Received: 6 October 2023 | Revised: 6 March 2024 | Accepted: 24 March 2024

DOI: 10.1002/cne.25612

RESEARCH ARTICLE

JCN RESEARCH IN SYSTEMS NEUROSCIENCE THE JOURNAL OF COMPARATIVE NEUROLOGY WILEY

Histological characterization and development of mesial surface sulci in the human brain at 13–15 gestational weeks through high-resolution histology

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Funding information
The Pratiksha Trust; Office of the Principal Scientific Adviser, Government of India

Abstract

Cellular-level anatomical data from early fetal brain are sparse yet critical to the understanding of neurodevelopmental disorders. We characterize the organization of the human cerebral cortex between 13 and 15 gestational weeks using high-resolution whole-brain histological data sets complimented with multimodal imaging. We observed the heretofore underrecognized, reproducible presence of infolds on the mesial surface of the cerebral hemispheres. Of note at this stage, when most of the cerebrum is occupied by lateral ventricles and the corpus callosum is incompletely developed, we postulate that these mesial infolds represent the primordial stage of cingulate, callosal, and calcarine sulci, features of mesial cortical development. Our observations are based on the multimodal approach and further include histological three-dimensional reconstruction that highlights the importance of the plane of sectioning. We describe the laminar organization of the developing cortical mantle,

including these infolds from the marginal to ventricular zone, with Nissl, hematoxylin and eosin, and glial fibrillary acidic protein (GFAP) immunohistochemistry. Despite the absence of major sulci on the dorsal surface, the boundaries among the orbital, frontal, parietal, and occipital cortex were very well demarcated, primarily by the cytoarchitecture differences in the organization of the subplate (SP) and intermediate zone (IZ) in these locations. The parietal region has the thickest cortical plate (CP), SP, and IZ, whereas the orbital region shows the thinnest CP and reveals an extra cell-sparse layer above the bilaminar SP. The subcortical structures show intensely GFAP-immunolabeled soma, absent in the cerebral mantle. Our findings establish a normative neurodevelopment baseline at the early stage.

KEYWORDS
cerebral cortex, developing sulci, fetal brain, histology, neurodevelopment RRID:AB_2799963, RRID:AB_2799098, RRID:AB_11179983, RRID:SCR_002285, RRID:SCR_001622

1 | INTRODUCTION

Approximately 2–3 weeks after conception, a spatially heterogeneous and temporally orchestrated complex process leads to the formation of the brain, for which key, though incomplete, histological reference data sets have been produced (Bayer & Altman, 2005, 2007; Ding et al., 2022; Hansen et al., 2010; Kostovic & Rakic, 1990; Rakic, 1988, 2003). The molecular and cellular framework of the brain during this early intrauterine developmental period guides the eventual organization of the adult brain (Rakic, 1988, 2007; Rubenstein & Rakic, 1999; Ding et al., 2022). However, a complete understanding of the morphological details of this structural framework in human neurodevelopment remains limited (Molnár, 2011; Bitar & Barry, 2020).

There are significant knowledge deficits concerning the neural development of the human fetus, particularly between 12 and 15 gestational weeks (GW). Presently, the most comprehensive histological data set for 12–16 GW is the developmental atlas by Bayer and Altman (2005), which consists of 20 sections from a single 13.5 GW brain specimen. Fifteen postconceptional week (PCW)/17 GW digitized fetal brain data from the Allen Brain Institute (Ding et al., 2022) contains 46 Nissl plates from one cerebral hemisphere. For age categories below 20 GW, magnetic resonance imaging (MRI) lacks resolution, and most data are from 20 GW and later stages (Huang et al., 2006, 2009; Zhang et al., 2013; Gholipour et al., 2017). High-sampling-density cellular-level histological data may reveal important information regarding the stages of neural development of eventually permanent brain regions as well as of transient cellular features (Vasung et al., 2019; Junaković et al., 2023), highlighting regional chronological variability in maturation. The relative scarcity of high-quality postmortem tissue makes the whole-brain histology of the developing human brain particularly challenging (Vasung et al., 2019; Šimić et al., 2022). In this article, we report our findings with a significantly higher sampling density (details to follow) than these oft-cited available references during this critical developmental window (see below).

