Title: Serotonin expression in the song circuitry of adult male zebra finches

Ping K. Yip[1], Magdalena Schmitzberger[1], Mohammed Al-Hasan[1], Julia George[2], Elina Tripoliti[3], Adina T. Michael-Titus[1], David Clayton[2] and John V. Priestley[1]

[1]Queen Mary University of London, Barts and The London School of Medicine and Dentistry, Blizard Institute, 4 Newark St, London, E1 2AT

[2]Queen Mary University of London, School of Biological and Chemical Sciences, G.E. Fogg building, Mile End Road, London E1 4NS

[3]UCL, Institute of Neurology, Department of Movement and Clinical Neurosciences, 33 Queen Square, London, WC1N 3BG

Corresponding author:

Dr Ping K. Yip
Centre for Neuroscience, Surgery and Trauma,
Blizard Institute,
Barts and The London School of Medicine and Dentistry,
Queen Mary University of London,
London E1 2AT, U.K.

Keywords: zebra finches, serotonin, song nuclei, peri-song nuclei, immunohistochemistry, speech

Abstract
Serotonin is an important neurotransmitter of the brain, but its role in song control remains to be fully demonstrated. Using male zebra finches (*Taeniopygia guttata*) that have song learning and production capabilities, we analysed the serotonin expression levels in the song nuclei and adjacent areas (peri-song nuclei) using immunohistochemistry. Key song nuclei were identified using combinations of Hoechst, choline acetyltransferase, and a neurofilament (NN18) marker in reference to the ZEBrA atlas. Mean serotonin expression was highest in interfacial nucleus (Nif) and lower in the other song nuclei in the following order (in order of highest first): interfacial nucleus (Nif) > Area X > dorsomedial part of the intercollicular nucelus (DM) > robust nucleus of the archistriatum (RA) > lateral magnocellular nucleus of the anterior neostriatum (LMAN) > ventral respiratory group (VRG) > dorsolateral nucleus of the medial thalamus (DLM) > the nucleus HVC (proper name) > tracheosyringeal motor nucleus (nXIIts). However, the mean serotonin expression (in order of highest first) in the peri-song nuclei regions was: peri-DM > peri-nXIIts > supra-peri-HVC > peri-RA > peri-DLM > peri-area X > infra-peri-HVC > peri-VRG > peri-LMAN > peri-Nif. Interestingly, serotoninergic fibers immunostained for serotonin or the serotonin transporter can be found as a basket-like peri-neuronal structure surrounding cholinergic cell bodies, and appear to form contacts onto dopaminergic neurones. In summary, serotonin fibers are present at discrete song nuclei, and peri-song nuclei regions, which suggest serotonin may have a direct and/or modulatory role in song control.

Introduction

The role of serotonin [5-hydroxytryptamine (5-HT)], particularly in modulation rather than mediation of motor activity, has been studied extensively (Jacobs and Fornal, 1997). Although serotoninergic fibres innervate α-motor neurones and secondary motor structures, they also project to non-motor targets such as the ascending auditory system of various species, including guinea pig, cat, bush baby and bat (Hurley and Pollak, 1999, Thompson et al., 1994). Furthermore, a recent clinical study suggested the involvement of the serotonin transporter gene (*SLC6A4*) in encoding accurate subcortical speech sounds in humans (Selinger et al., 2016). However, to date, the serotonergic system has not been well studied in speech.

Songbirds, including zebra finches are one of the few non-human taxa that possess the capacity for vocal learning, which presents a useful experimental model to address the human song and speech system (Bolhuis et al., 2010, Dong and Clayton, 2009). Most notably, the forebrain of the songbird supports cognitive abilities and vocal production, which are similar to those of humans although they differ structurally (Jarvis et al., 2005). Recently, it has been shown that there is an independently evolved convergent gene expression in specific brain regions for song and speech in songbirds and humans (Pfenning et al., 2014).

The male, but not the female zebra finches exhibit a unique system of interconnected brain nuclei specialised for song production and perception, referred to as the song system (Riebel et al., 2002). Different song nuclei in the adult male zebra finches are involved in the ascending and descending pathways by which vocal stimuli are recognized and processed (Vates et al., 1996). The song system is composed of two distinct circuits, the song motor pathway and the premotor anterior forebrain pathway and both involve different groups of song nuclei (Mooney, 2009). Moreover, the auditory input to the motor and premotor pathways is provided by particular nuclei, including the interfacial nucleus (Nif), nucleus uvaeformis (Uva) and the avalanche nucleus (Av) within the caudal mesopallium (CM) (Reiner et al., 2004, Vates et al., 1996, Akutagawa and Konishi, 2005, Akutagawa and Konishi, 2010). Furthermore, researchers have also suggested surrounding or adjacent regions of certain song nuclei such as $LMAN_{shell}$ and paraHVC are important in vocal learning and production (Bottjer and Altenau, 2010, Foster and Bottjer, 1998).

Serotonin projections involved in modulating various processes in mammals could also play a comparable role in birds, such as modulating brain circuitry involved in bird song (Wood et al., 2011) or involved in regulating neuroblast migration (Garcia-Gonzalez et al., 2017). Furthermore, it has been previously shown using high performance liquid chromatography with electrochemical detection that serotonin levels are different between different vocal control nuclei of castrated male zebra finches (Barclay and Harding, 1988). Recently, it has been shown that cholinergic signaling is required for song learning (Puzerey et al., 2018) and central vocal control

(Sadananda, 2004), and the dopaminergic system in the ventral tegmental area is involved in singing-related activity (Yanagihara and Hessler, 2006). Therefore, we used immunohistochemistry to identify serotonergic fibres, and their relationship to cholinergic and dopaminergic neurons in song and adjacent areas (peri-song nuclei) of adult male zebra finches.

Methods and materials

Tissue collection

Adult male zebra finches (*Taeniopygia guttata*) used in the study were housed at a large free-flight social aviary at Queen Mary University of London with a 14 h light-10 h dark cycle, and food and water were available *ad libitum*. The animal research was performed under the UK Animals (Scientific Procedures) Act of 1986 and in accordance with the European Union regulations under Directive 2010/63/EU on the protection of animals used for scientific purposes. Zebra finches were overdosed with sodium pentobarbital, then intracardially perfused with 0.9 % saline followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer for fixation. After the brain was carefully dissected out from the skull, it was post-fixed in 4 % PFA for a further 2 h, then cryoprotected in 20 % sucrose for at least 3 days at 4 ^{o}C until further processed.

The left hemisphere of each zebra finch brain was cut serially into 20 µm thick sections in the sagittal plane from midline in the medial-lateral direction onto Trajan Series 3 slides using a cryostat. Once the brain sections were cut and dried onto the slides, they were stored at -20 ^{o}C until required.

Standard immunohistochemistry

The immunostaining procedure was carried out as previously described (Bell et al., 2019). Briefly, the slides were washed 3 x 5 minutes in 10 mM phosphate-buffered saline (PBS) then incubated for 30 minutes at 80 °C in pre-heated antigen unmasking solution (H-3300, Vector Labs) to reveal antigen. After allowing to cool down for 15 minutes at room temperature, the slides were washed 3 x 5 minutes in 10 mM PBS, then incubated with skimmed milk solution 2 % (w/v) in PBS for at least 30 minutes. Sections were incubated overnight at room temperature with primary antibodies; rabbit anti-serotonin (1:1000, Immunostar, Cat. No. 20080) and mouse anti-neurofilament 160 kDa (1:100, clone NN18, Sigma-Aldrich Cat. No. MAB5254) or goat anti-choline acetyltransferase (1:100, Millipore (UK) Ltd, Cat. No. AB144P). Next day, the slides were rinsed in 3 x 5 minutes PBS, then incubated for 2 hours at room temperature in a dark box with either donkey anti-mouse Fluor® 594 (1:500, Abcam, Cat No. ab150108) and donkey anti-rabbit Alexa Fluor® 488 (1:500, Abcam, Cat. No. ab150076) or donkey anti-goat Alexa Fluor® 594 (1:500, ThermoFisher, Cat No. A-11058). Thereafter, the sections were incubated with Hoechst

33342 (0.01 mg/ml, Sigma-Aldrich) for 5 minutes, then rinsed 3 x 5 minutes in 10 mM PBS before coverslipped with Vectashield mounting medium (Vector Labs, Cat No. H-1000).

Immunohistochemistry using tyramide signal amplification system

The tyramide signal amplification system was used to enable double immunohistochemistry to be carried out using two primary antibodies raised from the same host species (Brouns et al., 2002, Yip et al., 2019). Briefly, slides were incubated in 0.3 % hydrogen peroxide for 20 min to remove endogenous peroxidase, followed by a pre-heated antigen unmasking solution for 30 min. Thereafter, the sections were initially blocked with 2 % skimmed milk solution for 30 min, then incubated overnight with rabbit anti-serotonin (1:10,000, Immunostar, Cat. No. 20080), a dilution which the standard immunohistochemical protocol using only a fluorescent secondary antibody is not able to detect (data not included), but is detected by the very sensitive tyramide signal amplification system. The next day, slides were incubated in donkey anti-rabbit biotin (1:400, Jackson ImmunoResearch Europe Ltd, Cat No. 711-065-152) for 2 h, followed by the Avidin-Biotin Complex (Vector Labs, Vectastain ABC Elite Kit, Cat. No. PK-6100) for 30 min, then biotinyl tyramide (1:75, NEN Life Sciences, TSA Biotin Tyramide Reagent Pack, Cat. No. SAT700001EA) for 10 min exactly. ExtrAvidin® -FITC (1:400, Sigma-Aldrich, Cat. No. E2761) was added onto the slides for 2 h, before incubation with goat anti-choline acetyltransferase (1:100, Millipore (UK) Ltd, Cat. No. AB144P), or rabbit anti-tyrosine hydroxylase (1:500, Sigma-Aldrich, Cat. No. T8700), or rabbit anti-serotonin transporter (1:500, Immunostar, Cat. No. 24330) overnight. The next day, slides were incubated with donkey anti-goat Alexa Fluor® 594 (1:500, Abcam, Cat No. ab150132) or donkey anti-rabbit Alexa Fluor® 594 (1:500, Abcam, Cat No. ab150076) for 2 h. After 5 min incubation with Hoechst, slides were coverslipped with Vectashield mounting medium. It should be noted that between each step, slides were washed with 10 mM PBS at 3 x 5 min.

Microscopy

To identify the song nuclei, whole serial sagittal sections stained with Hoechst and/or immunostained for neurofilament 160 kDa (NN18) or choline acetyltransferase (ChAT) or tyrosine hydroxylase were taken at x4 magnification and merged using Photoshop CS5 Software. This information was then used to identify the location of the song nuclei in the rostrocaudal, ventrodorsal and mediolateral planes with reference to the online Zebra Finch Expression Brain Atlas (ZEBrA) (http://www.zebrafinchatlas.org/resources/songbird-neuroanatomy). Song nuclei and corresponding peri-song nuclei regions were viewed using an Axioskop-2 fluorescence microscope (Carl Zeiss, U.K.) and captured at x20 magnification using a Hamamatsu CCD digital camera (Hamamatsu) via HiPic v9.1 software. The different fluorescent staining distinctly

demarks the different song nuclei. True co-localization of serotonin and tyrosine hydroxylase within neuronal cell bodies and nerve fibers was examined using Z-stack images captured with a Zeiss LSM 710 confocal microscope and ZENlite software (Zeiss, Cambridge, UK).

