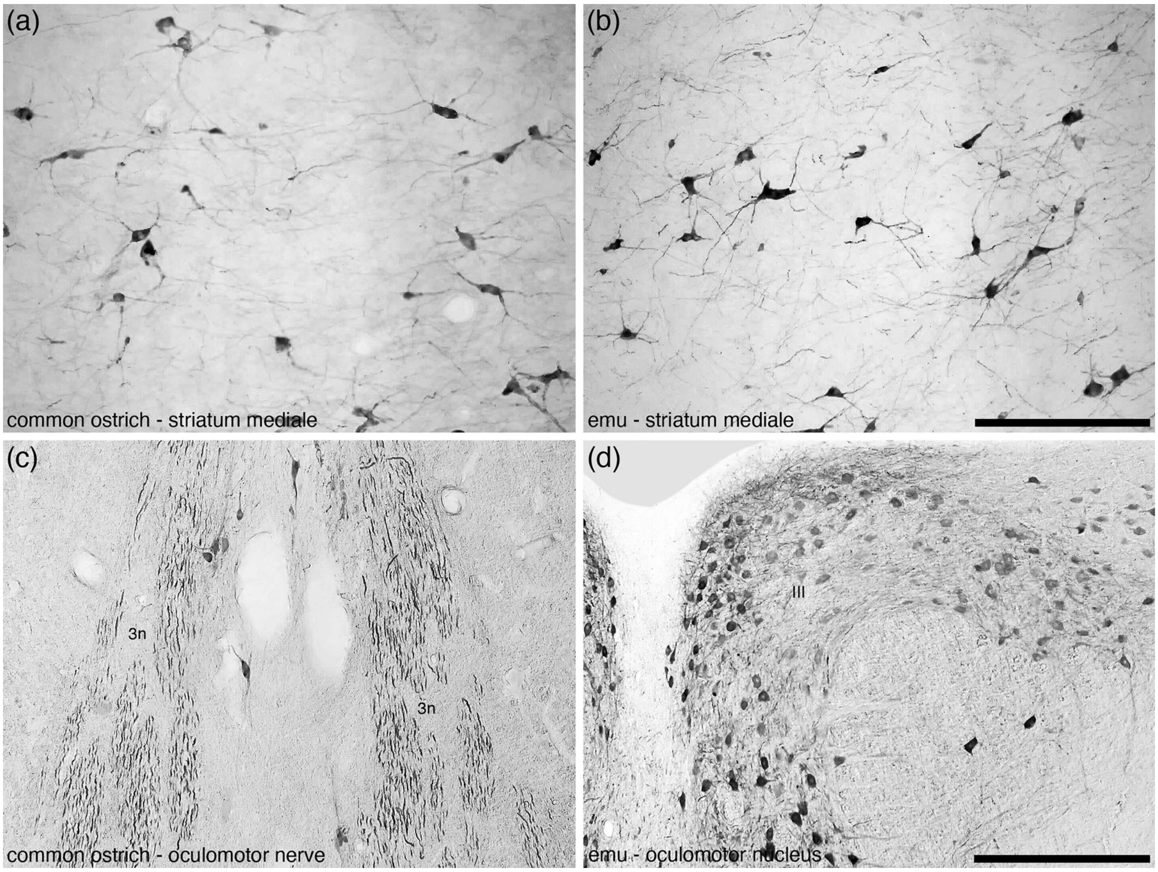

FIGURE 6 Photomicrographs of neurons and axons in the (a, b) striatum mediale, (c) oculomotor nerve, and (d) oculomotor nucleus in the ostrich (a, c) and emu (b, d) that were immunoreactive for the ChAT antibody used in the current study. No cholinergic neurons were observed in the hypothalamus of either the ostrich or emu but were clearly labeled by the antibody in other regions of the brain (also see Figure 5). In the case of the cholinergic neurons of the striatum mediale depicted here (a, b), these were taken from the same sections in which no immunostaining was evident in the hypothalamus, acting as an internal control. In all images, the dorsal is to the top and medial to the left, apart from (c) where the midline is found in the center of the image. Scale bar in (b) = 250 μm and applies to (a) and (b). Scale bar in (d) = 500 μm and applies to (c) and (d) oculomotor nucleus (III); oculomotor nerve (3n).

well as many distantly related vertebrates, including lamprey (*Lampetra fluviatilis* and *Petromyzon marinus*) (Pombal et al., 2001), the Senegal bichir (*Polypterus senegalus*) (López et al., 2013), the African clawed frog (*Xenopus laevis*) (López et al., 2002), and chicken (González-Cabrera et al., 2015), indicating that this antibody reliably identifies hypothalamic cholinergic neurons across a broad range of vertebrate species. When these lines of evidence are combined, it appears reasonable to conclude that hypothalamic cholinergic neurons are indeed absent in the ostrich and emu.

