BRAIN EVOLUTION: CLUES FROM AQUATIC ORGANISMS

EDITED BY: Paolo De Girolamo, Jean-Pierre Bellier and Livia D'Angelo
PUBLISHED IN: Frontiers in Neuroanatomy

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88966-962-2
DOI 10.3389/978-2-88966-962-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

BRAIN EVOLUTION: CLUES FROM AQUATIC ORGANISMS

Topic Editors:
Paolo De Girolamo, University of Naples Federico II, Italy
Jean-Pierre Bellier, Shiga University of Medical Science, Japan
Livia D'Angelo, University of Naples Federico II, Italy

Citation: De Girolamo, P., Bellier, J.-P., D'Angelo, L., eds. (2021). Brain Evolution: Clues from Aquatic Organisms. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-962-2

Table of Contents

04 ***Editorial: Brain Evolution: Clues From Aquatic Organisms***
Paolo de Girolamo, Jean-Pierre Bellier and Livia D'Angelo

06 ***Galanin in an Agnathan: Precursor Identification and Localisation of Expression in the Brain of the Sea Lamprey* Petromyzon marinus**
Daniel Sobrido-Cameán, Luis Alfonso Yáñez-Guerra, Francesco Lamanna, Candela Conde-Fernández, Henrik Kaessmann, Maurice R. Elphick, Ramón Anadón, María Celina Rodicio and Antón Barreiro-Iglesias

17 ***Zebrafish Astroglial Morphology in the Olfactory Bulb Is Altered With Repetitive Peripheral Damage***
Jackson Scheib and Christine Byrd-Jacobs

28 ***Using Teleost Fish to Discern Developmental Signatures of Evolutionary Adaptation From Phenotypic Plasticity in Brain Structure***
Zachary J. Hall and Vincent Tropepe

34 ***Brain and Behavioral Asymmetry: A Lesson From Fish***
Maria Elena Miletto Petrazzini, Valeria Anna Sovrano, Giorgio Vallortigara and Andrea Messina

56 ***Understanding the Molecular and Cell Biological Mechanisms of Electrical Synapse Formation***
E. Anne Martin, Abagael M. Lasseigne and Adam C. Miller

69 ***Calsequestrins New Calcium Store Markers of Adult Zebrafish Cerebellum and Optic Tectum***
Sandra Furlan, Marina Campione, Marta Murgia, Simone Mosole, Francesco Argenton, Pompeo Volpe and Alessandra Nori

81 ***The Melanocortin System in Atlantic Salmon (*Salmo salar *L.) and Its Role in Appetite Control***
Tharmini Kalananthan, Floriana Lai, Ana S. Gomes, Koji Murashita, Sigurd Handeland and Ivar Rønnestad

96 ***Cerebrotypes in Cephalopods: Brain Diversity and Its Correlation With Species Habits, Life History, and Physiological Adaptations***
Giovanna Ponte, Morag Taite, Luciana Borrelli, Andrea Tarallo, A. Louise Allcock and Graziano Fiorito

frontiers in Neuroanatomy

EDITORIAL
published: 07 May 2021
doi: 10.3389/fnana.2021.683489

Editorial: Brain Evolution: Clues From Aquatic Organisms

Paolo de Girolamo[1], *Jean-Pierre Bellier*[2] *and Livia D'Angelo*[1]*

[1] Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naples, Italy, [2] Molecular Neuroscience Research Center, Shiga University of Medical Science, Otsu, Japan

Keywords: cephalopods, agnathans, teleosts, brain structure and function, evolutionary adaptation

Editorial on the Research Topic

Brain Evolution: Clues From Aquatic Organisms

Aquatic species have served as models in brain evolution to address questions on the origin of the nervous system for a long time. The aquatic environment and its inhabitants have offered the possibility to understand how animals progressed from a simple nerve net to a complex centralized nervous system (Arendt et al., 2016), and how the environment has contributed to boosting the functional diversity of brain structures, shapes, and sizes, all reflecting peculiarities in species-specific sensory perception, central processing, and behavioral responses. Although fish are unequivocally the first group of organisms which come to mind when thinking about the aquatic environment, much of what we know today about brain evolution has also arisen from aquatic invertebrates, i.e., cephalopods. This collection of articles discusses topics ranging from neuropeptidergic systems responsible for different behaviors, to electric synapses formation, and neuroplasticity, in both invertebrates and vertebrates. A hierarchical clustering approach has been used to document the brain diversity of about 30 cephalopod species, correlated to the species habitats and physiological environmental adaptations, opening new avenues in evolutionary neurobiology and ecomorphology to reveal the biological basis of sensory orientation, cognitive potential, and motor abilities (Ponte et al.). Across the vertebrate tree, agnathans (lamprey) occupy the base representing intriguing models to understand early brain evolution in vertebrates. Remarkably, lamprey display complex neuropeptidergic systems and essential components of neuronal communication in the animal brain controlling and regulating several conserved behaviors, such as appetite. As an example, galanin, a neuropeptide known to regulate many physiological processes, including feeding and nociception in mammals, displays a wider expression pattern in the larval brain which is restricted to the ventral pallium, lateral hypothalamus, and prethalamus in the adult brain of sea lamprey *Petromyzon marinus* (Sobrido-Cameán et al.). Neuropeptidergic systems controlling food intake behavior are among the most studied in teleost fish, due to the high degree of conservation in vertebrates and the relevant impact in aquaculture (Amodeo et al., 2018). This is indeed the case of Atlantic Salmon (*Salmo salar* L.), a teleost species providing the unique opportunity for studying vertebrate genome evolution after an autotetraploid whole genome duplication over a period that is long enough to reveal long-term evolutionary patterns, but short enough to give a high-resolution picture of the process (Lien et al., 2016). For instance, the study on the Atlantic Salmon reports that the melanocortin-4 receptor is present as multiple paralogs (a1, a2, b1, and b2) and all paralogs are relatively well-conserved with the human homolog, sharing at least 63% amino acid sequence identity. It is worth noting that the mRNA expression of mc4r paralogs was not changed in the hypothalamus or in other highly expressed regions between the fed and fasted state of young salmon specimens (Kalananthan et al.).

OPEN ACCESS

Edited by:
Paul Manger,
University of the Witwatersrand, South Africa

Reviewed by:
Ayanda Ngwenya,
University of the Witwatersrand, South Africa

Correspondence:
Livia D'Angelo
livia.dangelo@unina.it

Received: *21 March 2021*
Accepted: *01 April 2021*
Published: *07 May 2021*

Citation:
de Girolamo P, Bellier J-P and D'Angelo L (2021) Editorial: Brain Evolution: Clues From Aquatic Organisms. Front. Neuroanat. 15:683489. doi: 10.3389/fnana.2021.683489

Studies of the teleost brain have contributed greatly toward our understanding of the processes of brain evolution and how environmental factors, such as sensory experience, modulate brain region sizes (Hall and Tropepe). We have already learnt much about vertebrate brain evolution from the comparative neurobiological approach relying on embryonic gene expression and mature neuroanatomy, with emphasis also on brain asymmetries and lateralization (Miletto Petrazzini et al.). The combination of neuroanatomical and behavioral analyses, imaging, and cutting-edge molecular genetic techniques represents a powerful approach to investigate gene-by-environment interaction effects, how genetically encoded asymmetry may change across the lifespan, and how anatomical asymmetries are linked to behavior. Among teleost fish, zebrafish at both the larval and adult stages, are extensively used in central nervous system research by targeting various brain disorders (Stewart et al., 2014). Zebrafish possess some common (shared), as well as some specific molecular biomarkers and features of neuroglia development and neuroplasticity. It is worth noting that zebrafish do not possess typical glial-like morphology, rather they show a morphology reminiscent of astroglia. However, experiments conducted on olfactory bulbs display that these structures have a location and function similar to the mammalian astrocyte (Scheib and Byrd-Jacobs). Identification and combination of molecular markers for a specific Ca2+ store and its neuronal-type association are accurately reported in the adult zebrafish brain, where calsequestrin, a calcium binding protein, is localized on the neuronal endoplasmic reticulum (Furlan et al.). Remarkably, the two isoforms identified (Casq1 and Casq2) are differentially localized in the zebrafish brain with virtually no overlapping. In addition, they are helpful to understand adaptive neuronal function to the aquatic habitat. Moreover, zebrafish are a well-established *in vivo* model for investigating synapses within the elaborate architecture of neurons. Pieces of evidence have been discussed on the cell biological mechanisms that develop, maintain, and regulate electrical synapses and mechanistic relationships between electrical and chemical synapse formation (Martin et al.). In conclusion, neuroscientists will find useful information regarding the brains of aquatic species, and how understanding these features contributes to our understanding of brain evolution, hopefully leading to further important discoveries in both aquatic and non-aquatic neurosciences.

AUTHOR CONTRIBUTIONS

PG, J-PB, and LD'A contributed to the whole conception. LD'A wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

ACKNOWLEDGMENTS

Guest Editors acknowledge all authors who have contributed with their valuable manuscripts.