Quantitative analysis

The serotonin levels in the song nuclei and peri-song nuclei regions were analysed using a customized script for the ImageJ software (National Institutes of Health, v1.52). The experimenter was blind to the serotonin expression as the imaging location was decided directly from the neurofilament 160 kDa (NN18), choline acetyltransferase (ChAT), tyrosine hydroxylase and/or Hoechst staining. Depending on the size of the song nuclei, 1 to 4 regions of interest of 200 square pixels from at least 2 sections were analysed from 3 male zebra finches. The results of the binary overlay generated by the user by thresholding the image created a number of pixels above the threshold, which were expressed as mean ± standard error of the mean (SEM) in arbitrary units. Given that the immunostaining between the different animals and different batches of immunostaining was similar, and that the microscope settings to capture the images were the same for each particular immunostaining for all animals and brain regions, no adjustments for threshold differences between different sections were carried out. Stereological analysis was not carried out as the aim was not to generate an absolute value for immunostained cell bodies and/or fibers, but to determine the relative differences across the different brain regions, using the same criteria. One-way ANOVA with post-hoc Tukey's multiple comparisons test was used to compare the relative intensity of serotonin immunoreactivity between regions (Table 1). Values of $p < 0.05$ were considered statistically significant. All statistical analyses were conducted using GraphPad Prism 7 (GraphPad Software, USA).

Results

Identification of song nuclei

Based on published literature and atlases, the following regions were considered to be involved in the major song circuitries present in the male zebra finches (Fig. 1): area X, dorsolateral nucleus of the anterior thalamus (DLM), HVC (proper name), Dorsomedial part of the intercollicular nucleus (DM), interfacial nucleus (Nif), lateral magnocellular nucleus of the anterior nidopallium (LMAN), robust nucleus of the arcopallium (RA), ventral respiratory group (VRG), ventral tegmental area (VTA), tracheosyringeal motor nucleus (nXIIts). Using the different fluorescent immunostaining of ChAT (Fig.1B-E), and immunostaining with NN18 (Fig. 1F-I) and serotonin (Fig. 1J-M), clear and distinct cytoarchitectural identification of the individual nuclei currently known to be involved in the zebra finches song circuitries were identified.

Serotonin in song nuclei

For the investigation of serotonin levels involved in song acquisition and production, different song nuclei of male zebra finches were analysed. Serotonin expression was present in some cell bodies (Fig. 2A-B), but was predominantly in the form of nerve fibers at the different levels that we analysed throughout the brain (Fig. 2C-D). The immunostaining observed was confirmed as true serotonin immunostaining based on dual immunostaining with the serotonin transporter (Fig. 3A-L). This showed that serotonin immunoreactive axons were also immunoreactive for the serotonin transporter. In an additional control, there was a lack of staining in the absence of the serotonin primary antibody (Fig. 3M-O). There were significant differences in the serotonin levels among the different song nuclei (Fig. 4A-J). The mean expression of serotonin was significantly highest in the Nif compared to other song nuclei. Similarly, DM being another nucleus in the song motor pathway also expressed high mean serotonin expression, but was not significantly higher than other song nuclei (Fig. 4A-J). However, the level of mean serotonin expression in other nuclei of the song motor pathway was low, in particularly the mesencephalic song nucleus nXIIts, which had the significantly lowest mean serotonin levels of all song nuclei. VRG was another song motor pathway nucleus that had significantly lower mean serotonin expression than Nif and Area X. The nucleus that projects directly to VRG and indirectly to the nXIIts is the RA, which has a moderate mean amount of serotonin expression. Both of the major song control nuclei, RA and HVC, were only significantly less in mean serotonin levels than Nif, with HVC having an additional reduction compared to area X, DM and RA. In the anterior forebrain pathway, area X expressed a high level of mean serotonin expression but was only significantly higher than DLM, LMAN, HVC, nXIIts, and VRG. Interestingly, the mean serotonin levels in another anterior forebrain pathway nucleus, LMAN, was lower, but not significantly different from area X. In summary, the order of mean serotonin expression (highest expression level first) was: Nif > area X > DM > RA > LMAN > VRG > DLM > HVC > nXIIts.

Serotonin expression in the peri-song nuclei

Serotonergic fibres were also observed throughout the non-song nuclei regions, in particular directly surrounding or adjacent to the song nuclei, which we have termed the peri-song nuclei (Fig. 5A-J). The level of mean serotonin expression was highest in the peri-song nucleus area surrounding the DM, a nucleus of the song motor pathway that is part of the intercollicular nucleus (ICo). It was significantly higher in serotonin expression than all the other peri-song nuclei regions analysed (Fig. 5J). The next highest mean serotonin expression was at the peri-nXIIts, which was also part of the song motor pathway. it was significantly different in mean serotonin expression from the other regions, apart from the region directly above the HVC termed

supra-peri-HVC, peri-RA and peri-DM song nuclei. Interestingly, the region directly below the HVC termed infra-peri-HVC, was significantly lower in mean serotonin expression than peri-DM, peri-nXIIts, and supra-peri-HVC. The peri-song regions surrounding the other three song nuclei involved in the song motor pathway namely VRG and Nif, expressed low levels of mean serotonin expression. Peri-VRG and peri-Nif were all significantly lower in mean serotonin levels than peri-DM, peri-nXIIts, supra-peri-HVC, and peri-area x. The other peri-song nuclei regions within the anterior forebrain pathway at the rostral brain region were low in mean serotonin levels, such that peri-area X and peri-LMAN were both significantly lower than peri-DM, peri-nXIIts, and supra-peri-HVC, with the addition of peri-RA for peri-LMAN. In summary, the order of mean serotonin expression (highest expression level first) was: peri-DM > peri-nXIIts > supra-peri-HVC > peri-RA > peri-DLM > peri-area X > infra-peri-HVC > peri-VRG > peri-LMAN > peri-Nif.

Interaction of serotonergic and cholinergic neurones in brainstem

Choline acetyltransferase (ChAT) is a marker used to identify cholinergic neurones that have been studied in the selective vocal motor system in zebra finches (Zuschratter and Scheich, 1990). Therefore, double-immunofluorescence staining of serotonin and ChAT will enable the identification of serotonin innervation of cholinergic cells, and of serotonin coexistence with acetylcholine. One particular brain area of interest in the zebra finches was the brain stem, which contains large clusters of cholinergic neurones (Fig. 6). At the nXIIts, there was limited serotonin immunoreactivity in cell bodies that strongly expressed ChAT immunostaining (Fig. 6A - D). In contrast, there was strong serotonin expression at the lingual division of the hypoglossal nucleus, with the formation of a basket-like peri-neuronal structure surrounding ChAT immunopositive cell bodies (Fig. 6E). A similar, but less strong basket-like peri-neuronal serotonin staining was also observed at the nucleus supraspinalis (Fig. 6F). Interestingly, regions of serotonin-labelled cell bodies or nerve fibers around nXIIts and nucleus supraspinalis had no co-localisation with ChAT immunopositive staining (Fig. 6G-J''). In summary, the data suggest that serotonergic fibers can innervate directly the cell bodies of cholinergic neurones in selected brain regions.

Co-localization of serotonin within dopaminergic neurones in the song nuclei

Serotonin and dopamine are synthesised in neurones via simple two-step pathways from their precursors tyrosine and tryptophan, respectively (Fernstrom, 1990). Tyrosine hydroxylase is a key enzyme to convert tyrosine to L-Dopa, a precursor for dopamine, which enables this marker to be used in the identification of dopaminergic neurones. Interestingly, it has been shown that serotonin can accumulate in dopaminergic neurones (Mossner et al., 2006, Zhou et al., 2002) and L-Dopa-derived dopamine can be detected within serotonergic fibers (Yamada et al., 2007). Therefore, it would be of interest to study the co-localization of serotonin and tyrosine hydroxylase labelling within fibres and/or cell bodies in the song nuclei (Fig. 7).

The mean percentage of serotonin and tyrosine hydroxylase co-localization was highest in the ventral tegmental area (VTA), a region enriched in dopaminergic cell bodies involved in the mesocorticolimbic projection (Fig. 7A, K). Although the co-expression in VTA was higher than in all the song nuclei, the mean co-expression of serotonin and tyrosine hydroxylase was only 7.5 % (Fig. 7K, 9A). Furthermore, serotonin fibers formed bouton-like contacts onto dopaminergic cell bodies (Fig. 9C-F). Within the song nuclei, the RA and LMAN had the largest mean percentage co-expression of serotonin and tyrosine hydroxylase, albeit low at 1.1%, which was significantly different from the anterior and posterior nXIIts and VRG. Posterior-nXIIts had the lowest mean co-expression of serotonin and tyrosine hydroxylase staining compared to the other song nuclei, with a value of 0.1 %. Other song nuclei within the anterior forebrain pathway, including Area X and DLM, were non-significantly lower than LMAN. There was no significant difference from other song nuclei, including Nif, DM and HVC located within the song motor pathway. In summary, the order of mean percentage serotonin and tyrosine hydroxylase co-localization expression in the song nuclei (highest expression level first) was: RA > LMAN > DLM > Nif > DM > HVC > area X > anterior-nXIIts > VRG > posterior-nXIIts.

Co-labelled serotonin and tyrosine hydroxylase neurones within the peri-song nuclei

In contrast to the VTA, the peri-VTA had only 0.2 % co-expression of serotonin and tyrosine hydroxylase compared to the anterior-peri-nXIIts with the highest value of 2.8 % (Fig. 8A & K, 9B). The peri-anterior nXIIts had significantly higher mean co-expression of serotonin and tyrosine hydroxylase compared to all peri-song nuclei, except posterior-peri-nXIIts and peri-VRG. The lowest mean co-expression of serotonin and tyrosine hydroxylase was at the peri-Nif, but was not significantly different from the peri-DM within the song motor pathway. Within the anterior forebrain pathway, peri-DLM had the highest mean co-expression of serotonin and tyrosine hydroxylase, but was not significantly different from the peri-area X and peri-LMAN. The peri-song nuclei areas around the major song control nuclei RA and HVC, did not express significant differences in the mean percentage of serotonin and tyrosine hydroxylase co-expression. In summary, the order of mean serotonin and tyrosine hydroxylase co-localization in the peri-song nuclei (highest expression level first) was: anterior-peri-nXIIts > posterior-peri-nXIIts > peri-VRG > peri-DM > peri-DLM > supra peri-HVC > peri-RA > infra peri-HVC > peri-areaX > peri-LMAN > peri-Nif.

Discussion

The purpose of this study was to identify the serotonergic system within the song and peri-song nuclei that are involved in the development and production of bird song. Therefore, the mean

expression of serotonin in the brain stem and the co-expression of serotonin and tyrosine hydroxylase distribution were examined. The results indicated significant differences in the local density of serotonergic cell bodies and nerve fibres across different brain regions of the healthy adult male zebra finches. Moreover, a general trend of high serotonin in the song nuclei is associated with a low mean serotonin expression in the peri-song nuclei regions, and vice versa. Interestingly, the mean serotonin expression in the song and peri-song nuclei regions of the song motor pathway were greater than in the anterior forebrain circuitry. Results from this study have also shown that a low degree of coexistence of serotonin within dopaminergic neurones occurs in the songbird, most notably in the midbrain song nucleus and the brainstem peri-song nuclei areas.