4.3 | Absence of hypothalamic cholinergic neurons in common ostrich and emu: Potential functional sequelae

If we accept that cholinergic neurons are indeed absent from the hypothalamus in the ostrich and emu, this must have functional consequences. As indicated earlier, the ostrich is known to have an unusual sleep physiology (Lesku et al., 2011) that has similarities to that observed in monotremes (Siegel et al., 1996, 1998, 1999). Another commonality between these very distantly related species is the apparent absence of hypothalamic cholinergic neurons (Manger et al., 2002; current study). In general, during sleep in both mammals and birds, the entire brain is found to be in the same state, be it non-REM or REM sleep. In contrast to this, in both monotremes and ostriches, the forebrain and hindbrain can be in different states, with the forebrain exhibiting the physiology of non-REM sleep while the hindbrain appears to be in REM sleep.

Woolf (1991) has proposed that the cholinergic neurons, having dendrodendritic, axodendritic, and gap-junction connections between their distal dendrites, have the potential to form a globally organized system. This globally organized aspect of the cholinergic system may potentially be used to derive and maintain global brain states, such as those typically seen during sleep and wake in birds and mammals. However, a hiatus in this global cholinergic system, such as the absence of hypothalamic cholinergic neurons seen in monotremes, the ostrich and emu, may then allow for two different "global" states to be present simultaneously in the forebrain and the hindbrain. Thus, a hypothalamic hiatus in the cholinergic system may be the anatomical indicator/correlate of the unusual sleep states observed in the ostrich and monotremes, although it should be noted that the sleep electrophysiology of ostriches and monotremes is not identical, as ostrich show periods of single and dual "global" brain states (Lesku et al., 2011). It is possible that this variation in ostriches may be mediated by a different, currently undetermined, neural system. Interestingly, the tinamou (*Eudromia elegans*), a close relative of the ostrich and emu, does not show the unusual sleep states observed in the ostrich (Tisdale et al., 2017). Thus, one might predict that in the tinamou, the hypothalamic cholinergic neurons will be present, a prediction that can be tested when appropriately prepared tinamou brains become available. In addition, it may be predicted that emus will sleep like ostriches, another prediction that can be readily tested when emus are available for polysomnographic recordings.

Another example of an unusual cholinergic system and variations in the expression of sleep is found in the rock hyrax (*Procavia capensis*) (Gravett et al., 2009, 2012). While not having different sleep states occurring simultaneously in different parts of the brain, hyrax expresses an unusual sleep state, *somnus innominatus*, that shows a mixture of non-REM and REM sleep physiology across the entire brain. This underscores the importance of the cholinergic system in the generation of global brain states. These comparative anatomical and sleep studies have the potential to provide supportive evidence for the global cholinergic concept proposed by Woolf (1991). If this global cholinergic concept is supported by additional comparative anatomical and sleep studies in species that are not regularly studied, this will provide a very important framework for our understanding of one broad function of the cholinergic system and possible dysfunctions associated with the global cholinergic system.

AUTHOR CONTRIBUTION

PM, JAL, NRC, and PRM conceptualized the study. PM and PRM obtained and prepared the brains used in this study. PM and PRM performed the staining and analysis. PM and PRM wrote the initial draft of the manuscript, with all authors contributing to the editing and improvement of the early drafts of the manuscript. All authors had full access to all data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis.

ACKNOWLEDGMENTS

This work was supported by Ajman University Research Grant: 2022-IRG-MED-3 (PM).

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.

ORCID

Pedzisai Mazengenya https://orcid.org/0000-0003-4733-1563
Paul R. Manger https://orcid.org/0000-0002-1881-2854

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/cne.25587.