To this point, the 12–16 GW age range is marked by important changes in the appearance of the interhemispheric mesial surface due to the structural reorganization of the large super ventricle of the embryonic brain into the two lateral ventricles (Taketani et al., 2015; Li et al., 2019), the appearance of the corpus callosum (CC), the callosal sulcus, and the deepening of the cingulate sulcus (Bayer & Altman, 2005; Huang et al., 2009; Ding et al., 2022). Thus, the beginning of the fetal stage is characterized by early-stage convolutions that result in the formation of longitudinal and lateral sulci/fissures (Chi et al., 1977; Naidich et al., 1994). While the development of sulci on the dorsal cortical surface has been studied extensively, the formation of sulci on the mesial surface in humans is less well understood. It is important to characterize and quantify the development of these mesial sulci, such as the callosal, marginal, calcarine and cingulate sulci, because they may be used as diagnostic criteria among certain neurodevelopmental disorders (Malinger et al., 2003; Hetts et al., 2006; Vasung et al., 2019). In addition to the lack of data in this regard, there are discrepancies in the chronological timelines for the development of the known dorsal surface and mesial surface sulci, depending on the methodology used, whether in utero and ex vivo MRI (1.5, 3, 7 T) or classical macro- and microscopic examinations (Chi et al., 1977; Hansen et al., 1993; Huang et al., 2009; Zhang et al., 2013). Early human fetal brain development, at 12–15 GW (early second trimester), is characterized by the presence of a significantly thinner germinal matrix in subcortical structures than in the developing cerebral mantle. Compared to the developing cerebral cortex, this indicates a more advanced stage of maturation (Bayer & Altman, 2005; Stiles & Jernigan, 2010; Vasung et al., 2019). Subsequently, the developing cortical mantle undergoes active neurogenesis, characterized by the prominent ventricular and subventricular zones (VZ and SVZ), especially the outer SVZ (OSVZ) (Kriegstein et al., 2006; Hansen et al., 2010), resulting in the formation and organization of the future neocortex.

At this critical developmental stage, the radial glial cells that are produced by the neuroepithelial cells play a dual role—providing the

scaffold for the migration of cells upward toward the developing cortical plate (CP) (Rakic, 1972; Sidman & Rakic, 1973) and actively contributing to neurogenesis and gliogenesis via asymmetric and symmetric division (Kriegstein et al., 2006; Hansen et al., 2010).

Here we apply our novel methodology, including postmortem in-skull structural MRI and block-face imaging, of serial sections across the entire brain (Karthik et al., 2023) to supplement our detailed histological analyses of the cortical cytoarchitecture of the mesial surface in relation to the early sulcal roots, laminar organization of the developing cerebral cortex, and the pattern of development of radial glial cells in the cerebral cortex and subcortical regions in 13–15 GW human fetal brain.

2 | MATERIALS AND METHODS

2.1 | Specimen procurement

Four de-identified specimens from 13 to 15 GW (Specimens 1–4, S1–S4) were obtained from the Department of Pathology at MediScan Systems Pvt. Ltd (MediScan) and Saveetha Medical College and Hospital (SMCH), Chennai, India. The specimens were collected after due consent from the next of kin, in accordance with the Declaration of Helsinki, along with the medical history and investigative records, which included prenatal ultrasound scans. All four brain specimens had no significant maternal history and were diagnosed as non-pathological based on prenatal ultrasound, postmortem gross examination, and postmortem MRI. S1 (15 GW) was used to show gross anatomical structures, specifically the mesial surface infolds, prior to histological processing. We have performed detailed histological processing (S2–S4) by staining with hematoxylin and eosin (H&E) as well as immunohistochemistry (IHC) glial fibrillary acidic protein (GFAP), synaptophysin and neuronal nuclear antigen (NeuN), which showed no cellular changes to indicate any form of pathology in all key structures, including the developing cerebral cortex, CC, thalamus, brainstem, and cerebellum. Of particular note, the histologic (cellular level) data were derived from high-resolution microscopy for 500+ sections examined from each specimen. The Institute Ethics Committee (IEC) of the Indian Institute of Technology, Madras (IITM) granted ethical approval for the acquisition of these specimens and their subsequent processing and analysis (IEC/2021-01/MS/06). PCW (or postfertilization) is generally understood to be 2 weeks "later" than gestational age, which is determined by the time of the maternal last menstrual period (i.e., postmenstrual age). Thus, a gestational age of 13 weeks corresponds to a postconceptional age of 11 weeks. Note that we chose to conform to gestational weeks (GW) so as to facilitate comparison with the Bayer and Altman atlases using that terminology. After extraction, the mass and dimensions of each specimen were measured and recorded: S2, 13 GW mass: 7.0 g, anterior–posterior (AP): 3.2 cm, dorsal–ventral (DV): 1.5 cm, right–left (RL): 2.7 cm. S3, 14 GW mass: 8.6 g, AP: 4.4 cm, DV: 2.2 cm, RL: 2.8 cm. S4, 15 GW mass: 6.2 g, AP: 3.0 cm, DV: 3.0 cm, RL: 2.5 cm.