Serotonin plays a role in motor and sensory processing in birds, such as modulating the brain circuitry involved in bird song, as seen in the raphe nucleus (brainstem) of mammals. Serotonergic fibres are important for learning and memory, neural development, perception, as well as the modulation of anxiety, mood, and sleep (Wood et al., 2011). Therefore, the importance of the serotonergic system is indicated by being conserved through a wide variety of vertebrates, including mammals and avian species (Wood et al., 2011). Mounting evidence suggests that serotonin plays an essential role in associative learning in rodents, with a dominant role of the serotonin receptors in this process (Harvey, 2003, Williams et al., 2002). In this study, a widespread distribution of labelled serotonergic fibres and neurones was observed in precise clusters in both the motor and pre-motor pathways, and surrounding tissues. Similarly, different serotonin levels in song nuclei have been previously demonstrated in castrated male zebra finches using the HPLC technique (Barclay and Harding, 1988). Barclay and Harding (1988) showed the order of mean serotonin expression (highest expression level first) was: DM > Nif > HVC = RA > LMAN > area X. Our study revealed mean serotonin expression (highest expression level first) was: Nif > DM > area X > RA > LMAN >HVC. Therefore, the two studies showed agreement regarding DM and Nif, which contained the highest serotonin level. However, there is a difference concerning HVC and area X. The reason is not known, but the difference could be due to the effect of castration, as altered serotonin levels were demonstrated in castrated dogs (Salavati et al., 2018).

This current study and the study by Barclay and Harding (1988) demonstrated that DM and Nif had the highest mean serotonin expression in the song nuclei. Nif is the first site of auditory gating and provides the source of input to the HVC (Akutagawa & Konishi 2005). A recent study has shown using neuroanatomical tract-tracing that the uvaeform nucleus (Uva) projects to the Nif and then continues to the HVC (Akutagawa & Konishi 2005). The intercollicular nucleus (ICo) is the equivalent to the mammalian inferior colliculus and is considered as a vocalization area found in a variety of bird species (Seller 1981, Kingsbury, Kelly 2011). Although the telencephalic

vocal control nucleus RA projects to the DM and nXIIts (Vicario, 1991), the moderate mean serotonin expression within the RA only correlates with the moderate serotonin expression observed in the DM, but not the low serotonin expression in the nXIIts. Although the reason is unclear for the latter, one possible explanation may be that serotonin is more concentrated within the fibers rather than the cell bodies. It has been shown that serotonin within serotonergic cells is readily transported from cell bodies into nerves of *Aplysia* by selective transport (Goldman and Schwartz, 1974). However, sparse clusters of serotonin immunopositive cell bodies were observed in selected zebra finch brain regions, especially in the midbrain region. At the peri-song nuclei, the highest mean serotonin expression was observed at the peri-DM and peri-nXIIts. Interestingly, the peri-DM that consists of the ICo also receives input from the RA (Vicario, 1991). Furthermore, the peri-nXIIts containing the suprahypoglossal area also receives projections from the RA in catbirds, which has been suggested to be involved with the expiratory motor neurones within the thoracolumbar spinal cord (Wild et al., 2000). However, the other peri-song nuclei in zebra finches with serotonin fibers terminating at these locations are currently not known. RA plays an essential role in the central regulation of the learned bird song, and it is involved in the control of the avian vocal organ via nXIIts (Abarbanel et al., 2004, Spiro et al., 1999). A recent study using *in vitro* single- unit and whole-cell electrophysiology observed that serotonin can exert a powerful excitatory stimulation on projection neurons of RA (Wood et al., 2011). The projection neurons in RA are considered equivalent to the mammalian layer V cortical pyramidal neurons, which are similarly excited by serotonin via the activation of 5-HT2 receptors as demonstrated using *in situ* hybridisation and pharmacological manipulations (Wood et al., 2011). Furthermore, they demonstrated that RA projecting neurones express the HTR2A and HTR2C, but not HTR2B serotonin receptor genes (Wood et al., 2011). Data from the zebra finch atlas website also showed that HTR2A and HTR2C gene expression were strongly expressed in other song nuclei, including area X and DLM, respectively (www.zebrafinchatlas.org/gene_display). According to our study, area X contains a moderate amount of serotonin expression, which suggests that the serotonin fibers in this basal ganglia structure may play a role in motor exploration for vocal learning (Hisey et al., 2018, Kojima et al., 2018, Xiao et al., 2018). Interestingly, the 5-HT2A agonist TCB-2, and the 5-HT2C agonist WAY 161,503 can increase and decrease motor activity, respectively (Halberstadt and Geyer, 2010, Halberstadt et al., 2013, Wolf and Schutz, 1997). Data from the zebra finch atlas website showed that HTR2B gene is expressed moderately by the HVC and is weakly distributed throughout the mesopallium (www.zebrafinchatlas.org/gene_display/HTR2B).

Serotonin in cholinergic neurones within the brain stem of zebra finches

This study has demonstrated that serotonin neurones do not co-express ChAT, but the serotonin fibers can make distinct basket-like formations around ChAT positive cell bodies. Although the

reason for this interaction is not known, it is possibly indicative of of serotonin modulation of cholinergic neurones. Recently, it has been demonstrated that serotonin can attenuate the cholinergic neuronal activity in rodent brains (Luebke et al., 1992, Sparks et al., 2017). Therefore, given that the cholinergic system is involved in the central vocal control, the serotonin system could modulate vocal acquisition, retention and motor control of song production (Sadananda, 2004).

Serotonin in dopaminergic neurones within the zebra finches song system

Several studies have reported the aberrant accumulation of serotonin in dopaminergic neurons (Mossner et al., 2006, Zhou et al., 2002) and of L-DOPA-derived dopamine within serotonergic fibers (Reiner et al., 2004). Data from this study suggests that all song nuclei and peri-song nuclei regions express limited serotonin within dopaminergic neurones. However, a quantitative analysis of the level of serotonin co-localised with tyrosine hydroxylase showed that this percentage, although low, differs considerably between various brain areas. The highest percentage (7.5%) of serotonin and tyrosine hydroxylase co-expression was observed in the midbrain nucleus VTA. The VTA in mammals and the songbird midbrain contain homologous dopaminergic neurons that project diffusely to telencephalic brain regions and play crucial roles in learning, motivation, and motor control (Gale et al., 2008). Interestingly, the peri-nXIIts and peri-VRG regions express the highest tyrosine hydroxylase and serotonin co-localisation ranging from 2.2 - 2.8%. It is likely that in the brain of normal zebra finches, there is limited dopamine biosynthesis in serotonergic neurones, and this is more likely to be in axonal terminals as shown in figure 9C-F. Although the role of serotoninergic fibers on the dopaminergic system is not known, it has been demonstrated that serotonin can potentiate dopamine inhibition of VTA in rat brain (Brodie and Bunney, 1996). Dopamine released by the dopaminergic terminals of the VTA-area X pathway influences information processing in the forebrain by altering the input and output functions of the medium spiny neurons (Ding and Perkel, 2004).

In summary, our results showed that various song nuclei and peri-song nuclei have different serotonin expression. Since serotonin expression was higher within the song motor pathway than the anterior forebrain pathway, this suggests that serotonin is involved in song production rather than song learning and plasticity (Moore et al., 2011). Furthermore, as the immunostaining is predominantly in fibers, it suggests they are from projection neurones of other song nuclei, such as RA projecting into the DM and peri-nXIIts. In summary, serotonin protein and genes in selective song and peri-song nuclei highlight the importance of the serotonergic system via a direct and/or modulatory role in song production in songbirds.

Acknowledgements

The authors would like to thank Dr Belén Martín-Martín of BALM facility for their assistance with the confocal microscope and ZENlite software.

Contributions

The authors have made the following declarations about their contributions: Conceived and designed the experiments: PKY, JVP. Performed the experiments and analysed the data: PKY, MS, JG. Contributed reagents/materials/analysis tools: PKY, DC. Wrote the paper: PKY, MS, JG, ET, AMT, DC, JVP.

Funding

No funding was received towards this work.

Competing financial interests

The authors declare no competing financial interests.

References

ABARBANEL, H. D., GIBB, L., MINDLIN, G. B. & TALATHI, S. 2004. Mapping neural architectures onto acoustic features of birdsong. *J Neurophysiol,* 92**,** 96-110.

AKUTAGAWA, E. & KONISHI, M. 2005. Connections of thalamic modulatory centers to the vocal control system of the zebra finch. *Proc Natl Acad Sci U S A,* 102**,** 14086-91.

AKUTAGAWA, E. & KONISHI, M. 2010. New brain pathways found in the vocal control system of a songbird. *J Comp Neurol,* 518**,** 3086-100.

BARCLAY, S. R. & HARDING, C. F. 1988. Androstenedione modulation of monoamine levels and turnover in hypothalamic and vocal control nuclei in the male zebra finch: steroid effects on brain monoamines. *Brain Res,* 459**,** 333-43.

BELL, Z. W., LOVELL, P., MELLO, C. V., YIP, P. K., GEORGE, J. M. & CLAYTON, D. F. 2019. Urotensin-related gene transcripts mark developmental emergence of the male forebrain vocal control system in songbirds. *Sci Rep,* 9**,** 816.

BOLHUIS, J. J., OKANOYA, K. & SCHARFF, C. 2010. Twitter evolution: converging mechanisms in birdsong and human speech. *Nat Rev Neurosci,* 11**,** 747-59.

BOTTJER, S. W. & ALTENAU, B. 2010. Parallel pathways for vocal learning in basal ganglia of songbirds. *Nat Neurosci,* 13**,** 153-5.

BRODIE, M. S. & BUNNEY, E. B. 1996. Serotonin potentiates dopamine inhibition of ventral tegmental area neurons in vitro. *J Neurophysiol,* 76**,** 2077-82.