REFERENCES

Amodeo, P., D'Aniello, E., Defranoux, F., Marino, A., D'Angelo, L., Ghiselin, M. T., et al. (2018). The suitability of fishes as models for studying appetitive behavior in vertebrates. *Res. Probl. Cell Differ.* 65, 423–438. doi: 10.1007/978-3-319-92486-1_19

Arendt, D., Tosches, M. A., and Marlow, H. (2016). From nerve net to nerve ring, nerve cord and brain evolution of the nervous system. *Nat. Rev. Neurosci.* 17, 61–72. doi: 10.1038/nrn.2015.15

Lien, S., Koop, B. F., Sandve, S. R., Miller, J. R., Kent, M. P., Nome, T., et al. (2016). The Atlantic salmon genome provides insights into rediploidization. *Nature* 533, 200–205. doi: 10.1038/nature17164

Stewart, A. M., Braubach, O., Spitsbergen, J., Gerlai, R., and Kalueff, A. V. (2014). Zebrafish models for translational neuroscience research: from tank to bedside. *Trends Neurosci.* 37, 264–278. doi: 10.1016/j.tins.2014.02.011

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroanatomy

BRIEF RESEARCH REPORT
published: 13 September 2019
doi: 10.3389/fnana.2019.00083

Galanin in an Agnathan: Precursor Identification and Localisation of Expression in the Brain of the Sea Lamprey *Petromyzon marinus*

*Daniel Sobrido-Cameán[1], Luis Alfonso Yáñez-Guerra[2], Francesco Lamanna[3], Candela Conde-Fernández[1], Henrik Kaessmann[3], Maurice R. Elphick[2], Ramón Anadón[1], María Celina Rodicio[1] and Antón Barreiro-Iglesias[1]**

[1] Department of Functional Biology, CIBUS, Faculty of Biology, Universidade de Santiago de Compostela, Santiago de Compostela, Spain, [2] School of Biological and Chemical Sciences, Queen Mary University of London, London, United Kingdom, [3] Center for Molecular Biology of Heidelberg University (ZMBH), DKFZ-ZMBH Alliance, Heidelberg, Germany

Galanin is a neuropeptide that is widely expressed in the mammalian brain, where it regulates many physiological processes, including feeding and nociception. Galanin has been characterized extensively in jawed vertebrates (gnathostomes), but little is known about the galanin system in the most ancient extant vertebrate class, the jawless vertebrates or agnathans. Here, we identified and cloned a cDNA encoding the sea lamprey (*Petromyzon marinus*) galanin precursor (*PmGalP*). Sequence analysis revealed that *PmGalP* gives rise to two neuropeptides that are similar to gnathostome galanins and galanin message-associated peptides. Using mRNA *in situ* hybridization, the distribution of *PmGalP*-expressing neurons was mapped in the brain of larval and adult sea lampreys. This revealed *PmGalP*-expressing neurons in the septum, preoptic region, striatum, hypothalamus, prethalamus, and displaced cells in lateral areas of the telencephalon and diencephalon. In adults, the laterally migrated *PmGalP*-expressing neurons are observed in an area that extends from the ventral pallium to the lateral hypothalamus and prethalamus. The striatal and laterally migrated *PmGalP*-expressing cells of the telencephalon were not observed in larvae. Comparison with studies on jawed vertebrates reveals that the presence of septal and hypothalamic galanin-expressing neuronal populations is highly conserved in vertebrates. However, compared to mammals, there is a more restricted pattern of expression of the galanin transcript in the brain of lampreys. This work provides important new information on the early evolution of the galanin system in vertebrates and provides a genetic and neuroanatomical basis for functional analyses of the galanin system in lampreys.

Keywords: lamprey, galanin, telencephalon, hypothalamus, striatum, neuropeptides

OPEN ACCESS

Edited by:
Livia D'Angelo,
University of Naples Federico II, Italy

Reviewed by:
Hiromasa Funato,
Toho University, Japan
Pierre-Yves Risold,
University of Franche-Comté, France

***Correspondence:**
Antón Barreiro-Iglesias
anton.barreiro@usc.es

Received: *29 July 2019*
Accepted: *02 September 2019*
Published: *13 September 2019*

Citation:
Sobrido-Cameán D, Yáñez-Guerra LA, Lamanna F, Conde-Fernández C, Kaessmann H, Elphick MR, Anadón R, Rodicio MC and Barreiro-Iglesias A (2019) Galanin in an Agnathan: Precursor Identification and Localisation of Expression in the Brain of the Sea Lamprey Petromyzon marinus. Front. Neuroanat. 13:83. doi: 10.3389/fnana.2019.00083

Abbreviations: B3, rhombencephalic Müller cell 3; Ch, optic chiasm; DCN, dorsal column nucleus; DHyp, dorsal hypothalamus; dLP, lateral pallium, dorsal part; fr, fasciculus retroflexus; Ha, habenula; Hyp, hypothalamus; IS, isthmus; lHa, left habenula; LHyp, lateral hypothalamus; LP, lateral pallium; M1-3, giant Müller cells 1 to 3; Ma, Mauthner neuron; MI, giant isthmic neuron (i.e., I1 neuron); MLFn, nucleus of the medial longitudinal fascicle; MP, medial pallium; NH, neurohypophysis; OB, olfactory bulb; ON, optic nerve; OT, optic tectum; ot, optic tract; P, pineal organ; PC, posterior commissure; PO, preoptic nucleus; PoC, postoptic commissure nucleus; PoR, postoptic recess; PT, pretectum; PTh, prethalamus; PTN, posterior tubercle nucleus; Rh, rhombencephalon; rHa, right habenula; SC, spinal cord; ShL, subhippocampal lobe; Sp, septum; Str, striatum; Th, thalamus; TS, torus semicircularis; VHyp, ventral hypothalamus; vLP, lateral pallium, ventral part; Vm, trigeminal motor nucleus; Xm, vagal motor nucleus; zl, zona limitans intrathalamica.

INTRODUCTION

The neuropetide galanin was named as such because in most species it contains an N-terminal glycine and a C-terminal alanine (Tatemoto et al., 1983). The mature galanin peptide comprises 29–30 residues and is cleaved from a pro-peptide precursor that also generates the longer galanin message-associated peptide (GMAP; 60 residues in humans). The N-terminal part of the mature galanin peptide is crucial for its biological activity and is highly conserved in jawed vertebrates. Galanin is expressed in the central and peripheral nervous systems and signals via three receptor subtypes to regulate many physiological processes, including feeding, arousal/sleep, learning and memory, pituitary hormone release, nerve regeneration, stress/anxiety, nociception/pain and thermoregulation (for reviews see Lang et al., 2007, 2015; Šípková et al., 2017).

The galanin pro-peptide has been identified biochemically or genetically in many jawed vertebrates, including mammalian and non-mammalian species, and the galaninergic system has been extensively characterized in the brain of jawed vertebrates (for reviews see Mensah et al., 2010; Lang et al., 2015). In mammals, including humans, galanin is widely expressed in the brain with galanin-expressing neuronal populations present in the telencephalon, hypothalamus and brainstem (Rökaeus et al., 1984; Skofitsch and Jacobowitz, 1985; Kaplan et al., 1988; Cortés et al., 1990; Elmquist et al., 1992; Kordower et al., 1992; Palkovits and Horváth, 1994; Cheung et al., 2001; Pérez et al., 2001; for a review see Jacobowitz et al., 2004). In amphibians, reptiles and birds, the telencephalon, hypothalamus, mesencephalon and rhombencephalon also contain galanin-expressing neurons (Lázár et al., 1991; Olivereau and Olivereau, 1992; Józsa and Mess, 1993; Jiménez et al., 1994). However, in fishes the expression of galanin appears to be more restricted to telencephalic and hypothalamic areas (Vallarino et al., 1991; Unniappan et al., 2004; Adrio et al., 2005; Rodríguez Díaz et al., 2011; for a review see Mensah et al., 2010).

In contrast to jawed vertebrates, there is very little information on the galanin system of jawless vertebrates or agnathans, which include lampreys. Agnathans occupy a key phylogenetic position at the base of the vertebrate tree, which makes them interesting models to understand the early evolution of neuropeptidergic systems in vertebrates. In addition, lampreys have complex life cycles with very different larval and adult stages in terms of their anatomy and feeding behavior, which provides an excellent model to understand the roles that a given neuropeptidergic system plays in different behavioral circumstances in the same species.

Only a few studies have looked at the organization of the galanin system in lampreys (Buchanan et al., 1987; Jiménez et al., 1996; Pombal and Puelles, 1999; Yáñez et al., 1999; Bosi et al., 2004). These studies were conducted using antibodies generated against porcine galanin and reported the distribution of galanin-like immunoreactivity in the spinal cord (Buchanan et al., 1987), brain (Jiménez et al., 1996), and parapineal organ (Yáñez et al., 1999) of adult lampreys. Galanin-like-immunoreactive (ir) fibers, but not immunoreactive neurons, are present in the spinal cord of adult lampreys, mainly in its lateral region (Buchanan et al., 1987). In the brain, galanin-like-ir neurons are present in the telencephalon, hypothalamus and prethalamus, but not in the mesencephalon or rhombencephalon (Jiménez et al., 1996). Galanin-like-ir fibers have been also described in different brain regions, including the prosencephalon, mesencephalon and rhombencephalon (Jiménez et al., 1996), and the parapineal organ (Yáñez et al., 1999) of adult lampreys. However, the galanin precursor transcript/peptide has not yet been identified in lampreys and the roles of galanin in the sea lamprey CNS are not known.

Here, we report the identification of the galanin precursor transcript of the sea lamprey *Petromyzon marinus* (*PmGalP*). Sequence analyses revealed that this pro-peptide contains galanin and GMAP peptide sequences. We also report the pattern of expression of *PmGalP* in the CNS of both larval and adult animals by means of *in situ* hybridization (ISH). Our results confirmed the presence of the known galanin-expressing periventricular neuronal populations of lampreys, but we also discovered the existence of other *PmGalP*-expressing neuronal populations, including the presence of laterally migrated neurons in the diencephalon and hypothalamus. Our results provide a genetic and neuroanatomical basis for future functional studies on the role of galanin and GMAP in the CNS of lampreys.

MATERIALS AND METHODS

Animals

Larval (n = 10) and adult (downstream migrating young adults, n = 2; upstream migrating adults, n = 3) sea lampreys, *P. marinus* L., were used for this study. Downstream migrating young adults and larvae (ammocoete: lengths comprised between 80 and 120 mm, 4–7 years old) were collected from the River Ulla (Galicia, Spain) with permission from the *Xunta de Galicia*. Upstream migrating adults were acquired from local suppliers. Adults were fixed freshly, and larvae were maintained in aquaria containing river sediment and with appropriate feeding, aeration and temperature conditions until the day of use. Before all experiments, animals were deeply anesthetized with 0.1% tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, United States) in fresh water and killed by decapitation. All experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and the *Conselleriìa do Medio Rural e do Mar* of the *Xunta de Galicia* (License Ref. JLPV/IId) and were performed in accordance with European Union and Spanish guidelines on animal care and experimentation.