2.1.1 | Histological workflow

This section describes our specimen procurement, histological processing, and data acquisition methodology (Figure 1). The histology workflow has been optimized for developing human brains and adapted from a similar workflow on small animal brains such as rodents and marmosets (Pinskiy et al., 2015; Lin et al., 2019). Within a postmortem interval (PMI) of 2–4 h, whole fetal specimens were immersed in a fixative solution of 20% formalin. Following specimen acquisition, structural MRIs (1.5 or 3 T) were collected in situ before autopsy and extraction (details in the following section). The brains were extracted from the skull after 2 weeks and stored by immersing in 4% paraformaldehyde (PFA) in 0.01 M phosphate buffer (PB), pH 7.4 at room temperature.

2.1.2 | In-situ magnetic resonance imaging (MRI)

MRI was performed on the brains within the cranium using 1.5 T (version 5.3.1/5.3.1.4, Philips Medical Systems) for S2 at Saveetha Medical College and Hospital, India, and for S3 and S4 using 3 T (version DV28.0_R05_2.34.a, Signa Architect, GE medicals) at Sri Ramachandra Institute of Higher Education and Research, India, (Figures 1a and 2). The imaging was performed using finger matrix coils with 16 channels. For S2, a *T*1-weighted turbo field echo sequence was acquired with 110 slices, yielding a matrix size of 256 × 256 × 110 (parameters: repetition time 7 ms; echo time 3 ms; flip angle 8°; field of view 170 mm; slice thickness 1 mm without overlap among images). For S3 and S4, a *T*1-weighted gradient MP-RAGE sequence was acquired, with 88 and 64 slices (parameters: repetition time 2500 ms; echo time 3 ms; flip angle 8°; field of view 100 mm; slice thickness 1 mm without overlap), yielding a matrix dimension of 512 × 512 × 88 (S3) and 512 × 512 × 64 (S4). MRI data were used for the three-dimensional (3D) reconstruction of stacked histological sections, as shown in Figure 2.

2.2 | Cryoprotection

Prior to sectioning, the specimens were cryoprotected using gradient sucrose, beginning with 10% sucrose in 4% PFA for 2–4 days, 20% sucrose in 0.01 M PB solution (PBS), for 3–4 days, and 30% sucrose in 0.01 M PBS for 5–7 days at 4°C (Figure 1a). The volume of the immersion solution used was approximately five times that of the specimen, and the endpoint of each cryoprotection stage was marked by the specimen's complete equilibrium in the solution.

2.3 | Freezing

Combining in situ MRIs and 3D surface scans (Einscan Pro, SHINING 3D Technology GmbH) of the extracted brain, we designed 3D-printed customized brain molds for each specimen. These brain molds allowed

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planes, suggests that the plane of section used to delineate these infolds is significant and may necessitate a reevaluation of the chronology of the developing sulci in humans. Other structural studies, notably MRI, have reported comparable infolds in sagittal images, where with 7T MRI, 16 GA was described as the most intensive point for sulcal emergence (Hansen et al., 1993; Zhang et al., 2013; Wang et al., 2015).