BROUNS, I., VAN NASSAUW, L., VAN GENECHTEN, J., MAJEWSKI, M., SCHEUERMANN, D. W., TIMMERMANS, J. P. & ADRIAENSEN, D. 2002. Triple immunofluorescence staining with antibodies

raised in the same species to study the complex innervation pattern of intrapulmonary chemoreceptors. *J Histochem Cytochem,* 50**,** 575-82.
DING, L. & PERKEL, D. J. 2004. Long-term potentiation in an avian basal ganglia nucleus essential for vocal learning. *J Neurosci,* 24**,** 488-94.
DONG, S. & CLAYTON, D. F. 2009. Habituation in songbirds. *Neurobiol Learn Mem,* 92**,** 183-8.
FERNSTROM, J. D. 1990. Aromatic amino acids and monoamine synthesis in the central nervous system: influence of the diet. *J Nutr Biochem,* 1**,** 508-17.
FOSTER, E. F. & BOTTJER, S. W. 1998. Axonal connections of the high vocal center and surrounding cortical regions in juvenile and adult male zebra finches. *J Comp Neurol,* 397**,** 118-38.
GALE, S. D., PERSON, A. L. & PERKEL, D. J. 2008. A novel basal ganglia pathway forms a loop linking a vocal learning circuit with its dopaminergic input. *J Comp Neurol,* 508**,** 824-39.
GARCIA-GONZALEZ, D., KHODOSEVICH, K., WATANABE, Y., ROLLENHAGEN, A., LUBKE, J. H. R. & MONYER, H. 2017. Serotonergic Projections Govern Postnatal Neuroblast Migration. *Neuron,* 94**,** 534-549 e9.
GOLDMAN, J. E. & SCHWARTZ, J. H. 1974. Cellular specificity of serotonin storage and axonal transport in identified neurones of Aplysia californica. *J Physiol,* 242**,** 61-76.
HALBERSTADT, A. L. & GEYER, M. A. 2010. LSD but not lisuride disrupts prepulse inhibition in rats by activating the 5-HT(2A) receptor. *Psychopharmacology (Berl),* 208**,** 179-89.
HALBERSTADT, A. L., POWELL, S. B. & GEYER, M. A. 2013. Role of the 5-HT(2)A receptor in the locomotor hyperactivity produced by phenylalkylamine hallucinogens in mice. *Neuropharmacology,* 70**,** 218-27.
HARVEY, J. A. 2003. Role of the serotonin 5-HT(2A) receptor in learning. *Learn Mem,* 10**,** 355-62.
HISEY, E., KEARNEY, M. G. & MOONEY, R. 2018. A common neural circuit mechanism for internally guided and externally reinforced forms of motor learning. *Nat Neurosci,* 21**,** 589-597.
HURLEY, L. M. & POLLAK, G. D. 1999. Serotonin differentially modulates responses to tones and frequency-modulated sweeps in the inferior colliculus. *J Neurosci,* 19**,** 8071-82.
JACOBS, B. L. & FORNAL, C. A. 1997. Serotonin and motor activity. *Curr Opin Neurobiol,* 7**,** 820-5.
JARVIS, E. D., GUNTURKUN, O., BRUCE, L., CSILLAG, A., KARTEN, H., KUENZEL, W., MEDINA, L., PAXINOS, G., PERKEL, D. J., SHIMIZU, T., STRIEDTER, G., WILD, J. M., BALL, G. F., DUGAS-FORD, J., DURAND, S. E., HOUGH, G. E., HUSBAND, S., KUBIKOVA, L., LEE, D. W., MELLO, C. V., POWERS, A., SIANG, C., SMULDERS, T. V., WADA, K., WHITE, S. A., YAMAMOTO, K., YU, J., REINER, A., BUTLER, A. B. & AVIAN BRAIN NOMENCLATURE, C. 2005. Avian brains and a new understanding of vertebrate brain evolution. *Nat Rev Neurosci,* 6**,** 151-9.
KOJIMA, S., KAO, M. H., DOUPE, A. J. & BRAINARD, M. S. 2018. The Avian Basal Ganglia Are a Source of Rapid Behavioral Variation That Enables Vocal Motor Exploration. *J Neurosci,* 38**,** 9635-9647.
LUEBKE, J. I., GREENE, R. W., SEMBA, K., KAMONDI, A., MCCARLEY, R. W. & REINER, P. B. 1992. Serotonin hyperpolarizes cholinergic low-threshold burst neurons in the rat laterodorsal tegmental nucleus in vitro. *Proc Natl Acad Sci U S A,* 89**,** 743-7.
MOONEY, R. 2009. Neurobiology of song learning. *Curr Opin Neurobiol,* 19**,** 654-60.
MOORE, J. M., SZEKELY, T., BUKI, J. & DEVOOGD, T. J. 2011. Motor pathway convergence predicts syllable repertoire size in oscine birds. *Proc Natl Acad Sci U S A,* 108**,** 16440-5.
MOSSNER, R., SIMANTOV, R., MARX, A., LESCH, K. P. & SEIF, I. 2006. Aberrant accumulation of serotonin in dopaminergic neurons. *Neurosci Lett,* 401**,** 49-54.
PFENNING, A. R., HARA, E., WHITNEY, O., RIVAS, M. V., WANG, R., ROULHAC, P. L., HOWARD, J. T., WIRTHLIN, M., LOVELL, P. V., GANAPATHY, G., MOUNCASTLE, J., MOSELEY, M. A., THOMPSON, J. W., SODERBLOM, E. J., IRIKI, A., KATO, M., GILBERT, M. T., ZHANG, G., BAKKEN, T., BONGAARTS, A., BERNARD, A., LEIN, E., MELLO, C. V., HARTEMINK, A. J. & JARVIS, E. D. 2014. Convergent

transcriptional specializations in the brains of humans and song-learning birds. *Science,* 346**,** 1256846.

PUZEREY, P. A., MAHER, K., PRASAD, N. & GOLDBERG, J. H. 2018. Vocal learning in songbirds requires cholinergic signaling in a motor cortex-like nucleus. *J Neurophysiol,* 120**,** 1796-1806.

REINER, A., PERKEL, D. J., MELLO, C. V. & JARVIS, E. D. 2004. Songbirds and the revised avian brain nomenclature. *Ann N Y Acad Sci,* 1016**,** 77-108.

RIEBEL, K., SMALLEGANGE, I. M., TERPSTRA, N. J. & BOLHUIS, J. J. 2002. Sexual equality in zebra finch song preference: evidence for a dissociation between song recognition and production learning. *Proc Biol Sci,* 269**,** 729-33.

SADANANDA, M. 2004. Acetylcholinesterase in central vocal control nuclei of the zebra finch (Taeniopygia guttata). *J Biosci,* 29**,** 189-200.

SALAVATI, S., MOGHEISEH, A., NAZIFI, S., SHOJAEE TABRIZI, A., TAHERI, P. & KOOHI, F. 2018. Changes in sexual hormones, serotonin and cortisol concentrations following oral administration of melatoninin in castrated and intact dogs. *J. Vet. Behav.,* 27**,** 27-34.

SELINGER, L., ZARNOWIEC, K., VIA, M., CLEMENTE, I. C. & ESCERA, C. 2016. Involvement of the Serotonin Transporter Gene in Accurate Subcortical Speech Encoding. *J Neurosci,* 36**,** 10782-10790.

SPARKS, D. W., TIAN, M. K., SARGIN, D., VENKATESAN, S., INTSON, K. & LAMBE, E. K. 2017. Opposing Cholinergic and Serotonergic Modulation of Layer 6 in Prefrontal Cortex. *Front Neural Circuits,* 11**,** 107.

SPIRO, J. E., DALVA, M. B. & MOONEY, R. 1999. Long-range inhibition within the zebra finch song nucleus RA can coordinate the firing of multiple projection neurons. *J Neurophysiol,* 81**,** 3007-20.

THOMPSON, G. C., THOMPSON, A. M., GARRETT, K. M. & BRITTON, B. H. 1994. Serotonin and serotonin receptors in the central auditory system. *Otolaryngol Head Neck Surg,* 110**,** 93-102.

VATES, G. E., BROOME, B. M., MELLO, C. V. & NOTTEBOHM, F. 1996. Auditory pathways of caudal telencephalon and their relation to the song system of adult male zebra finches. *J Comp Neurol,* 366**,** 613-42.

VICARIO, D. S. 1991. Organization of the zebra finch song control system: II. Functional organization of outputs from nucleus Robustus archistriatalis. *J Comp Neurol,* 309**,** 486-94.

WILD, J. M., WILLIAMS, M. N. & SUTHERS, R. A. 2000. Neural pathways for bilateral vocal control in songbirds. *J Comp Neurol,* 423**,** 413-26.

WILLIAMS, G. V., RAO, S. G. & GOLDMAN-RAKIC, P. S. 2002. The physiological role of 5-HT2A receptors in working memory. *J Neurosci,* 22**,** 2843-54.

WOLF, W. A. & SCHUTZ, L. J. 1997. The serotonin 5-HT2C receptor is a prominent serotonin receptor in basal ganglia: evidence from functional studies on serotonin-mediated phosphoinositide hydrolysis. *J Neurochem,* 69**,** 1449-58.

WOOD, W. E., LOVELL, P. V., MELLO, C. V. & PERKEL, D. J. 2011. Serotonin, via HTR2 receptors, excites neurons in a cortical-like premotor nucleus necessary for song learning and production. *J Neurosci,* 31**,** 13808-15.

XIAO, L., CHATTREE, G., OSCOS, F. G., CAO, M., WANAT, M. J. & ROBERTS, T. F. 2018. A Basal Ganglia Circuit Sufficient to Guide Birdsong Learning. *Neuron,* 98**,** 208-221 e5.

YAMADA, H., AIMI, Y., NAGATSU, I., TAKI, K., KUDO, M. & ARAI, R. 2007. Immunohistochemical detection of L-DOPA-derived dopamine within serotonergic fibers in the striatum and the substantia nigra pars reticulata in Parkinsonian model rats. *Neurosci Res,* 59**,** 1-7.

YANAGIHARA, S. & HESSLER, N. A. 2006. Modulation of singing-related activity in the songbird ventral tegmental area by social context. *Eur J Neurosci,* 24**,** 3619-27.

YIP, P. K., BOWES, A. L., HALL, J. C. E., BURGUILLOS, M. A., IP, T. R., BASKERVILLE, T., LIU, Z. H., MOHAMED, M., GETACHEW, F., LINDSAY, A. D., NAJEEB, S. U., POPOVICH, P. G., PRIESTLEY, J. V. & MICHAEL-

TITUS, A. T. 2019. Docosahexaenoic acid reduces microglia phagocytic activity via miR-124 and induces neuroprotection in rodent models of spinal cord contusion injury. *Hum Mol Genet*.
ZHOU, F. C., LESCH, K. P. & MURPHY, D. L. 2002. Serotonin uptake into dopamine neurons via dopamine transporters: a compensatory alternative. *Brain Res,* 942**,** 109-19.
ZUSCHRATTER, W. & SCHEICH, H. 1990. Distribution of choline acetyltransferase and acetylcholinesterase in the vocal motor system of zebra finches. *Brain Res,* 513**,** 193-201.

Figure 1: Song circuitries of the male zebra finches.

(A) Schematic diagram represents sagittal sections (~0.0 mm and ~1.7 mm from midline) of a male zebra finch brain, illustrating the pathways and their main respective song circuitries based on published literature and atlases. The song system is composed of the anterior forebrain pathway (blue) and song motor pathway (red), which receive input from the auditory nuclei (green). The VTA-areaX pathway (yellow) contains a rich dopaminergic cell group in the VTA that influences song learning and production. The auditory nuclei consist of the Uva, Nif and Av. Area X, DLM, LMAN are the main song nuclei of the anterior forebrain pathway. The main song nuclei for the song motor pathway are HVC, RA, DM, nXIIts, and VRG. Activation of the nXIIts results in the movement of the syrinx and respiratory muscles (purple) to generate bird song. The arrows indicate the direction of neuronal innervation. Abbreviations: Av, avalanche nucleus; DLM, dorsal lateral nucleus of the anterior thalamus; HVC, proper name; DM, dorsomedial part of the intercollicular nucleus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; Nif, interfacial nucleus; nXIIts, hypoglossal motor nucleus; RA, robust nucleus of the arcopallium; Uva, nucleus uvaeformis; VRG, ventral respiratory group; VTA, ventral tegmental area. (B-M) Certain song nuclei can be distinctively identified using ChAT (B-E), NN18 (F-I) or serotonin (J-M). Scale bar 100 mm.

Figure 2. Serotonergic neurones in the brain of adult male zebra finches.