Cloning and Sequencing of the *PmGalP* cDNA

The *PmGalP* sequence was identified in a custom annotation of protein-coding genes (unpublished data) based on the *P. marinus* germline genome (Smith et al., 2018). This sequence was deposited in GenBank under accession number MK977616.

Larvae (n = 5) were anesthetized as indicated above and the brain and spinal cord were dissected out under sterile conditions. Total RNA was isolated from these tissues using the TriPure reagent (Roche, Mannheim, Germany). The first-strand cDNA synthesis reaction from total RNA

was catalyzed with Superscript III reverse transcriptase (Invitrogen, Waltham, MA, United States) using random primers (hexamers; Invitrogen). For polymerase chain reaction (PCR) cloning, specific oligonucleotide primers (forward: 5′-TCTGCGTGCCATCATCGACT-3′; reverse: 5′-TTACGCTTAGCTCGCCACGA-3′) were designed based on the *PmGalP* transcript sequence. The amplified fragments were cloned into pGEM-T easy vectors (Promega, Madison, WI, United States) using standard protocols and sequenced by GATC Biotech (Cologne, Germany) using Sanger sequencing, which confirmed the original sequence.

Alignment of the *PmGalP* Sequence With Galanin Precursor Sequences From Other Vertebrates and Phylogenetic Analyses

The amino acid sequence of the PmGalP (GenBank; MK977616) was obtained by translation of the cDNA sequence using ExPASy (Gasteiger et al., 2003), and the signal peptide was predicted using SignalP 4.0 (Petersen et al., 2011). The PmGalP sequence was aligned with galanin precursors from a variety of vertebrate species, including mammals, sauropsids, lobe-finned fishes, ray-finned fishes, and cartilaginous fishes (see section "**Supplementary File S1**" for a list of the sequences used). The alignments shown in **Figure 1B** and **Supplementary Figure S1** were performed using MAFFT (Katoh et al., 2017), with the number of maximum iterations set to 1000 to ensure an optimal alignment. The scoring matrix used was BLOSUM62. The alignment generated was highlighted using the software BOXSHADE[1] with 80% conservation as the minimum. Finally, the sequences were highlighted in phylum-specific colors: mammals (purple), sauropsids (orange), lobe-finned fishes (yellow), ray-finned fishes (green), cartilaginous fishes (pink), and agnathans (blue).

A phylogenetic analysis of galanin precursors was performed using the Neighbor-Joining method (Saitou and Nei, 1987). The amino acid sequences of full- length precursors (see section "**Supplementary File S1**" for a list of the sequences) were aligned using MAFFTT and a tree was generated, the *Ciona intestinalis* galanin-like peptide precursor was designated as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap (Efron et al., 1996) test (1000 replicates) are shown next to the branches. The substitution model used was Jones-Taylor-Thornton Gamma distributed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The phylogenetic analysis was conducted in MEGA7 (Kumar et al., 2016).

In situ Hybridisation

Templates for *in vitro* transcription were prepared by PCR amplification as follows. A 352-base pair (bp) fragment of the *PmGalP* sequence was obtained using the primers described but in this case, the reverse primer included the sequence of the universal T7 promoter (TAAGCTTTAATACGACTCACTATAGGGAGA). For the generation of sense probes, the sequence of the T7 promoter was included in the forward primers. Digoxigenin (DIG)-labeled riboprobes were synthesized using the amplified fragments as templates and following standard protocols using a T7 polymerase (Nzytech, Lisbon, Portugal).

The methods employed for mRNA *in situ* hybridisation were the same as previously described for tyrosine hydroxylase, a 5-HT1a receptor and a $GABA_B$ receptor (Barreiro-Iglesias et al., 2010; Cornide-Petronio et al., 2013; Romaus-Sanjurjo et al., 2016). Briefly, the brains/rostral spinal cords of larvae and young and mature adults were dissected out and fixed by immersion for 12 h in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C. Then, they were cryoprotected with 30% sucrose in PBS, embedded in Tissue-Tek® O.C.T.™ Compound (Sakura, Torrance, CA, United States), frozen in liquid nitrogen-cooled isopentane, and cut serially on a cryostat (14μm thickness) in transverse planes. Sections were mounted on Superfrost® Plus glass slides (Menzel, Brunswick, Germany). The sections were incubated with the *PmGalP* DIG-labeled antisense riboprobe (1μg/mL) at 70°C overnight in hybridization mix and treated with RNAse A (Sigma) in the post-hybridization washes. Then, the sections were incubated with a sheep anti-DIG antibody conjugated to alkaline phosphatase (1:2000; Roche) overnight at 4°C. Staining was conducted in BM Purple (Roche) at 37°C until the signal was clearly visible. No staining was detected when using sense probes. Finally, the sections were mounted in Mowiol® (Sigma).

Imaging

Photomicrographs were obtained with an BX51 microscope equipped with a DP71 digital camera (Olympus, Tokyo, Japan). Plates of photomicrographs and minimal bright/contrast adjustments were performed with Photoshop CS (Adobe). Drawings were done with CorelDraw 2019.

Nomenclature

For the nomenclature of brain regions and brain nuclei we followed the nomenclature used by our group in recent studies on the organization of different neuronal systems (including neuropeptidergic systems) in the sea lamprey brain (Barreiro-Iglesias et al., 2017; Fernández-López et al., 2017). In some instances, equivalencies to nomenclatures used by other authors are mentioned in the results and discussion. The readers should take into account that in lampreys most mature neurons are located in periventricular locations in the brain and do not migrate away from the ventricle.

RESULTS

Identification of *PmGalP* and Sequence Analysis

Analysis of *P. marinus* germline genome sequence data revealed the occurrence of a candidate galanin precursor in