REFERENCES

Brauth, S. E., Heaton, J. T., Roberts, T. F., & Liang, W. (2011) *Budgerigar brain atlas*. http://www.brauthlab.umd.edu/atlas.htm

Canavan, S. V., & Margoliash, D. (2020). Budgerigars have complex sleep structure similar to that of mammals. *PLoS Biology*, *18*, e3000929. https://doi.org/10.1371/journal.pbio.3000929

Dell, L. A., Kruger, J. L., Bhagwandin, A., Jillani, N. E., Pettigrew, J. D., & Manger, P. R. (2010). Nuclear organization of cholinergic, putative catecholaminergic and serotonergic systems in the brains of two megachiropteran species. *Journal of Chemical Neuroanatomy*, *40*, 177–195. https://doi.org/10.1016/j.jchemneu.2010.05.008

González-Cabrera, C., Garrido-Charad, F., Roth, A., & Marín, G. J. (2015). The isthmic nuclei providing parallel feedback connections to the avian tectum have different neurochemical identities: Expression of glutamatergic and cholinergic markers in the chick (*Gallus gallus*). *Journal of Comparative Neurology*, *523*, 1341–1358. https://doi.org/10.1002/cne.23739

Gravett, N., Bhagwandin, A., Fuxe, K., & Manger, P. R. (2009). Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the rock hyrax, *Procavia capensis*. *Journal of Chemical Neuroanatomy*, *38*, 57–74. https://doi.org/10.1016/j.jchemneu.2009.02.005

Gravett, N., Bhagwandin, A., Lyamin, O. I., Siegel, J. M., & Manger, P. R. (2012). Sleep in the rock hyrax, *Procavia capensis*. *Brain, Behavior and Evolution*, *79*, 155–169. https://doi.org/10.1159/000335342

Imam, A., Bhagwandin, A., Ajao, M. S., & Manger, P. R. (2018). The brain of the tree pangolin (*Manis tricuspis*). V. The diencephalon and hypothalamus. *Journal of Comparative Neurology*, *527*, 2413–2439. https://doi.org/10.1002/cne.24619

Izawa, E.-I., & Watanabe, S. (2007). A stereotaxic atlas of the brain of the jungle crow (*Corvus macrorhynchos*). In S. Watanabe, M. A. Hofman, & K. G. Daigaku (Eds.), *Integration of comparative neuroanatomy and cognition* (pp. 215–273). Keio University Press.

Kaiser, A., Alexandrova, O., & Grothe, B. (2011). Urocortin-expressing olivocochlear neurons exhibit tonotopic and developmental changes in the auditory brainstem and in the innervation of the cochlea. *Journal of Comparative Neurology*, *519*, 2758–2778. https://doi.org/10.1002/cne.22650

Kuenzel, W. J., & van Tienhoven, A. (1982). Nomenclature and location of avian hypothalamic nuclei and associated circumventricular organs. *Journal of Comparative Neurology*, *206*, 293–313. https://doi.org/10.1002/cne.902060309

Laux, A., Delalande, F., Mouheiche, J., Stuber, D., van Dorsselaer, A., Bianchi, E., Bezard, E., Poisbeau, P., & Goumon, Y. (2012). Localization of endogenous morphine-like compounds in the mouse spinal cord. *Journal of Comparative Neurology*, *520*, 1547–1561. https://doi.org/10.1002/cne.22811

Lesku, J. A., & Rattenborg, N. C. (2014). Avian sleep. *Current Biology*, *24*, R12–R14. https://doi.org/10.1016/j.cub.2013.10.005

Lesku, J. A., Meyer, L. C., Fuller, A., Maloney, S. K., Dell'Omo, G., Vyssotski, A. L., & Rattenborg, N. C. (2011). Ostriches sleep like platypuses. *PLoS ONE*, *6*, e23203. https://doi.org/10.1371/journal.pone.0023203

López, J. M., Smeets, W. J. A. J., & González, A. (2002). Choline acetyltransferase immunoreactivity in the developing brain of *Xenopus laevis*. *Journal* *of Comparative Neurology*, *453*, 418–434. https://doi.org/10.1002/cne.10419

López, J. M., Perlado, J., Morona, R., Northcutt, R. G., & González, A. (2013). Neuroanatomical organization of the cholinergic system in the central nervous system of a basal actinopterygian fish, the Senegal bichir *Polypterus senegalus*. *Journal of Comparative Neurology*, *521*, 24–49. https://doi.org/10.1002/cne.23155

Manger, P. R., Fahringer, H. M., Pettigrew, J. D., & Siegel, J. M. (2002). The distribution and morphological characteristics of cholinergic cells in the brain of monotremes as revealed by ChAT immunohistochemistry. *Brain, Behavior and Evolution*, *60*, 275–297. https://doi.org/10.1159/000067195