(A-D) Sagittal brain sections were immunostained with serotonin. (A-B) Serotonin immunostained cell bodies in insets are a higher magnification of dashed boxes. (C-D) Serotonin immunostained fibers in insets are a higher magnification of dashed boxes. Images represent sagittal planes at approximately A) 0.5 mm, B) 1.3 mm, C) 2.0 mm, and D) 2.7 mm from the midline. Scale bar = 1 mm.

Figure 3. Serotonin co-immunostaining with serotonin transporter in song nuclei and peri-song nuclei of adult male zebra finches.

(A-I) Representative images of various brain regions immunostained with serotonin (green) and serotonin transporter (red). (A-I) Strong co-expression of serotonin and serotonin transporter demonstrating presence of serotoninergic fibers (arrows) in the HVC and supra-peri-HVC (A-C), DM and peri-DM (D-F), and anterior peri-nXIIts (G-I). (J-L) Low expression of serotonin and serotonin transporter was observed in the nXIIts. (M-O) In the absence of any serotonin primary antibody, there was no positive immunostaining in areas containing rich serotoninergic fibers such as in the supra-peri-HVC as demonstrated with the presence of serotonin transporter immunostained fibers (arrowheads). Scale bar is 50 μm. Orientation of brain section is D = dorsal, V = ventral, P = posterior and A = anterior. See figure 1 legend for abbreviations.

Figure 4. Serotonin expression in song nuclei of adult male zebra finches.

(A-I) Representative images of various song nuclei immunostained with serotonin (green). In the song nuclei of the anterior forebrain pathway. There was a significantly lower serotonin expression observed in the area X (A), DLM (B), LMAN (C) HVC (D), RA (E) when compared to Nif (F). The song nuclei of the song motor pathway contained the highest serotonin expression in Nif (F), with lower in DM (G) and VRG (I), but the lowest of all song nuclei in nXIIts (H). (J) N = 3 zebra finch brains were studied for each song nuclei. Statistical analysis was carried out using one-way ANOVA followed by post hoc Tukey's test. Significance was indicated with: **** $p < 0.0001$ in comparison to Nif; †† $p < 0.01$, ††† $p < 0.001$, †††† $p < 0.0001$ in comparison to area X; $^{\#\#}$ $p < 0.01$, $^{\#\#\#\#}$ $p < 0.0001$ in comparison to DM; ^ $p < 0.05$, ^^^^ $p < 0.0001$ in comparison to RA; $^{\&\&\&}$ $p < 0.001$ in comparison to LMAN, ^{aa} $p < 0.01$ in comparison to DLM; ^{bb} $p < 0.01$ in comparison to VRG, and ^{c} $p < 0.05$ in comparison to HVC. Three zebra finches with at least two sections for each song nuclei per animal were analysed. Scale bar is 100 µm. Orientation of brain section is D = dorsal, V = ventral, P = posterior and A = anterior. See figure 1 legend for abbreviations.

Figure 5. Serotonin expression in peri-song nuclei of adult male zebra finches.

(A-I) Representative images of various peri-song nuclei regions immunostained with serotonin (green). In the peri-song nuclei regions of the anterior forebrain pathway, there was a significantly lower serotonin expression observed in the peri-area X (A), peri-DLM (C), peri-LMAN (C), infra peri-HVC (D), supra-peri-HVC (D'), and peri-RA (E) when compared to peri-DM (G). The peri-song nuclei regions of the song motor pathway containing serotonin expression were lowest in peri-Nif (F) and highest in peri-DM (G), with moderately high in peri-nXIIts (H) and low in peri-VRG (I). (J) Statistical analysis was carried out using one-way ANOVA followed by post hoc Tukey's test. Significance was indicated with **** $p < 0.0001$ in comparison to peri-DM; † $p < 0.05$, ††† $p < 0.001$, †††† $p < 0.0001$ in comparison to peri-nXIIts; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, $^{\#\#\#\#}$ $p < 0.0001$ in comparison to supra-peri-HVC; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$ in comparison to peri-RA. Three zebra finches with at least two sections for each song nuclei per animal were analysed. Scale bar is 100 µm. Orientation of brain section is D = dorsal, V = ventral, P = posterior and A = anterior. See figure 1 legend for abbreviations.

Figure 6. Various forms of interaction between serotoninergic and cholinergic neurones in the brainstem of zebra finches.

Choline acetyltransferase (ChAT) (A & C, red) and serotonin (B & C, green) immunopositive cell bodies and nerve fibers were observed in the brain stem of adult male zebra finches. Insets are areas of high magnification, shown in the dashed boxes in corresponding panels. (D-D'') Strong ChAT immunostained cell bodies with limited serotonin (arrows) immunostaining. (E-E'') Strong

ChAT immunostained cell bodies surrounded in a basket-like formation of strong serotonin (arrows) immunostained fibers. (F-F'') Strong ChAT immunostained cell bodies surrounded in a basket-like formation of moderate serotonin (arrows) immunostained fibers. (G-G'') Limited ChAT immunostaining with strong serotonin (arrows) immunostained fibers. (H-H'') Weak ChAT immunostained cell bodies and fibers are separate from strong serotonin immunostained cell bodies (arrows) and nerve fibers. (I-I'') Strong ChAT and serotonin immunostained fibers with limited coexpression. (J-J'') Weak ChAT and weak serotonin immunostained fibers. Scale bars in A-C are 100 mm, and D-J, D'-J' and D''-J'' are 50 mm. See figure 1 legend for abbreviations.

Figure 7. Serotonin immunostaining within dopaminergic neurones in song nuclei of adult male zebra finches.

(A-I) Representative images of various brain regions immunostained with serotonin (green) and tyrosine hydroxylase (red). (A) In the VTA, a rich dopaminergic nucleus demonstrated some co-expression of serotonin and tyrosine hydroxylase. (B-J) In the song nuclei of the zebra finches brain, low co-expression of serotonin and tyrosine hydroxylase was observed in the area X (B), DLM (C), LMAN (D), HVC (E), RA (F), Nif (G), DM (H), anterior nXIIts (I), posterior nXIIts (I') and VRG (J). (K) Analysis within the song nuclei showed significantly higher co-expression of serotonin within dopaminergic neurones in the RA and LMAN than the anterior and posterior nXIIts, and VRG. Statistical analysis was carried out using one-way ANOVA followed by post hoc Tukey's test. Significance was indicated with * $p < 0.05$, ** $p < 0.01$ in comparison to RA, and † $p < 0.05$, †† $p < 0.01$ in comparison to LMAN. Scale bar is 50 µm. Orientation of brain section is D = dorsal, V = ventral, P = posterior and A = anterior. See figure 1 legend for abbreviations.

Figure 8. Serotonin immunostaining within dopaminergic neurones in peri-song nuclei of adult male zebra finches.

(A-J) Representative images of various peri-nuclear brain regions immunostained with serotonin (green) and tyrosine hydroxylase (red). (A) In the peri-VTA, dopaminergic fibers have minimal co-expression of serotonin and tyrosine hydroxylase. (B-F) In the peri-song nuclei of the anterior forebrain pathway, there was a significantly lower co-expression of serotonin and tyrosine hydroxylase observed in the peri-area X (B), peri-DLM (C), peri-LMAN (D), supra-peri-HVC (E), Infra-peri-HVC (E'), peri-RA (F), peri-Nif (G), peri-DM (H), in comparison to anterior peri-nXIIts (I), posterior peri-nXIIts (I') and peri-VRG (J). (K) Highest co-expression of serotonin and tyrosine hydroxylase was observed in the peri-song nuclei: anterior peri-nXIIts, posterior peri-nXIIts, and peri-VTG with no statistical difference between these regions. Statistical analysis was carried out using one-way ANOVA followed by post hoc Tukey's test. Significance was indicated with: *** $p < 0.001$, **** $p < 0.0001$, in comparison to anterior peri-nXIIts; †† $p < 0.01$, ††† $p < 0.001$ in comparison to posterior peri-nXIIts, and $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ in comparison to peri-VRG.

Scale bar is 50 μm. Orientation of brain section is D = dorsal, V = ventral, P = posterior and A = anterior. See figure 1 legend for abbreviations.

Figure 9. Expression of serotonin within dopaminergic neurones in the VTA of adult male zebra finches.

(A-B) Z-stack confocal microscope images of serotonin (green) and tyrosine hydroxylase (red) immunostaining at the VTA (A) and peri-VTA (B). Co-expression of serotonin and tyrosine hydroxylase reveals a yellow colour in some nerve fibers (arrow). (C-F) Serotonin fibers (green) formed bouton-like connections onto a tyrosine hydroxylase positive cell body (white arrows) and dendrites (white arrowheads) at different z planes. Scale bars for A-B are 50 μm and for C-F are 25 mm.

Fig.1

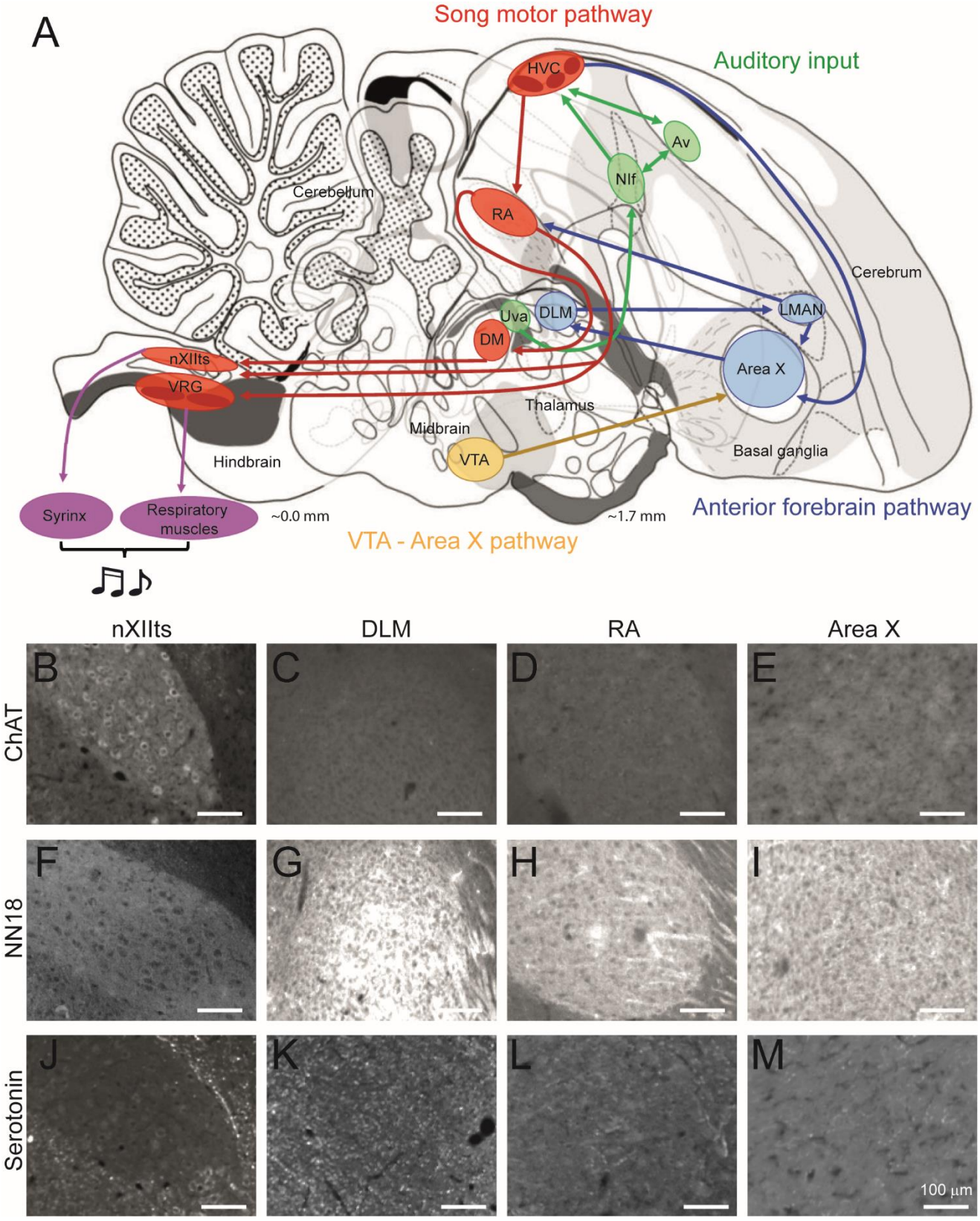

A
Song motor pathway
Auditory input
HVC
Av
NIf
RA
Cerebellum
Cerebrum
Uva
DLM
LMAN
DM
nXIIts
VRG
Area X
Thalamus
Midbrain
VTA
Hindbrain
Basal ganglia
Syrinx
Respiratory muscles
~0.0 mm
~1.7 mm
Anterior forebrain pathway
VTA - Area X pathway
nXIIts
DLM
RA
Area X
ChAT
NN18
Serotonin
B
C
D
E
F
G
H
I
J
K
L
M
100 µm