[1] www.ch.embnet.org/software/BOX_form.html

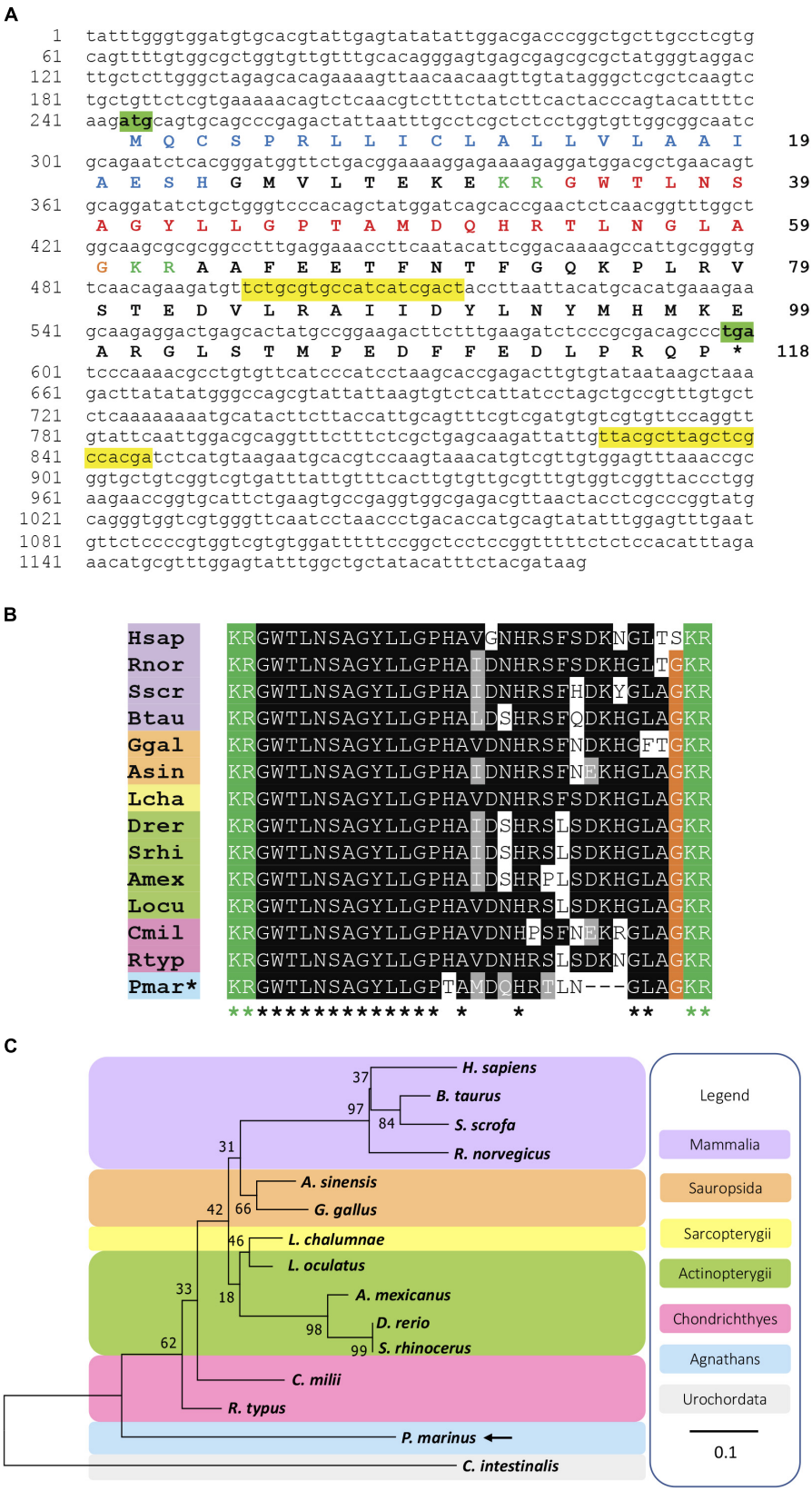


FIGURE 1 | Continued

FIGURE 1 | Identification of a galanin precursor in the sea lamprey *Petromyzon marinus*. **(A)** Nucleotide sequence (lower case) of a transcript that encodes the *Petromyzon marinus* galanin precursor (PmGalP; upper case). The start and stop codons are highlighted in green. The predicted signal peptide sequence is shown in blue and dibasic cleavage sites are shown in green. The putative galanin peptide derived from the precursor protein is shown in red, with the C-terminal glycine that is substrate for amidation shown in orange. The primers used for cloning of a fragment of *PmGalP* cDNA are highlighted in yellow. **(B)** Alignment of a region of PmGalP, including the galanin peptide bounded by dibasic cleavage sites, with the corresponding region of galanin precursor proteins from other vertebrate species. Conserved residues are highlighted, with conservation in more than 70% of sequences shown in black and with conservative substitutions shown in gray. **(C)** Neighbor-joining tree showing relationships of galanin-type precursors in selected chordate species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis was conducted in MEGA 7. The urochordate galanin-like sequence from *Ciona intestinalis* (Cint) was used to root the tree and is highlighted in gray. Species names in the alignment **(B)** are as follows: Hsap (*Homo sapiens*), Btau (*Bos taurus*), Rnor (*Rattus norvegicus*), Sscr (*Sus scrofa*), Ggal (*Gallus gallus*), Asin (*Alligator sinensis*), Lcha (*Latimeria chalumnae*), Srhi (*Sinocyclocheilus rhinocerous*), Drer (*Danio rerio*), Amex (*Astyanax mexicanus),* Locu *(Lepisosteus oculatus),* Rtyp *(Rhincodon typus)*, Cmil (*Callorhinchus milii*), Pmar (*Petromyzon marinus*). Additionally, in the alignment **(B)** and the phylogenetic tree **(C)**, species names are highlighted in taxon-specific colors: purple (mammals), orange (sauropsids), yellow (lobe-finned fishes), green (ray-finned fishes), pink (cartilaginous fishes), blue (agnathans). The accession numbers and the alignment of the sequences used to build this phylogenetic tree are shown in **Supplementary File S1**.

P. marinus (*PmGalP*; GenBank accession number MK977616). PmGalP is a 118-residue protein (**Figure 1**) with a 23-residue signal peptide, a 26 residue galanin-like peptide bounded by dibasic cleavage sites (**Figure 1A**) and a 56-residue galanin-associated peptide-like sequence that spans from the second dibasic cleavage site to the C-terminus of the precursor (**Supplementary Figure S1**).

The sequence of the predicted C-terminally amidated mature peptide was aligned with galanin-type peptides from other vertebrates, including mammals, sauropsids, lobe-finned fishes, ray-finned fishes, and cartilaginous fishes. Comparison of the *P. marinus* galanin with gnathostome galanins revealed both similarities and differences. Comprising 26 residues, *P. marinus* galanin is shorter than gnathostome galanins, which are 29 or 30 residues in length (**Figures 1A,B**). However, the first thirteen residues of *P. marinus* galanin are identical to gnathostome galanins (**Figure 1B**). The residue at position 14 (histidine, H) is conserved in all gnathostome galanins, whereas in *P. marinus* galanin this position is occupied by a threonine (T) residue, which is a non-conservative substitution. Positions 15 to 21 in *P. marinus* galanin have conservative substitutions with respect to gnathostome galanins, but by comparison with human galanin positions 22 and 23 in *P. marinus* galanin have non-conservative substitutions of Phenylalanine (F) with Leucine (L), and of Serine (S) with Asparagine (N), respectively. However, this feature is not unique to *P. marinus* galanin, because differences at position 22 are also seen in all the ray-finned fishes and in the cartilaginous fish *Rhincodon typus* and differences at position 23 are also seen in two sauropsids and in the cartilaginous fish *Callorhinchus milii*. Residues at positions 24 to 26 in gnathostome galanins are missing in *P. marinus* galanin but the C-terminal GLAamide of *P. marinus* galanin is a highly conserved feature of most gnathostome galanins (**Figure 1B**).

Based on an alignment of PmGalP with fourteen other galanin-type precursor protein sequences, a phylogenetic reconstruction was made using the neighbor-joining method with the galanin-type precursor from the urochordate *C. intestinalis* used to root the tree. The phylogenetic analysis of precursors shows that the PmGalP occupies a position in the tree consistent with the basal phylogenetic position of agnathans in vertebrate phylogeny (**Figure 1C**).

Distribution of *PmGalP*-Expressing Neuronal Populations in the Lamprey Brain

The expression of the *PmGalP* transcript in the CNS of the sea lamprey was analyzed using mRNA *in situ* hybridisation. Expression of *PmGalP* was restricted to the prosencephalon and no expression was detected in the mesencephalon, rhombencephalon or spinal cord of both larval (**Figure 2**) and adult (**Figure 3**) sea lampreys.

Larvae

The distribution of *PmGalP*-positive (*PmGalP*+) neurons was analyzed in larvae with body lengths between 80 and 120 mm (**Figure 2**). *PmGalP*+ neurons were found in two telencephalic regions (**Figures 2B,C,F,G**). The most rostral population of *PmGalP*+ cells was found in a periventricular location in the septum (septocommissural preoptic area of Pombal et al., 2009; **Figures 2B,F**). Strongly stained *PmGalP*+ neurons were also found in the preoptic nucleus (**Figures 2C,G**). This preoptic population appeared as a caudal continuation of the septal population.

In the alar diencephalon, a group of strongly stained *PmGalP*+ cells was observed in the rostral part of the prethalamus (prosomere 3; see Pombal et al., 2009). In this region, most of the *PmGalP*+ cells are located in the periventricular area (**Figures 2D,H**), but some laterally displaced *PmGalP*+ cells were also observed (**Figures 2D,H**). In the hypothalamus, numerous *PmGalP*+ cells were observed in the periventricular area of the infundibular recess (ventral hypothalamus; **Figures 2E,I**). Some of these cells showed a strongly stained dendrite crossing the ependymal layer, suggesting that they are cerebrospinal fluid-contacting cells (**Figure 2I**). Some laterally displaced *PmGalP*+ cells were also present in this hypothalamic region (**Figures 2E,I**).

Adults

We investigated possible changes in the *PmGalP*+ populations after metamorphosis and during sexual maturation by analyzing brains of young downstream (about 17 cm in length) and mature upstream (about 85 cm in length) migrating adult sea lampreys (**Figure 3**). The general distribution of *PmGalP*+ cells

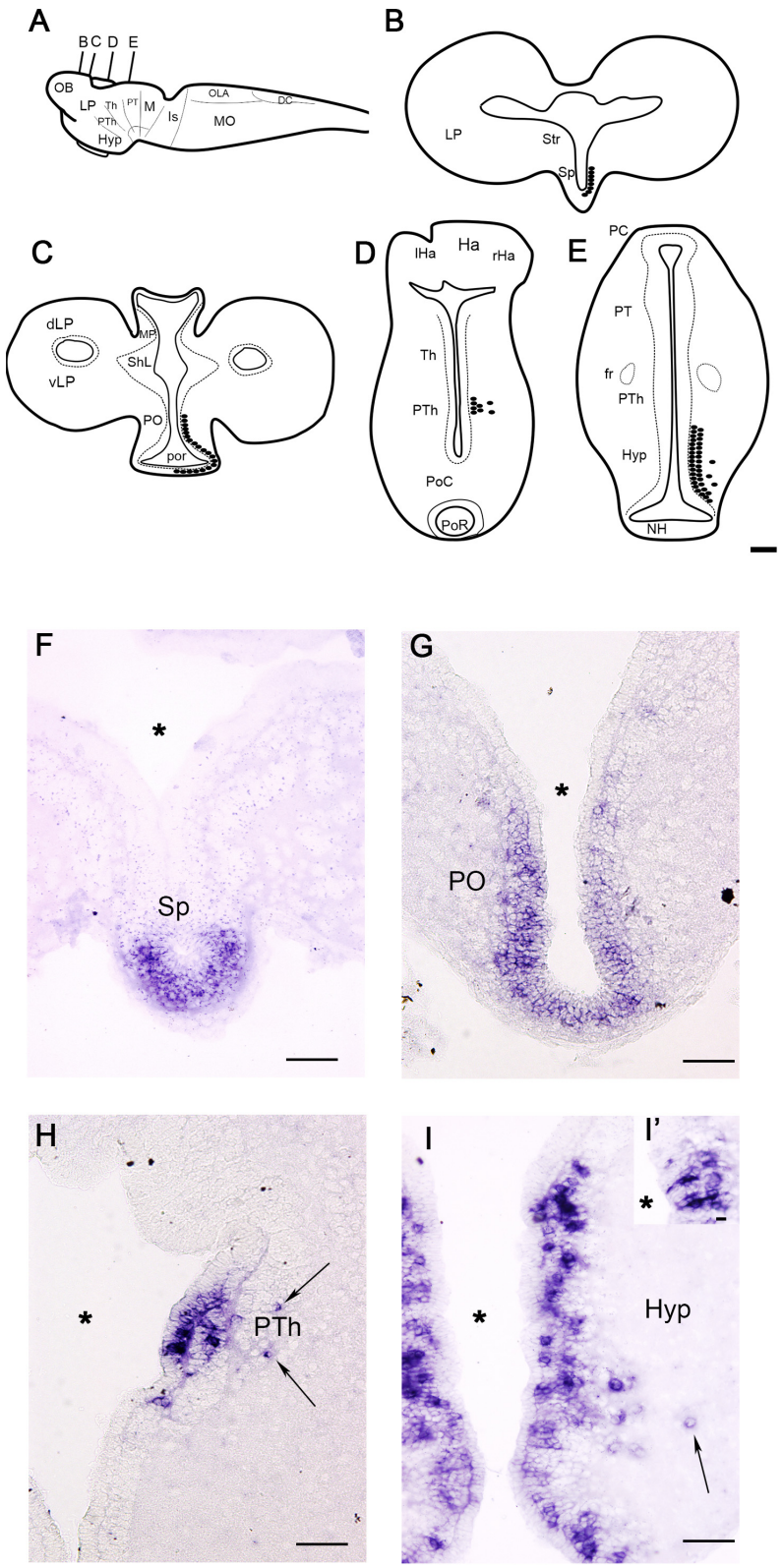


FIGURE 2 | Schematic drawings **(A–E)** and photomicrographs **(F–I)** of sections of the larval sea lamprey brain showing the distribution of *PmGalP* expressing neurons. For abbreviations, see list. The plane of section of schematic drawings **B–E** is indicated in **A**. Arrows indicate the presence of laterally migrated cells. The asterisks indicate the ventricles. A detail of CSF-c cells of the hypothalamus is shown in **I'**. Dorsal is to the top. Scale bars: 100 μm.