Manger, P. R., Pillay, P., Maseko, B. C., Bhagwandin, A., Gravett, N., Moon, D. J., Jillani, N., & Hemingway, J. (2009). Acquisition of brains from the African elephant (*Loxodonta africana*): perfusion-fixation and dissection. *Journal of Neuroscience Methods*, *179*, 16–21. https://doi.org/10.1016/j.jneumeth.2009.01.001

Medina, L., & Reiner, A. (1994). Distribution of choline acetyltransferase immunoreactivity in the pigeon brain. *Journal of Comparative Neurology*, *342*, 497–537. https://doi.org/10.1002/cne.903420403

Mu, P., & Huang, Y. H. (2019). Cholinergic system in sleep regulation of emotion and motivation. *Pharmacological Research*, *143*, 113–118. https://doi.org/10.1016/j.phrs.2019.03.013

Platt, B., & Reidel, G. (2011). The cholinergic system, EEG and sleep. *Behavioral Brain Research*, *221*, 499–504. https://doi.org/10.1016/j.bbr.2011.01.017

Pombal, M. A., Marín, O., & González, A. (2001). Distribution of choline acetyltransferase-immunoreactive structures in the lamprey brain. *Journal of Comparative Neurology*, *431*, 105–126. https://doi.org/10.1002/1096-9861(20010226)431:1⟨105::AID-CNE1058⟩3.0.CO;2-P

Reiner, A., Perkel, D. J., Bruce, L. L., Butler, A. B., Csillag, A., Kuenzel, W., Medina, L., Paxinos, G., Shimizu, T., Striedter, G., Wild, M., Ball, G. F., Durand, S., Güntürkün, O., Lee, D. W., Mello, C. V., Powers, A., White, S. A., Hough, G., & Kubikova, L. (2004). Revised nomenclature for avian telencephalon and some related brainstem nuclei. *Journal of Comparative Neurology*, *473*, 377–414. https://doi.org/10.1002/cne.20118

Roberts, T. F., Hall, W. S., & Brauth, S. E. (2002). Organization of the avian basal forebrain: Chemical anatomy in the parrot (*Melopsittacus undulatus*). *Journal of Comparative Neurology*, *454*, 383–408. https://doi.org/10.1002/cne.10456

Siegel, J. M., Manger, P. R., Nienhuis, R., Fahringer, H. M., & Pettigrew, J. D. (1996). The echidna *Tachyglossus aculeatus* combines REM and non-REM aspects in a single sleep state: Implications for the evolution of sleep. *Journal of Neuroscience*, *16*, 3500–3506. https://doi.org/10.1523/JNEUROSCI.16-10-03500.1996

Siegel, J. M., Manger, P. R., Nienhuis, R., Fahringer, H. M., & Pettigrew, J. D. (1998). Monotremes and the evolution of rapid eye movement sleep. *Philosophical Transactions of the Royal Society of London B, Biological Science*, *353*, 1147–1157. https://doi.org/10.1098/rstb.1998.0272

Siegel, J. M., Manger, P. R., Nienhuis, R., Fahringer, H. M., Shalita, T., & Pettigrew, J. D. (1999). Sleep in the platypus. *Neuroscience*, *91*, 391–400. https://doi.org/10.1016/s0306-4522(98)00588-0

Tisdale, R. K., Vyssotski, A. L., Lesku, J. A., & Rattenborg, N. C. (2017). Sleep-related electrophysiology and behavior of tinamous (*Eudromia elegans*): Tinamous do not sleep like ostriches. *Brain, Behavior and Evolution*, *89*, 249–261. https://doi.org/10.1159/000475590

Woolf, N. J. (1991). Cholinergic systems in mammalian brain and spinal cord. *Progress in Neurobiology*, *37*, 475–524. https://doi.org/10.1016/0301-0082(91)90006-m

Woolf, N. J., & Butcher, L. L. (2011). Cholinergic systems mediate action from movement to higher consciousness. *Behavioural Brain Research*, *221*, 488–498. https://doi.org/10.1016/j.bbr.2009.12.046

How to cite this article: Mazengenya, P., Lesku, J. A., Rattenborg, N. C., & Manger, P. R. (2024). Apparent absence of hypothalamic cholinergic neurons in the common ostrich and emu: implications for global brain states during sleep. *Journal of Comparative Neurology*, *532*, e25587. https://doi.org/10.1002/cne.25587