Fig. 2

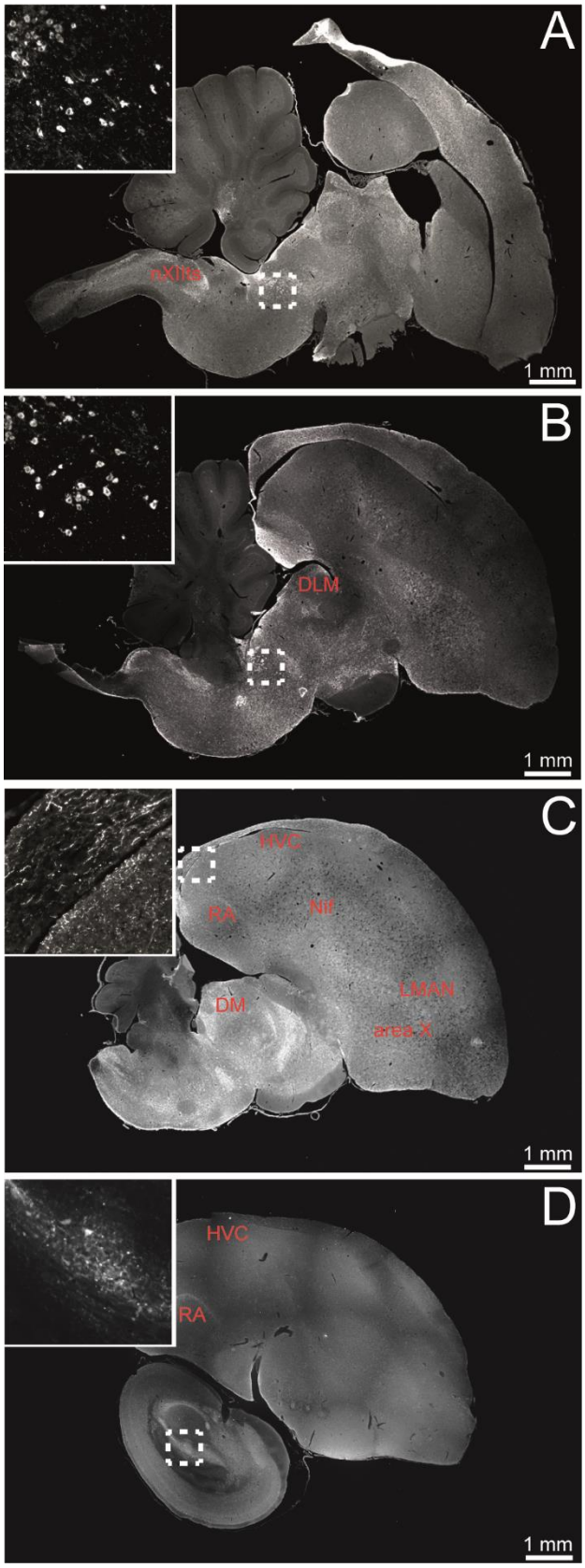


Fig. 3

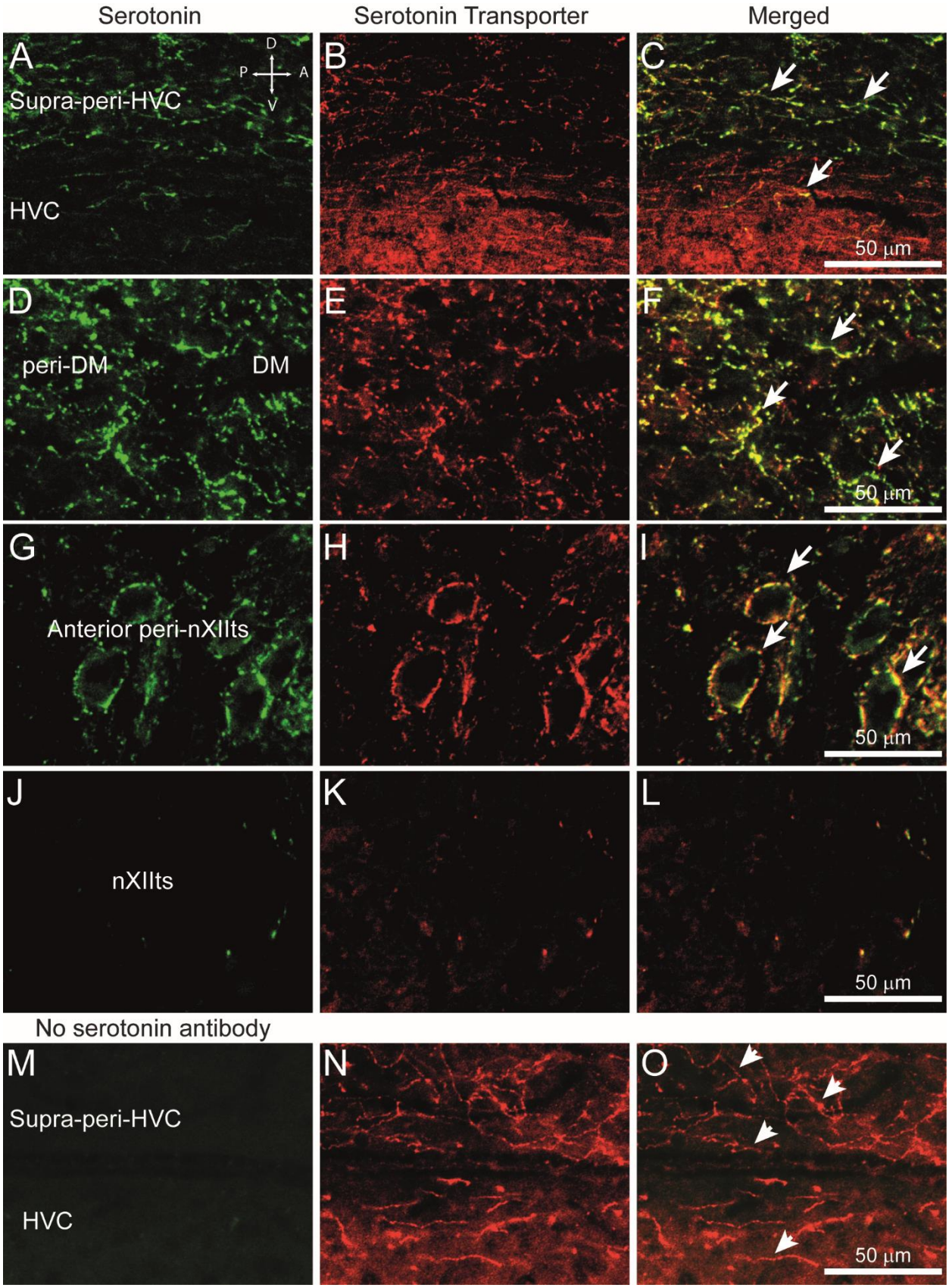
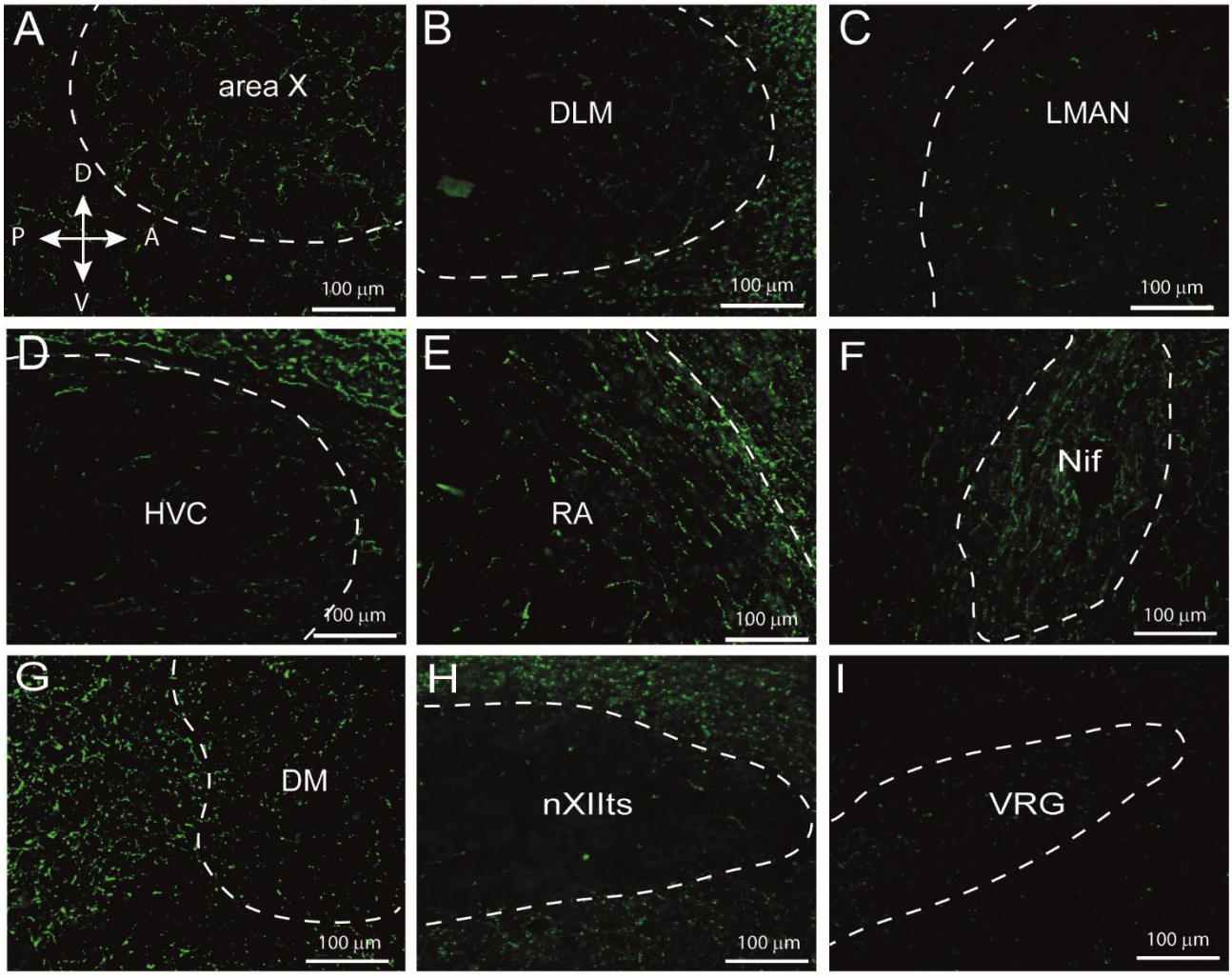