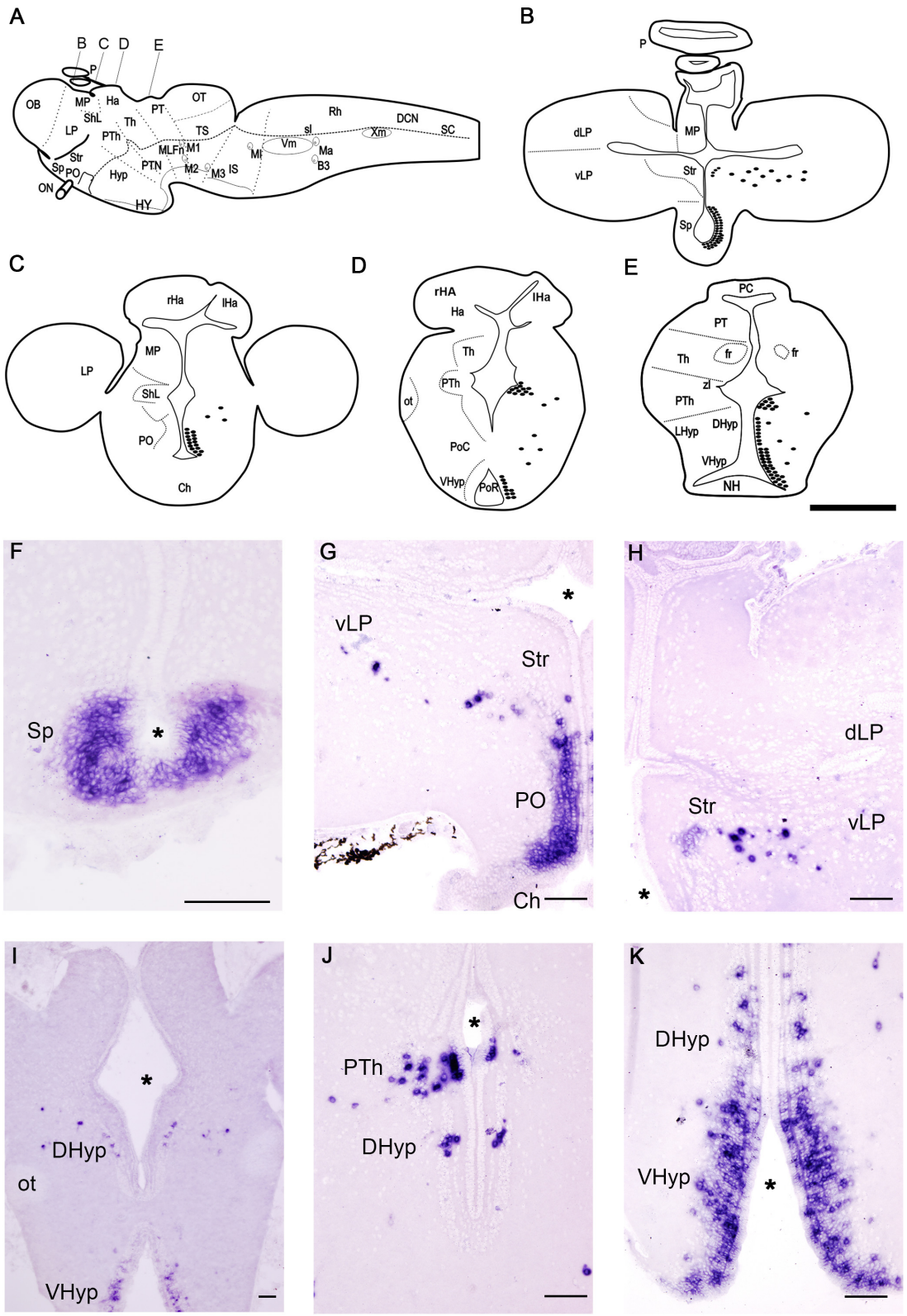


FIGURE 3 | Schematic drawings **(A–E)** and photomicrographs **(F–K)** of sections of the adult sea lamprey brain showing the distribution of *PmGalP* expressing neurons. For abbreviations, see list. **I** is a photomicrograph of an upstream migrating adult sea lamprey, the rest of the photomicrographs are from young adults. The plane of section of schematic drawings **B–E** is indicated in **A**. The asterisks indicate the ventricles. Dorsal is to the top. Scale bars: 100 μm.

in young and mature adult lampreys is similar and therefore the description below of *PmGalP*+ cells in adult lampreys is based on our analysis of both young and mature animals.

As in larvae, the most rostral *PmGalP*+ population was observed in the periventricular area of the septum (**Figures 3B,F**). The preoptic nucleus of adult sea lampreys also contained strongly stained *PmGalP*+ cells (**Figures 3C,G**). Interestingly, a new and conspicuous population of *PmGalP*+ cells was found dispersed in the adult telencephalon in a region ranging from the area lateral to the dorsal part of the preoptic nucleus to the ventral part of the lateral pallium (**Figures 3B,C,G,H**). In adult lampreys, some weakly stained *PmGalP*+ cells were also observed in the characteristic cell band of the striatum (**Figures 3B,G,H**).

In the adult sea lamprey diencephalon, a *PmGalP*+ population was also found in the prethalamus. These *PmGalP*+ cells were found in the rostral prethalamus as in larvae, but they also extended more caudally in adults (**Figures 3D,E,J**). Laterally displaced *PmGalP*+ cells were also present in the prethalamus (**Figures 3D,E,J**). These displaced cells of the prethalamus appeared to be in continuity with those of the telencephalon (see previous paragraph). In the dorsal and ventral hypothalamus of adult lampreys, a large group of *PmGalP*+ cells was observed in periventricular layers around both the post-optic and the infundibular recesses. In the ventral hypothalamus, these cells were strongly stained and occupied three to four compact rows of cells. In the dorsal hypothalamus, we observed the presence of fewer *PmGalP*+ *cells* and these were less densely packed (**Figures 3E,J,K**). As in larvae, laterally displaced *PmGalP*+ cells were also observed in the hypothalamus, although these cells were more numerous than in larvae (**Figures 3E,I–K**).

DISCUSSION

Galanin is a 29-residue neuropeptide in vertebrates (30 residues in humans) with numerous endocrine activities. Exogenously administered galanin has many biological actions, including inhibition of acetylcholine and insulin release, stimulation of feeding, modulation of spinal nociceptive flexor reflexes, inhibition of gastric acid secretion and reduction of alcohol consumption (Ch'ng et al., 1985; Amiranoff et al., 1989; Xu et al., 1990, 1995; Crawley, 1995; Kask et al., 1995; Millón et al., 2019). The amino acid sequence of gnathostome galanins is in general very conserved, as they only differ in five amino acid residues. Notably, most of these differences are in the C-terminal region from residues 16 to 30, whereas residues 1–15 are highly conserved (Fisone et al., 1989; Land et al., 1991; Mensah et al., 2010). In this study, we report the identification of a galanin precursor in the agnathan *P. marinus* (PmGalP). PmGalP contains a predicted C-terminally amidated peptide comprising 26 residues, which is 3 to 4 residues shorter than galanins found in other vertebrates. An alignment of the *P. marinus* galanin with galanins from gnathostomes shows that the lamprey galanin is the most divergent of the sequences reported thus far in vertebrates, with several non-conservative amino acid substitutions. Furthermore, *P. marinus* galanin does not align completely with gnathostome galanins in the C-terminal region, due its shorter length. However, the first thirteen residues are identical to those in gnathostome galanins and residues 15 to 21 comprise a combination of conserved and non-conserved residues (**Figure 1B**).

Interestingly, receptor binding assays and *in vivo* pharmacological experiments have demonstrated that the N-terminal region of galanins is the most important region for the activation of galanin receptors and subsequent biological actions. Experiments using different fragments of galanins demonstrated that synthetic galanin containing only the first 15 or 16 residues, GAL(1–15) and GAL(1–16), binds to galanin receptors with affinity in the nanomolar range, with a fivefold lower affinity compared to full-length galanin. In contrast, synthetic galanin containing residues 17–29 of galanin, GAL(17–29), has 10,000-fold lower affinity compared to galanin. This suggests that the C-terminal residues 17–29 contribute very little to receptor binding and activation (Fisone et al., 1989; Lagny-Pourmir et al., 1989; Gallwitz et al., 1990). Furthermore, *in vivo* analysis of the inhibitory effects of galanin on gastric acid secretion in rats revealed that N-terminal fragments of galanin (GAL 1–10) and (GAL 1–15) retain approximately 60% of the activity of full-length galanin, whilst a C-terminal fragment (GAL 15–29) had no bioactivity when tested at the same dose ranges as galanin and the fragment (GAL 9–29) retained only 5% of activity of full-length galanin (Rossowski and Coy, 1989; Mungan et al., 1992). These findings are consistent with the finding that the N-terminal 13-amino acid residues of galanin are conserved in vertebrates, including *P. marinus*, whereas the C-terminal region of galanins is much more variable and most notably in *P. marinus*. Therefore, the divergence in the C-terminal region of *P. marinus* galanin by comparison with gnathostome galanins likely reflects lack of selection pressure because this region is less important than the N-terminal region for receptor activation and bioactivity.