The development of primary sulci in humans and monkeys follows a particular chronological order, with delays indicative of developmental disorders (Chi et al., 1977; Hansen et al., 1993; Fukunishi et al., 2011). Based on histological data from previous studies (Chi et al., 1977; Bayer & Altman, 2005), the parieto-occipital, cingulate, calcarine, and olfactory sulci appear between 16 and 18 GW; however, MRI studies have suggested their earlier appearance (Hansen et al., 1993; Zhang et al., 2013). The histological sections of 13–15 GW brains examined in this study indicate that the medial surface sulci appear at this stage. Our findings demonstrate a temporal relationship between the formation of these folds and the maturation of the lateral ventricles, which is consistent with previous reports in human fetal and cynomolgus monkeys for the calcarine sulcus (Fukunishi et al., 2006; Kashima et al., 2008; Sawada et al., 2012; Li et al., 2019). In addition, previous studies show that as the brain develops, smaller cerebral fossa or sulcal roots fuse to produce larger sulci (Régis et al., 2005; Zhang et al., 2013). By 17 GW, there is a distinctive mesial cortical surface delineating the two lateral ventricles, a well-developed CC with both the rostrum and splenium apparent, and most importantly, a discernible cingulate sulcus (Bayer & Altman, 2005; Nishikuni & Ribas, 2013). From our data, we are able to show a much earlier timeline for the development of mesial surface sulci. These present findings contribute significantly to our understanding of the organization of developing sulci in the human brain. Further analysis of whole brains using similar approaches to ours at later developmental stages is needed to comprehensively show that these primordial sulci eventually become the sulci of the adult brain. Alternatively, some of these primordial sulci may be of a transitive nature. The developmental trajectory of sulci, as well as that of the CC, may serve as important benchmarks for the early diagnosis (12–17 GW) of neurodevelopmental disorders (Malinger et al., 2003; Sun & Hevner, 2014; Li et al., 2019).

4.2 | Laminar organization of the developing cerebral cortex 13–15 GW

Using the whole brain histology in the developing cerebral cortex, we present the detailed cytoarchitecture features to define the developing cerebral mantle in the occipital, parietal, frontal, orbital, and the temporal cortices. Despite the absence of dorsal surface sulci, the boundary among the occipital, parietal, frontal, and orbital regions could be demarcated by observing changes in the SP and IZ. Paraffin embedding for histological processing often causes high tissue shrinkage due to the dehydration process. In this study, we were able to measure the shrinkage pre- and post-histological processing using brain dimensions from *in-skull* MRI and histological sections. The shrinkage in all three specimens was less than 5%, as reported in the large brain specimen (Kumarasami et al., 2023).

We observed variations in the thickness of the CP and SP in each of the five regions of the cerebral cortex, where we measured the thickness of each layer. The thickness measurements presented here are from 2D sections in the sagittal plane. Despite careful alignment, we cannot rule out the possibility of slight misalignments. However, it is important to highlight that all the sections were obtained in a continuous manner from a single whole-brain block. Hence, any misalignment will be propagated across the brain. The aim here was to compare relative thickness across different regions in the developing brain rather than the absolute values, and hence, we present our data from each specimen independently. The average thickness of combined CP and SP is similar to those previously reported from postmortem MRI studies in 13–15 GW (Huang et al., 2009). The cortical thickness in the adult human brain ranges from 1 to 4.5 mm (Fischl & Dale, 2000) obtained from MRI and 1.8 to 4.5 mm from histology, Big Brain (Wagstyl et al., 2020). The regional variations in the thickness of the CP and the SP at the early fetal stage may reflect the regional variations of the mature adult cerebral cortex; however, this is yet to be investigated in detail.