Serotonin
Serotonin Transporter
Merged
A
Supra-peri-HVC
HVC
D
P
A
V
B
C
50 μm
D
peri-DM
DM
E
F
50 μm
G
Anterior peri-nXIIts
H
I
50 μm
J
nXIIts
K
L
50 μm
No serotonin antibody
M
Supra-peri-HVC
HVC
N
O
50 μm

Fig. 4


A
area X
D
P
A
V
100 µm
B
DLM
100 µm
C
LMAN
100 µm
D
HVC
100 µm
E
RA
100 µm
F
Nif
100 µm
G
DM
100 µm
H
nXIIts
100 µm
I
VRG
100 µm

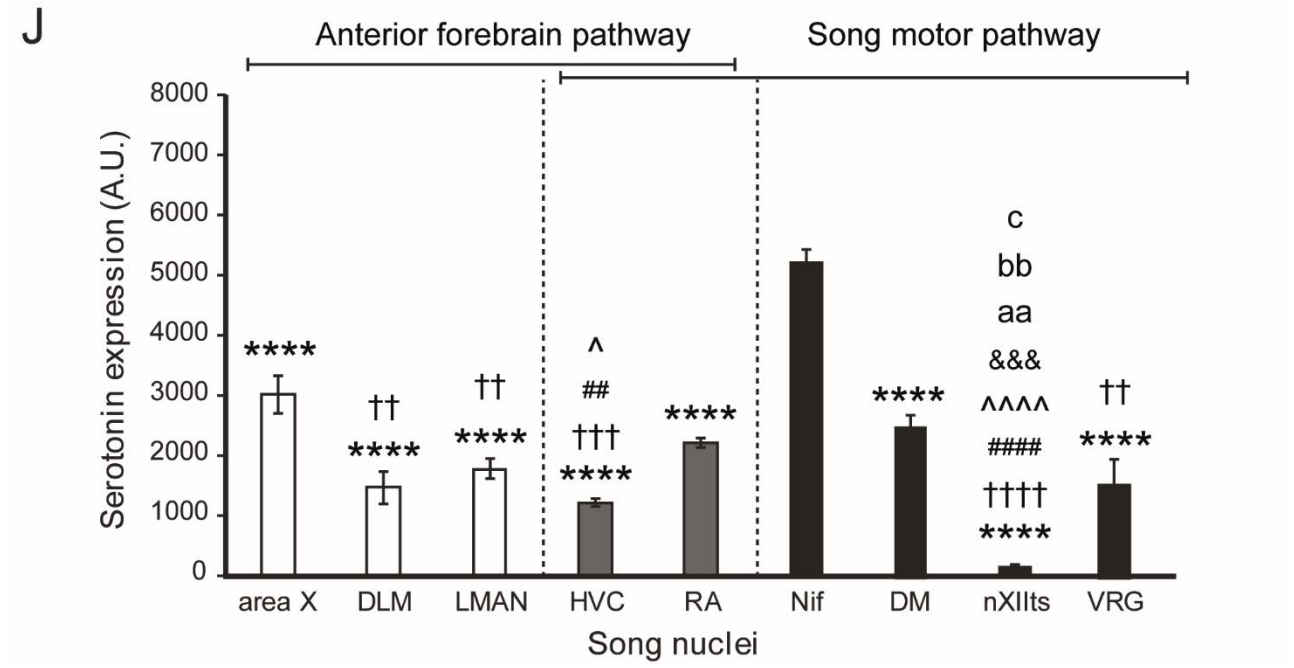

J
Anterior forebrain pathway
Song motor pathway
Serotonin expression (A.U.)
8000
7000
6000
5000
4000
3000
2000
1000
0
****
††
****
††
****
^
##
†††
****
****
****
c
bb
aa
&&&
^^^^
####
††††
****
††
****
area X
DLM
LMAN
HVC
RA
Nif
DM
nXIIts
VRG
Song nuclei

Fig. 5

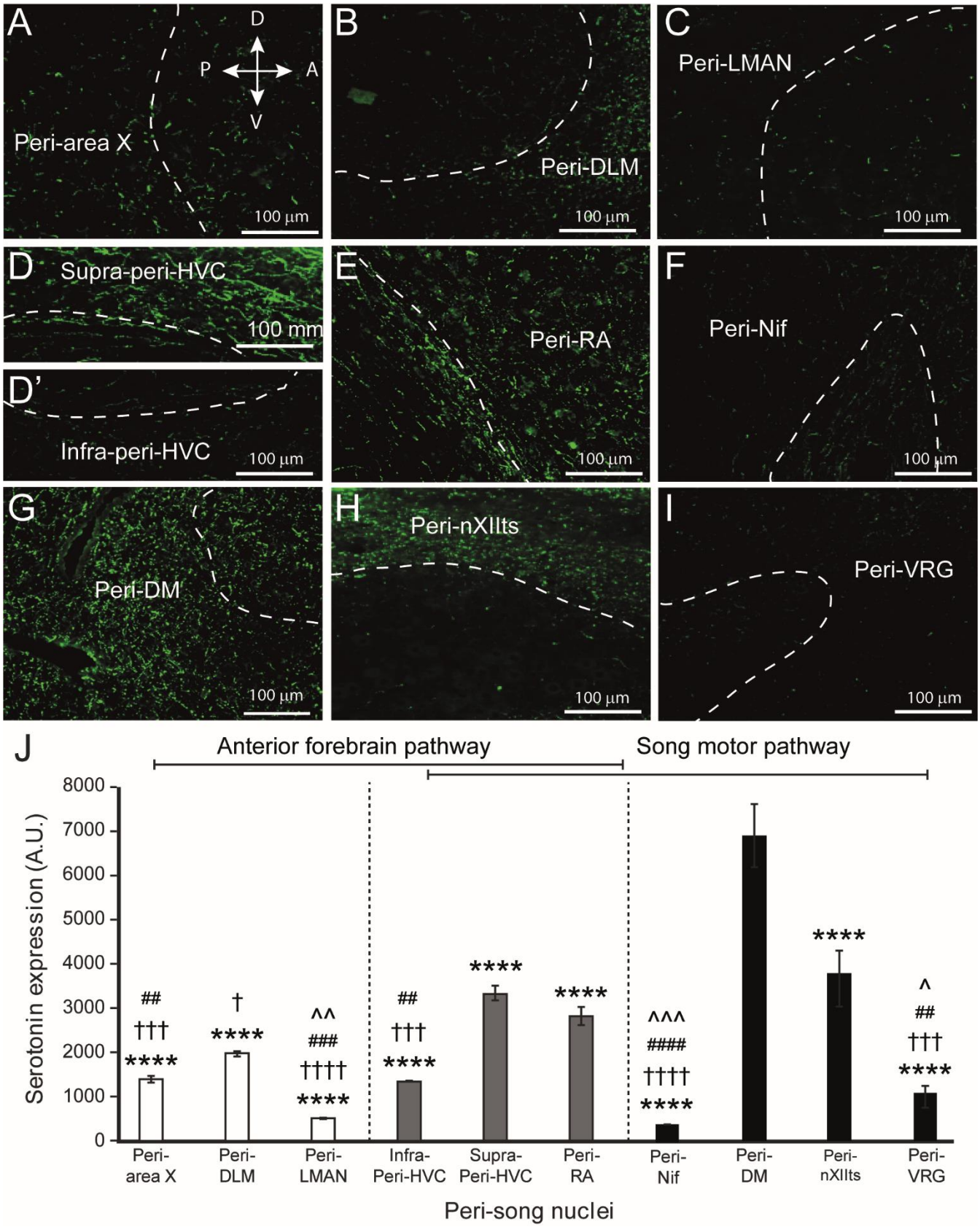

A
D
P
A
V
Peri-area X
100 µm
B
Peri-DLM
100 µm
C
Peri-LMAN
100 µm
D
Supra-peri-HVC
100 mm
D'
Infra-peri-HVC
100 µm
E
Peri-RA
100 µm
F
Peri-Nif
100 µm
G
Peri-DM
100 µm
H
Peri-nXIIts
100 µm
I
Peri-VRG
100 µm
J
Anterior forebrain pathway
Song motor pathway
Serotonin expression (A.U.)
8000
7000
6000
5000
4000
3000
2000
1000
0
Peri-area X
Peri-DLM
Peri-LMAN
Infra-Peri-HVC
Supra-Peri-HVC
Peri-RA
Peri-Nif
Peri-DM
Peri-nXIIts
Peri-VRG
Peri-song nuclei