Previous studies on the organization of the galaninergic system in the CNS of lampreys were performed only in adults and using antibodies generated against porcine galanin (Buchanan et al., 1987; Jiménez et al., 1996; Yáñez et al., 1999). Here, we generated specific riboprobes against the *PmGalP* and analyzed its expression in the CNS of larval and adult sea lampreys using *in situ* hybridisation. This confirmed the presence of previously reported (Jiménez et al., 1996) galanin-like-ir periventricular cell populations of the sea lamprey prosencephalon (septal, hypothalamic and prethalamic populations) and galanin-like-ir laterally migrated telencephalic cells. However, Jiménez et al. (1996) used an outdated neuroanatomical nomenclature in their immunohistochemical study, with the septal region identified as the *nucleus commissurae anterior* by these authors. Our analysis using *in situ* hybridisation also identified strong *PmGalP* expression in the preoptic area in continuation with the septal population, the presence of weakly stained striatal *PmGalP*+ cells and the presence of laterally migrated *PmGalP*+ cells in the prethalamus and hypothalamus. These galaninergic populations were not previously reported by Jiménez et al. (1996) in their immunohistochemical study. The reasons for these discrepancies might be related to the sensitivity of the

porcine antibodies used or to the differential accumulation of *PmGalP* transcripts and mature galanin peptide in the soma and fibers of galaninergic neurons. In addition, we extended our analyses to the larval brain showing that most of the galaninergic populations are already present before the metamorphosis, with the exception of the laterally migrated and striatal cells of the telencephalon, which were only present in adult lampreys.

The advantage of previous immunohistochemical studies is that they revealed the presence of extensive galanin-like-ir innervation of the brain (Jiménez et al., 1996), parapineal organ (Yáñez et al., 1999), and spinal cord (Buchanan et al., 1987). Our study confirms the lack of galaninergic cells in the spinal cord and brainstem, which suggests that the galanin-like-ir fibers of the lamprey spinal cord reported by Buchanan et al. (1987) must be of hypothalamic origin. The hypothalamus is the only brain region with *PmGalP* expressing neurons that also contains descending neurons that project to the spinal cord in lampreys (Barreiro-Iglesias et al., 2008). This should be experimentally confirmed in future hodological studies.

As noted by Jiménez et al. (1996), comparison with other vertebrates shows that the distribution of galaninergic neuronal populations in lampreys is similar to that of jawed fishes, since in both groups galanin-expressing neurons are mainly restricted to the prosencephalon. This is in striking contrast to amphibians, reptiles, birds and mammals, in which galaninergic cell populations are present also in the mesencephalon and rhombencephalon (see section "Introduction"). For example, in the brainstem of mammals, including humans, galanin expression is prominent in the locus coeruleus (Melander et al., 1986; Holets et al., 1988; Xu et al., 1998; Le Maître et al., 2013). Interestingly, tyrosine hydroxylase *in situ* hybridization and immunohistochemical studies indicate that lampreys do not have a locus coeruleus (Pierre et al., 1997; Barreiro-Iglesias et al., 2010), which suggests that these features evolved after the split of jawless and jawed vertebrates. So, evolution of the galaninergic system in vertebrates involved an increase in the number of mesencephalic and brainstem populations. Other neuronal systems, as serotonergic (Parent, 1984; Pierre et al., 1992) and glycinergic (Villar-Cerviño et al., 2008) systems, have also evolved with an increase in caudal populations. In contrast, present and previous results show that the presence of septal and hypothalamic galaninergic neuronal populations is a highly conserved character in all vertebrates (Goodson et al., 2004; Adrio et al., 2005). The galaninergic septal neurons have been implicated in the regulation of social behavior in birds and mammals (Goodson et al., 2004), whereas galaninergic hypothalamic neurons are mainly implicated in the regulation of feeding in fishes and mammals (Leibowitz et al., 1998; Sahu, 1998; Volkoff et al., 2005). Interestingly, both larval and adult lampreys have *PmGalP*+ neurons in their septum and hypothalamus. Therefore, the lamprey would be an interesting vertebrate model to investigate the roles of galanin in these brain regions in context of very different developmental stages in terms of social and feeding behaviors. Our study provides a molecular and neuroanatomical basis for future functional studies on the role of galanin and GAMP in these and other brain regions of lampreys.

DATA AVAILABILITY

The datasets generated for this study can be found in GenBank under accession number MK977616.

ETHICS STATEMENT

This animal study was reviewed and approved by the Bioethics Committee at the University of Santiago de Compostela and the Consellería do Medio Rural e do Mar of the *Xunta de Galicia* (License Ref. JLPV/IId).

AUTHOR CONTRIBUTIONS

DS-C, LY-G, FL, CC-F, and HK contributed to the acquisition of experimental data. DS-C, LY-G, ME, RA, MR, and AB-I contributed to the data analysis and interpretation, and drafting of the manuscript. AB-I contributed to the concept and design of the study. All authors have approved the final manuscript.

FUNDING

Grant sponsors: Spanish Ministry of Economy and Competitiveness and the European Regional Development Fund 2007–2013 (Grant number: BFU-2017-87079-P). LY-G was supported by a Ph.D. studentship awarded by the Mexican Council of Science and Technology (CONACyT studentship no. 418612), and Queen Mary University of London.

ACKNOWLEDGMENTS

The authors thank the staff of Ximonde Biological Station for providing the lampreys used in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnana.2019.00083/full#supplementary-material

FIGURE S1 | Alignment of selected galanin precursors from vertebrates used for the identification of signal peptides (underlined in blue), mature peptides (underlined in red), and galanin-associated peptides (underlined in purple). Conserved residues are highlighted. Conservation in more than 70% of sequences is highlighted in black, conservative substitutions are highlighted in gray. Species names are as follows: Hsap (*Homo sapiens*), Btau (*Bos taurus*), Rnor (*Rattus norvegicus*), Sscr (*Sus scrofa*), Ggal (*Gallus gallus*), Asin (*Alligator sinensis*), Lcha (*Latimeria chalumnae*), Srhi (*Sinocyclocheilus rhinocerous*), Drer (*Danio rerio*), Amex (*Astyanax mexicanus),* Locu *(Lepisosteus oculatus),* Rtyp *(Rhincodon typus)*, Cmil (*Callorhinchus milii*), and Pmar (*Petromyzon marinus*). Accession numbers are shown next to the names.

FILE S1 | Sequences used for the phylogenetic reconstruction in **Figure 1C**.

REFERENCES

Adrio, F., Rodríguez, M. A., and Rodríguez-Moldes, I. (2005). Distribution of galanin-like immunoreactivity in the brain of the Siberian sturgeon (*Acipenser baeri*). *J. Comp. Neurol.* 487, 54–74. doi: 10.1002/cne.20536

Amiranoff, B., Lorinet, A. M., Yanaihara, N., and Laburthe, M. (1989). Structural requirement for galanin action in the pancreatic beta cell line Rin m 5F. *Eur. J. Pharmacol.* 163, 205–207. doi: 10.1016/0014-2999(89)90421-4

Barreiro-Iglesias, A., Fernández-López, B., Sobrido-Cameán, D., and Anadón, R. (2017). Organization of alpha-transducin immunoreactive system in the brain and retina of larval and young adult Sea Lamprey (*Petromyzon marinus*), and their relationship with other neural systems. *J. Comp. Neurol.* 525, 3683–3704. doi: 10.1002/cne.24296

Barreiro-Iglesias, A., Laramore, C., Shifman, M. I., Anadón, R., Selzer, M. E., and Rodicio, M. C. (2010). The sea lamprey tyrosine hydroxylase: cDNA cloning and in situ hybridization study in the brain. *Neuroscience* 168, 659–669. doi: 10.1016/j.neuroscience.2010.04.025

Barreiro-Iglesias, A., Villar-Cerviño, V., Anadón, R., and Rodicio, M. C. (2008). Descending brain-spinal cord projections in a primitive vertebrate, the lamprey: cerebrospinal fluid-contacting and dopaminergic neurons. *J. Comp. Neurol.* 511, 711–723. doi: 10.1002/cne.21863

Bosi, G., Shinn, A. P., Giari, L., Arrighi, S., and Domeneghini, C. (2004). The presence of a galanin-like peptide in the gut neuroendocrine system of *Lampetra fluviatilis* and *Acipenser transmontanus*: an immunohistochemical study. *Tissue Cell* 36, 283–292. doi: 10.1016/j.tice.2004.04.004

Buchanan, J. T., Brodin, L., Hökfelt, T., Van Dongen, P. A., and Grillner, S. (1987). Survey of neuropeptide-like immunoreactivity in the lamprey spinal cord. *Brain Res.* 408, 299–302. doi: 10.1016/0006-8993(87)90392-1

Cheung, C. C., Hohmann, J. G., Clifton, D. K., and Steiner, R. A. (2001). Distribution of galanin messenger RNA-expressing cells in murine brain and their regulation by leptin in regions of the hypothalamus. *Neuroscience* 103, 423–432. doi: 10.1016/s0306-4522(01)00012-4

Ch'ng, J. L., Christofides, N. D., Anand, P., Gibson, S. J., Allen, Y. S., Su, H. C., et al. (1985). Distribution of galanin immunoreactivity in the central nervous system and the responses of galanin-containing neuronal pathways to injury. *Neuroscience* 16, 343–354. doi: 10.1016/0306-4522(85)90007-7

Cornide-Petronio, M. E., Anadón, R., Rodicio, M. C., and Barreiro-Iglesias, A. (2013). The sea lamprey tryptophan hydroxylase: new insight into the evolution of the serotonergic system of vertebrates. *Brain Struct. Funct.* 218, 587–593. doi: 10.1007/s00429-012-0412-7

Cortés, R., Villar, M. J., Verhofstad, A., and Hökfelt, T. (1990). Effects of central nervous system lesions on the expression of galanin: a comparative in situ hybridization and immunohistochemical study. *Proc. Natl. Acad. Sci. U.S.A.* 87, 7742–7746. doi: 10.1073/pnas.87.19.7742