The CP displays dense uniform Nissl labeling, which is thickest in the developing parietal. The underlying SP at 13–15 GW developing stage demonstrates the SP formation stage as described by Kostović and Rakić (1990), which consists of two sub-bands, except in the orbital cortex, where the upper band is cell-dense and the lower band is cell-sparse, with the greatest thickness in the developing parietal cortex. In addition, we identify an additional cell-sparse band between the CP and the SP, which appears to be unique to the orbital cortex. Kopić et al. (2023) have recently characterized this using a "deep projection neuronal marker" as a "double plate" specific to the orbitobasal cortex at PCW 13. We are unable to remark on the origins of this band based on our current data, but we hypothesize that it may form the bands of the future piriform cortex. Previously, SP was referred to as the layer or waiting zone where afferent fibers from the developing thalamus and other cortices initiate the formation of connections with the cortical network (Kostovic & Rakic, 1990; Kostović & Judaš, 2006; Kostović & Judas, 2010). As the SP develops, it thickens, and the cells disperse, paving the way for the formation of new connections. At the SP formation stage, it is believed that these afferent connections originate from neurons in the basal forebrain and thalamus, and only at a later stage (mid-fetal) from other regions of the cerebral cortex, as reported in the occipital and the parietal regions in the human fetal cortex (Kostovic & Rakic, 1990). The differences in origin and timing of these connections most likely account for the regional differences (frontal, orbital, and temporal) in SP appearance as observed in this study (Vasung et al., 2010, 2019). In conjunction with SP, the IZ exhibited alterations in the cellular organization and thickness of the occipital to the temporal cortex. In the developing occipital cortex, there are four sublayers of IZ, whereas in other cortical regions, there are only two or three sublayers. This is comparable to the 13.5 GW data from Bayer and Alman (2005), in which the IZ, also known as a stratified transitional field, differentiates further into six sublayers by 20 GW. Additionally, according to our findings, the border between the SP and IZ is most clearly delineated in the occipital region. In the developing parietal cortex, the IZ is significantly thicker, possibly reflecting the presence of active neuronal migration. The cytoarchitectural description and quantification

of developing cerebral cortical layers provide essential information to systematically evaluate regional maturation of the cerebral cortex by studying the maturation of CP and SP, the formation of thalamocortical and cortical connections, the disappearance of transitional layers, and neuronal and glial differentiation, at various gestational ages. MRI-based laminar organization can typically demarcate four layers in this age group (Huang et al., 2009; Wang et al., 2015). Morphometric data for the developing brain (postmortem) is available for 13–40 PCW (15–42 GW); however, the thickness analysis for four developing laminar compartments (CP, SP, IZ, and VZ) is reported only for 21–40 PCW.

4.3 | Maturation pattern of radial glia in 13–15 GW through IHC

Using IHC labeling for GFAP, we show that the radial glial processes are more prominent, with dense labeling, in the superior layer of the SVZ and the OSVZ of the cerebral cortex. This is comparable to findings that have been reported previously in humans (Hansen et al., 2010) and other species (Noctor et al., 2004). Importantly, we observed no difference in the GFAP labeling of processes and cells in the sulcal folds. The intensity and pattern of labeling were similar to other regions in the developing cortex, suggesting that the folds that we report here have a structure consistent in the developing cortical regions from VZ to MZ. We did not find any banded/angled radial glial processes in the lower end of SP as described by Kostović et al. (2019), 13–14 GW, and few scattered banded ones were observed in 15 GW. This difference could either be due to the difference in the plane of sectioning or because these were visualized using Golgi stain in the previous study. The inner walls of the ventricles are lined with neuronal epithelial cells that are the primary source of cortical neurons and glia (Rakic, 2003; Noctor et al., 2004; Sun & Hevner, 2014). The radial glial cells have a dual role: providing the scaffold for the migration of cells toward the developing CP and contributing to neurogenesis (Sidman & Rakic, 1973; Rakic, 1978, 1988; Kriegstein et al., 2006; Hansen et al., 2010). This radial glial cell in later stages also produces astrocytes and oligodendrocytes (Campbell & Götz, 2002; Rezaie et al., 2003). Previous studies have investigated the distribution and maturation of astrocytes around 16 GW (Wilkinson et al., 1990) and midgestational age (19–23 GW) using GFAP and other astrocyte markers like vimentin and S-100 (Rezaie et al., 2003; Howard et al., 2008; Holst et al., 2019) in the cerebral cortex. The spatial and temporal switching of RGC from neurogenesis to gliogenesis, astrocyte development, diversity, and proliferation are not well established in the human fetal brain due to difficulties in the retrieval of high-quality postmortem fetal tissue, variations in data from different brain regions, and the choice of radial glial and astrocyte markers (Wilkinson et al., 1990; Molofsky & Deneen, 2015; Holst et al., 2019; Sasaki et al., 1988).