Fig. 6

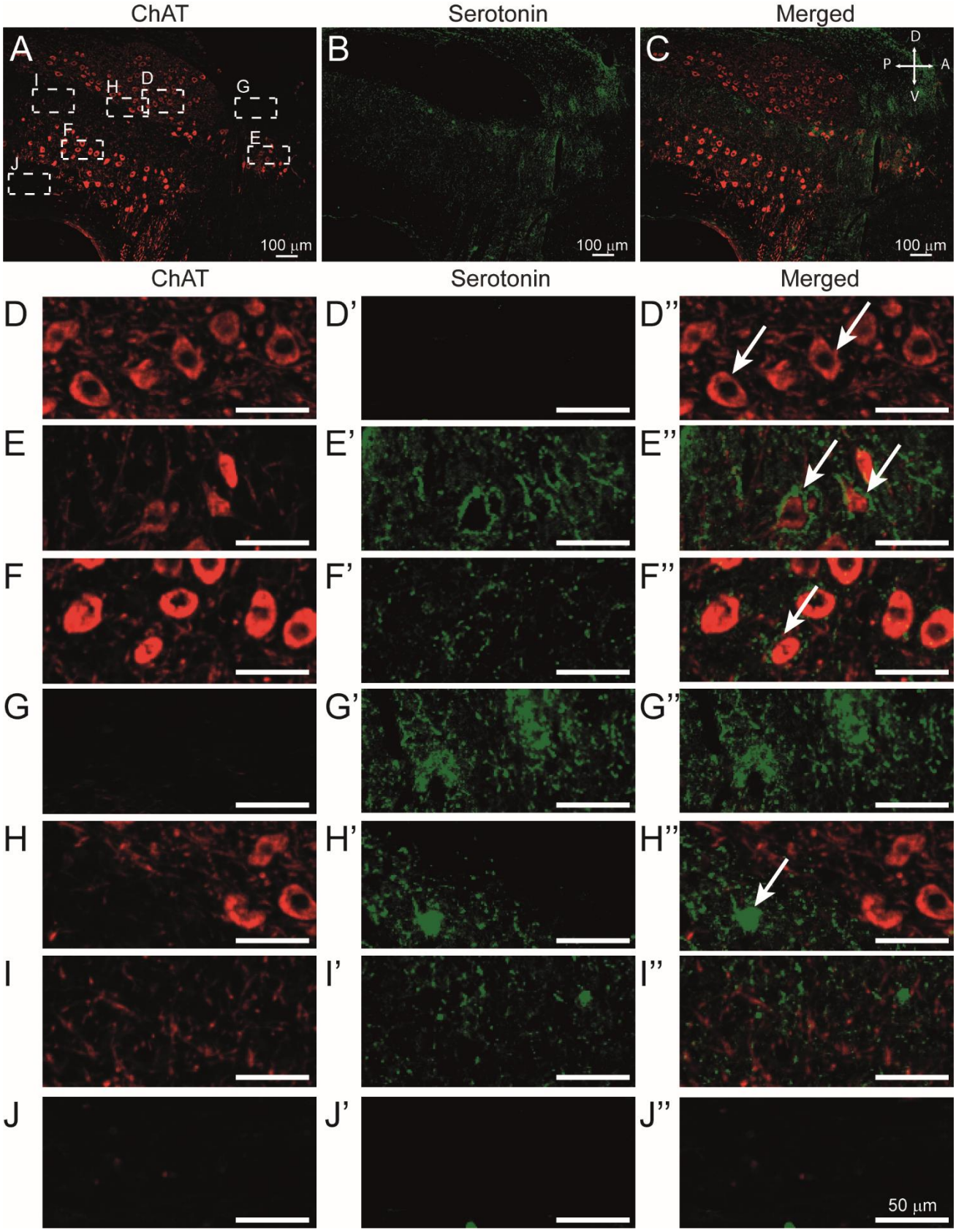

ChAT
Serotonin
Merged
A
B
C
I
H
D
G
F
E
J
D
P
A
V
100 µm
100 µm
100 µm
ChAT
Serotonin
Merged
D
D'
D''
E
E'
E''
F
F'
F''
G
G'
G''
H
H'
H''
I
I'
I''
J
J'
J''
50 µm

Fig. 7

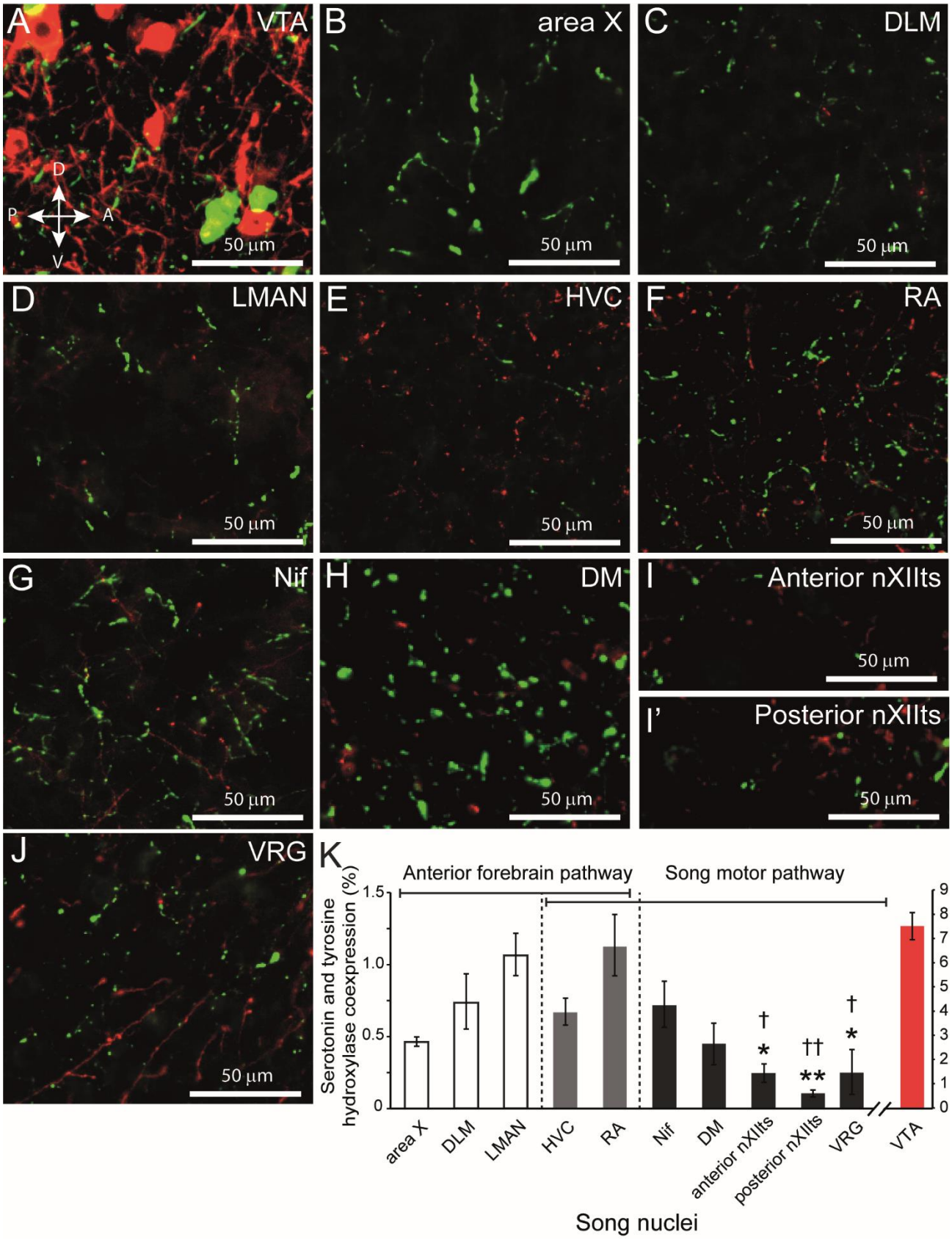

A
VTA
D
P
A
V
50 μm
B
area X
50 μm
C
DLM
50 μm
D
LMAN
50 μm
E
HVC
50 μm
F
RA
50 μm
G
Nif
50 μm
H
DM
50 μm
I
Anterior nXIIts
50 μm
I'
Posterior nXIIts
50 μm
J
VRG
50 μm
K
Anterior forebrain pathway
Song motor pathway
Serotonin and tyrosine hydroxylase coexpression (%)
1.5
1.0
0.5
0
9
8
7
6
5
4
3
2
1
0
†
*
††
**
†
*
area X
DLM
LMAN
HVC
RA
Nif
DM
anterior nXIIts
posterior nXIIts
VRG
VTA
Song nuclei

Fig. 8

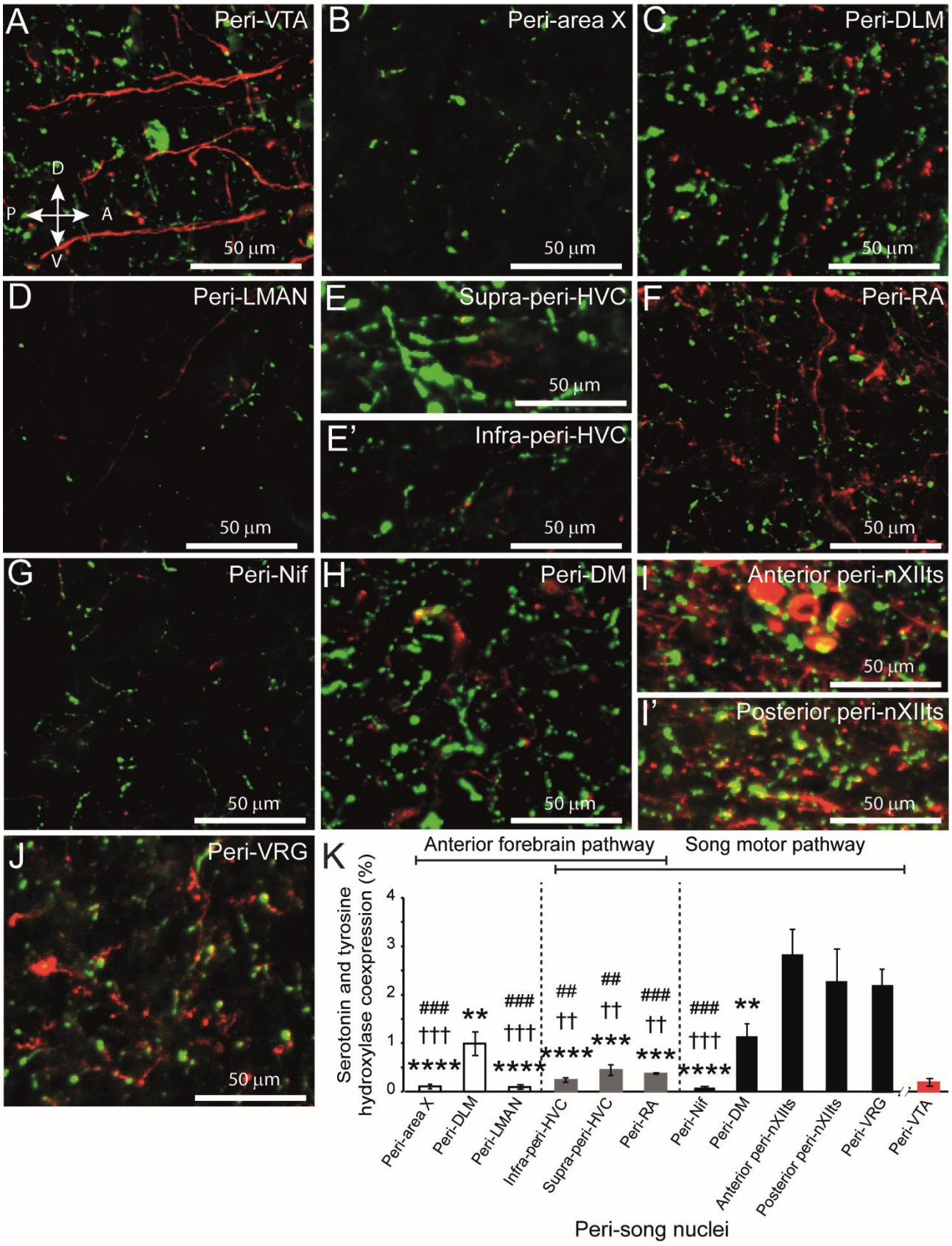

A
Peri-VTA
D
P
A
V
50 µm
B
Peri-area X
50 µm
C
Peri-DLM
50 µm
D
Peri-LMAN
50 µm
E
Supra-peri-HVC
50 µm
E'
Infra-peri-HVC
50 µm
F
Peri-RA
50 µm
G
Peri-Nif
50 µm
H
Peri-DM
50 µm
I
Anterior peri-nXIIts
50 µm
I'
Posterior peri-nXIIts
50 µm
J
Peri-VRG
50 µm
K
Anterior forebrain pathway
Song motor pathway
Serotonin and tyrosine hydroxylase coexpression (%)
Peri-area X
Peri-DLM
Peri-LMAN
Infra-peri-HVC
Supra-peri-HVC
Peri-RA
Peri-Nif
Peri-DM
Anterior peri-nXIIts
Posterior peri-nXIIts
Peri-VRG
Peri-VTA
Peri-song nuclei

Fig. 9