Crawley, J. N. (1995). Biological actions of galanin. *Regul. Pept.* 59, 1–16. doi: 10.1016/0167-0115(95)00083-n

Efron, B., Halloran, E., and Holmes, S. (1996). Bootstrap confidence levels for phylogenetic trees. *Proc. Natl. Acad. Sci. U.S.A.* 93, 7085–7090. doi: 10.1073/pnas.93.14.7085

Elmquist, J. K., Fox, C. A., Ross, L. R., and Jacobson, C. D. (1992). Galanin-like immunoreactivity in the adult and developing Brazilian opossum brain. *Brain Res. Dev. Brain Res.* 67, 161–179. doi: 10.1016/0165-3806(92)90217-k

Fernández-López, B., Sobrido-Cameán, D., Anadón, R., Rodicio, M. C., and Barreiro-Iglesias, A. (2017). Restricted co-localization of glutamate and dopamine in neurons of the adult sea lamprey brain. *J. Anat.* 231, 776–784. doi: 10.1111/joa.12674

Fisone, G., Berthold, M., Bedecs, K., Undén, A., Bartfai, T., Bertorelli, R., et al. (1989). N-terminal galanin-(1-16) fragment is an agonist at the hippocampal galanin receptor. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9588–9591. doi: 10.1073/pnas.86.23.9588

Gallwitz, B., Schmidt, W. E., Schwarzhoff, R., and Creutzfeldt, W. (1990). Galanin: structural requirements for binding and signal transduction in RINm5F insulinoma cells. *Biochem. Biophys. Res. Commun.* 172, 268–275. doi: 10.1016/s0006-291x(05)80204-9

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A. (2003). ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* 31, 3784–3788. doi: 10.1093/nar/gkg563

Goodson, J. L., Evans, A. K., and Lindberg, L. (2004). Chemoarchitectonic subdivisions of the songbird septum and a comparative overview of septum chemical anatomy in jawed vertebrates. *J. Comp. Neurol.* 473, 293–314. doi: 10.1002/cne.20061

Holets, V. R., Hökfelt, T., Rökaeus, A., Terenius, L., and Goldstein, M. (1988). Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. *Neuroscience* 24, 893–906. doi: 10.1016/0306-4522(88)90076-0

Jacobowitz, D. M., Kresse, A., and Skofitsch, G. (2004). Galanin in the brain: chemoarchitectonics and brain cartography–a historical review. *Peptides* 25, 433–464. doi: 10.1016/j.peptides.2004.02.015

Jiménez, A. J., Mancera, J. M., Pérez-Fígares, J. M., and Fernández-Llebrez, P. (1994). Distribution of galanin-like immunoreactivity in the brain of the turtle *Mauremys caspica*. *J. Comp. Neurol.* 349, 73–84. doi: 10.1002/cne.903490106

Jiménez, A. J., Mancera, J. M., Pombal, M. A., Pérez-Fígares, J. M., and Fernández-Llebrez, P. (1996). Distribution of galanin-like immunoreactive elements in the brain of the adult lamprey *Lampetra fluviatilis*. *J. Comp. Neurol.* 368, 185–197.

Józsa, R., and Mess, B. (1993). Galanin-like immunoreactivity in the chicken brain. *Cell Tissue Res.* 273, 391–399. doi: 10.1007/bf00312842

Kaplan, L. M., Spindel, E. R., Isselbacher, K. J., and Chin, W. W. (1988). Tissue-specific expression of the rat galanin gene. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1065–1069.

Kask, K., Langel, U., and Bartfai, T. (1995). Galanin–a neuropeptide with inhibitory actions. *Cell Mol. Neurobiol.* 15, 653–673. doi: 10.1007/bf02071130

Katoh, K., Rozewicki, J., and Yamada, K. D. (2017). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform.* doi: 10.1093/bib/bbx108 [Epub ahead of print].

Kordower, J. H., Le, H. K., and Mufson, E. J. (1992). Galanin immunoreactivity in the primate central nervous system. *J. Comp. Neurol.* 319, 479–500. doi: 10.1002/cne.903190403

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lagny-Pourmir, I., Lorinet, A. M., Yanaihara, N., and Laburthe, M. (1989). Structural requirements for galanin interaction with receptors from pancreatic beta cells and from brain tissue of the rat. *Peptides* 10, 757–761. doi: 10.1016/0196-9781(89)90109-5

Land, T., Langel, U., and Bartfai, T. (1991). Hypothalamic degradation of galanin(1-29) and galanin(1-16): identification and characterization of the peptidolytic products. *Brain Res.* 558, 245–250. doi: 10.1016/0006-8993(91)90775-q

Lang, R., Gundlach, A. L., Holmes, F. E., Hobson, S. A., Wynick, D., Hökfelt, T., et al. (2015). Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity. *Pharmacol. Rev.* 67, 118–175. doi: 10.1124/pr.112.006536

Lang, R., Gundlach, A. L., and Kofler, B. (2007). The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. *Pharmacol. Ther.* 115, 177–207. doi: 10.1016/j.pharmthera.2007.05.009

Lázár, G. Y., Liposits, Z. S., Tóth, P., Trasti, S. L., Maderdrut, J. L., and Merchenthaler, I. (1991). Distribution of galanin-like immunoreactivity in the brain of *Rana esculenta* and *Xenopus laevis*. *J. Comp. Neurol.* 310, 45–67. doi: 10.1002/cne.903100106

Le Maître, E., Barde, S. S., Palkovits, M., Diaz-Heijtz, R., and Hökfelt, T. G. (2013). Distinct features of neurotransmitter systems in the human brain with focus on the galanin system in locus coeruleus and dorsal raphe. *Proc. Natl. Acad. Sci. U.S.A.* 110, 536–545. doi: 10.1073/pnas.1221378110

Leibowitz, S. F., Akabayashi, A., and Wang, J. (1998). Obesity on a high-fat diet: role of hypothalamic galanin in neurons of the anterior paraventricular nucleus projecting to the median eminence. *J. Neurosci.* 18, 2709–2719. doi: 10.1523/jneurosci.18-07-02709.1998

Melander, T., Hökfelt, T., Rökaeus, A., Cuello, A. C., Oertel, W. H., Verhofstad, A., et al. (1986). Coexistence of galanin-like immunoreactivity with catecholamines, 5-hydroxytryptamine, GABA and neuropeptides in the rat CNS. *J. Neurosci.* 6, 3640–3654. doi: 10.1523/jneurosci.06-12-03640.1986

Mensah, E. T., Volkoff, H., and Unniappan, S. (2010). Galanin systems in non-mammalian vertebrates with special focus on fishes. *Exp. Suppl.* 102, 243–262. doi: 10.1007/978-3-0346-0228-0_17

Millón, C., Flores-Burgess, A., Castilla-Ortega, E., Gago, B., García-Fernandez, M., Serrano, A., et al. (2019). Central administration of galanin N-terminal fragment 1-15 decreases the voluntary alcohol intake in rats. *Addiction* 24, 76–87. doi: 10.1111/adb.12582

Mungan, Z., Ozmen, V., Ertan, A., Coy, D. H., Baylor, L. M., Rice, J. C., et al. (1992). Structural requirements for galanin inhibition of pentagastrin-stimulated gastric acid secretion in conscious rats. *Eur. J. Pharmacol.* 214, 53–57. doi: 10.1016/0014-2999(92)90095-l

Olivereau M., and Olivereau, J. M. (1992). Immunocytochemical localization of a galanin-like peptidergic system in the brain of two urodele and two anuran species (Amphibia). *Histochemistry* 98, 51–66. doi: 10.1007/bf00716937

Palkovits, M., and Horváth, S. (1994). Galanin immunoreactive neurons in the medulla oblongata of rats. *Acta Biol. Hung.* 45, 399–417.

Parent, A. (1984). Functional anatomy and evolution of monoaminergic systems. *Am. Zool.* 24, 783–790. doi: 10.1186/1741-7007-10-45

Pérez, S. E., Wynick, D., Steiner, R. A., and Mufson, E. J. (2001). Distribution of galaninergic immunoreactivity in the brain of the mouse. *J. Comp. Neurol.* 434, 158–185. doi: 10.1002/cne.1171

Petersen, T. N., Brunak, S., Heijne, G., and von Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. doi: 10.1038/nmeth.1701

Pierre, J., Mahouche, M., Suderevskaya, E. I., Repérant, J., and Ward, R. (1997). Immunocytochemical localization of dopamine and its synthetic enzymes in the central nervous system of the lamprey *Lampetra fluviatilis*. *J. Comp. Neurol.* 380, 119–135. doi: 10.1002/(sici)1096-9861(19970331)380:1<119::aid-cne9>3.3.co;2-j

Pierre, J., Repèrant, J., Ward, R., Vesselkin, N. P., Rio, J. P., Miceli, D., et al. (1992). The serotoninergic system of the brain of the lamprey, *Lampetra fluviatilis*: an evolutionary perspective. *J. Chem. Neuroanat.* 5, 195–219. doi: 10.1016/0891-0618(92)90046-s

Pombal, M. A., Megías, M., Bardet, S. M., and Puelles, L. (2009). New and old thoughts on the segmental organization of the forebrain in lampreys. *Brain Behav. Evol.* 74, 7–19. doi: 10.1159/000229009

Pombal, M. A., and Puelles, L. (1999). Prosomeric map of the lamprey forebrain based on calretinin immunocytochemistry, Nissl stain, and ancillary markers. *J. Comp. Neurol.* 414, 391–422. doi: 10.1002/(sici)1096-9861(19991122)414:3<391::aid-cne8>3.3.co;2-f

Rodríguez Díaz, M. A., Candal, E., Santos-Durán, G. N., Adrio, F., and Rodríguez-Moldes, I. (2011). Comparative analysis of Met-enkephalin, galanin and GABA immunoreactivity in the developing trout preoptic-hypophyseal system. *Gen. Comp. Endocrinol.* 173, 148–158. doi: 10.1016/j.ygcen.2011.05.012