In our study, the labeling of GFAP in the subcortical structures, including the thalamus and brain stem, was different from the immature cerebral cortex. The subcortical structures show cell somatic labeling consistent with differentiating astrocytes in this early age group similar to those reported in 19–23 GW in the developing human brain (Rezaie et al., 2003), but here we report as early as 13 GW, including the brainstem, which was observed at 17 GW (Wilkinson et al., 1990) with GFAP labeling compared to vimentin and S-100 (Wilkinson et al., 1990). At 13–15 GW, we did not find such labeling in the developing cerebral cortex. The regional developmental pattern and functional role of maturing astrocytes in the human central nervous system are critical in understanding various brain pathologies (Falcone et al., 2020; Vakilzadeh & Martinez-Cerdeño, 2023).

5 | CONCLUSIONS

Our histological processing pipeline, which has been optimized for the human fetal brain, provides robust cellular-level data to characterize the structural architecture of the developing human brain between 13 and 15 GW. The serial sections from the entire brain in the sagittal plane reveal the initiation of important future mesial surface sulci. The formation of these early cortical folds, along with the development of important fiber tracts, may provide the framework for the formation of specialized functional compartments and cytoarchitectonic areas in the cerebral cortex. This approach will be used to study the developing human brain at various gestational ages and generate comparative data sets using multimodal imaging, ranging from MRI to histology.

AUTHOR CONTRIBUTIONS

Richa Verma1: Conceptualization; data curation; formal analysis; methodology; project administration; visualization; writing—original draft. **Rajeswaran Rangasami**: Conceptualization; resources; data curation; formal analysis; methodology; project administration; visualization; writing—original draft. **Jaikishan Jayakumar**: Conceptualization; data curation; formal analysis; methodology; project administration; visualization; writing—original draft. **Rebecca Folkerth**: Writing—review and editing. **Paul R. Manger**: Writing—review and editing. **Goran Šimić**: Writing—review and editing. **Mihail Bota**: Data curation; writing—review and editing. **Moitrayee Majumder** Data curation; formal analysis. **Karthika Pandurangan**: Investigation; formal analysis. **Stephen Savoia**: Resources. **Srinivasa Karthik**: Resources. **Ramdayalan Kumarasami**: Resources. **G. Rohini**: Resources. **Sudha Vasudevan**: Resources. **Chitra Srinivasan**: Resources. **S. Lata**: Resources. **E. Harish Kumar**: Resources. **Jayaraman Kumutha**: Resources. **Suresh S.**: Resources. **Jayaraj Joseph**: Conceptualization; supervision; resources; methodology; fundingAcquisition. **Partha P Mitra**: Conceptualization; supervision; resources; methodology; fundingAcquisition; writing—review and editing. **Mohanasankar Sivaprakasam**: Conceptualization; supervision; resources; methodology; fundingAcquisition; writing—review and editing.

ACKNOWLEDGMENTS

The authors would like to acknowledge Prof. Z. Krsnik, Croatian Institute for Brain Research (CIBR), School of Medicine, University of Zagreb, for her valuable input in marking the boundaries of developing the cerebral cortex. We are thankful to all the technical staff from SGBC, IIT Madras for their contribution.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

FUNDING INFORMATION

This work was supported by funding from the Office of the Principal Scientific Adviser, Government of India, and the Pratiksha Trust. PPM is supported by the H N Mahabala Chair Professorship of the Indian Institute of Technology Madras.

INFORMED CONSENT STATEMENT

All postmortem specimens were obtained after due consent from the next of kin in accordance with the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/cne.25612.

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How to cite this article: Verma, R., Jayakumar, J., Folkerth, R., Manger, P. R., Bota, M., Majumder, M., Pandurangan, K., Savoia, S., Karthik, S., Kumarasami, R., Joseph, J., Rohini, G., Vasudevan, S., Srinivasan, C., Lata, S., Kumar, E. H., Rangasami, R., Kumutha, J., Suresh, S., … Sivaprakasam, M. (2024). Histological characterization and development of mesial surface sulci in the human brain at 13–15 gestational weeks through high-resolution histology. *Journal of Comparative Neurology*, *532*, e25612. https://doi.org/10.1002/cne.25612