Rökaeus, A., Melander, T., Hökfelt, T., Lundberg, J. M., Tatemoto, K., Carlquist, M., et al. (1984). A galanin-like peptide in the central nervous system and intestine of the rat. *Neurosci. Lett.* 47, 161–166. doi: 10.1016/0304-3940(84)90423-3

Romaus-Sanjurjo, D., Fernández-López, B., Sobrido-Cameán, D., Barreiro-Iglesias, A., and Rodicio, M. C. (2016). Cloning of the GABA(B) receptor subunits B1 and B2 and their expression in the central nervous system of the adult sea lamprey. *Front. Neuroanat.* 10:118. doi: 10.3389/fnana.2016.00118

Rossowski, W. J., and Coy, D. H. (1989). Inhibitory action of galanin on gastric acid secretion in pentobarbital-anesthetized rats. *Life Sci.* 44, 1807–1813. doi: 10.1016/0024-3205(89)90297-x

Sahu, A. (1998). Evidence suggesting that galanin (GAL), melanin-concentrating hormone (MCH), neurotensin (NT), proopiomelanocortin (POMC) and neuropeptide Y (NPY) are targets of leptin signaling in the hypothalamus. *Endocrinology* 139, 795–798. doi: 10.1210/en.139.2.795

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Šípková, J., Kramáriková, I., Hynie, S., and Klenerová, V. (2017). The galanin and galanin receptor subtypes, its regulatory role in the biological and pathological functions. *Physiol. Res.* 66, 729–740.

Skofitsch, G., and Jacobowitz, D. M. (1985). Immunohistochemical mapping of galanin-like neurons in the rat central nervous system. *Peptides* 6, 509–546. doi: 10.1016/0196-9781(85)90118-4

Smith, J. J., Timoshevskaya, N., Ye, C., Holt, C., Keinath, M. C., Parker, H. J., et al. (2018). The sea lamprey germline genome provides insights into programmed genome rearrangement and vertebrate evolution. *Nat. Genet.* 50, 270–277. doi: 10.1038/s41588-017-0036-1

Tatemoto, K., Rökaeus, A., Jörnvall, H., McDonald, T. J., and Mutt, V. (1983). Galanin - a novel biologically active peptide from porcine intestine. *FEBS Lett.* 164, 124–128. doi: 10.1016/0014-5793(83)80033-7

Unniappan, S., Cerdá-Reverter, J. M., and Peter, R. E. (2004). In situ localization of preprogalanin mRNA in the goldfish brain and changes in its expression during feeding and starvation. *Gen Comp Endocrinol.* 136, 200–207. doi: 10.1016/j.ygcen.2003.12.010

Vallarino, M., Feuilloley, M., Vandesande, F., and Vaudry, H. (1991). Immunohistochemical mapping of galanin-like immunoreactivity in the brain of the dogfish *Scyliorhinus canicula*. *Peptides* 12, 351–357. doi: 10.1016/0196-9781(91)90025-k

Villar-Cerviño, V., Barreiro-Iglesias, A., Anadón, R., and Rodicio, M. C. (2008). Distribution of glycine immunoreactivity in the brain of adult sea lamprey (*Petromyzon marinus*). Comparison with gamma-aminobutyric acid. *J. Comp. Neurol.* 507, 1441–1463. doi: 10.1002/cne.21634

Volkoff, H., Canosa, L. F., Unniappan, S., Cerdá-Reverter, J. M., Bernier, N. J., Kelly, S. P., et al. (2005). Neuropeptides and the control of food intake in fish. *Gen. Comp. Endocrinol.* 142, 3–19.

Xu, X. J., Wiesenfeld-Hallin, Z., Fisone, G., Bartfai, T., and Hökfelt, T. (1990). The N-terminal 1-16, but not C-terminal 17-29, galanin fragment affects the flexor reflex in rats. *Eur. J. Pharmacol.* 182, 137–141. doi: 10.1016/0014-2999(90)90502-w

Xu, X. J., Wiesenfeld-Hallin, Z., Langel, U., Bedecs, K., and Bartfai, T. (1995). New high affinity peptide antagonists to the spinal galanin receptor. *Br. J. Pharmacol.* 116, 2076–2080. doi: 10.1111/j.1476-5381.1995.tb16414.x

Xu, Z. Q., Shi, T. J., and Hökfelt, T. (1998). Galanin/GMAP- and NPY-like immunoreactivities in locus coeruleus and noradrenergic nerve terminals in the hippocampal formation and cortex with notes on the galanin-R1 and -R2 receptors. *J. Comp. Neurol.* 392, 227–251. doi: 10.1002/(sici)1096-9861(19980309)392:2<227::aid-cne6>3.0.co;2-4

Yáñez, J., Pombal, M. A., and Anadón, R. (1999). Afferent and efferent connections of the parapineal organ in lampreys: a tract tracing and immunocytochemical study. *J. Comp. Neurol.* 403, 171–189. doi: 10.1002/(sici)1096-9861(19990111)403:2<171::aid-cne3>3.0.co;2-m

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroanatomy

ORIGINAL RESEARCH
published: 11 February 2020
doi: 10.3389/fnana.2020.00004

Zebrafish Astroglial Morphology in the Olfactory Bulb Is Altered With Repetitive Peripheral Damage

*Jackson Scheib and Christine Byrd-Jacobs**

Department of Biological Sciences, Western Michigan University, Kalamazoo, MI, United States

Zebrafish do not possess the typical astrocytes that are found in mammalian systems. In some brain areas, this teleost has radial glia that appears to perform astrocyte-like functions, but these cells have not been described in the zebrafish olfactory bulb. Mammalian astrocytes facilitate neuroplasticity and undergo astrogliosis after insult. The role of these cells in the zebrafish olfactory system after the damage has been poorly explored. This is important to examine because zebrafish have a high degree of neuroplasticity and the olfactory bulb is a brain area renowned for plasticity. The goal of this study was to explore the potential role of zebrafish astrocytes in the olfactory bulb damage response, with a goal to exploit the high level of regeneration in this system. We found that anti-glial fibrillary acidic protein (GFAP) labels numerous processes in the zebrafish olfactory bulb that are concentrated in the nerve and glomerular layers (GL) and do not show radial glial-like morphology. We propose to term this astroglia, since their location and response to damage suggests that they are similar in function to the mammalian astrocyte. To induce repetitive peripheral damage to the olfactory organ, a wax plug was inserted into the nasal cavity of adult zebrafish every 12 h for up to 7 days; this crushes the olfactory organ and leads to degradation of olfactory sensory neuron axons that project to the olfactory bulb. After 1 day, we found a significant increase in astroglial labeling in the affected bulb when compared to the internal control bulb and astroglial branches appeared to increase in number and size. By the third day of plug insertions there was no significant difference in astroglial labeling between the affected bulb and the internal control bulb. These data lead us to believe that astrogliosis does occur in the presence of peripheral damage, but this process attenuates within 1 week and no glial scar is evident upon recovery from the damage. Further exploration of astrocytes in zebrafish, in particular this apparent attenuation of astrogliosis, has the potential to elucidate key differences in glial function between teleosts and mammals.

Keywords: astroglia, astrogliosis, zebrafish, olfactory bulb, GFAP, deafferentation

OPEN ACCESS

Edited by:
Livia D'Angelo,
University of Naples Federico II, Italy

Reviewed by:
Ruxandra F. Sirbulescu,
Massachusetts General Hospital and Harvard Medical School, United States
Fatemeh Chehrehasa,
Queensland University of Technology, Australia

****Correspondence:***
Christine Byrd-Jacobs
christine.byrd@wmich.edu

Received: *30 October 2019*
Accepted: *27 January 2020*
Published: *11 February 2020*

Citation:
Scheib J and Byrd-Jacobs C (2020) Zebrafish Astroglial Morphology in the Olfactory Bulb Is Altered With Repetitive Peripheral Damage. Front. Neuroanat. 14:4. doi: 10.3389/fnana.2020.00004

INTRODUCTION

Astrocytes are crucial cells in the central nervous system (CNS) that provide energy substrates to neurons and influence synaptic transmission and plasticity (Barros and Deitmer, 2010; Pérez-Alvarez and Araque, 2013; Baldwin and Eroglu, 2017). Since they play a role in neuroplasticity, which is necessary for recovery from injury to the CNS, their activity in the presence of damage is

crucial to understand. Insults to the CNS typically cause astrogliosis, which paradoxically can be both neuroprotective and neurotoxic in mammals and, if repetitive, may lead to the development of disease states (Sofroniew, 2009; Pekny et al., 2014; Burda et al., 2016; Kulbe and Hall, 2017; Sullan et al., 2018). Glial scarring may also occur during astrogliosis and is typically thought of as detrimental and neurotoxic, though some components of the glial scar may be beneficial, as it has been shown that it may be needed for axon regeneration (Anderson et al., 2016). Clearly, it would be beneficial to alter some features of astrogliosis and the subsequent scar formation to eliminate neurotoxic aspects and obtain maximum neuroplasticity to recover from injury. One way to approach this problem is to elucidate the cellular and molecular mechanism of neuroplasticity in regenerative model organisms, such as zebrafish, to find key differences in their physiology to exploit for medical intervention in mammals.

Adult zebrafish are renowned for their neuroplasticity and have been shown to recover and regenerate many nervous system structures after damage (Becker et al., 1997; Zupanc, 2008; März et al., 2011). While most cellular aspects of the CNS are comparable to mammalian systems, zebrafish lack permanent glial scaring, like other teleosts (Baumgart et al., 2012; Takeda et al., 2015; Vitalo et al., 2016). This suggests that astrogliosis in zebrafish differs from that in mammals, and this may influence their dynamic neuroplasticity and promote their ability to recover from nervous system insults. Thus, studying astrocytes and astrogliosis in zebrafish has the potential to identify novel physiological processes involved in CNS recovery from damage.

In particular, the adult zebrafish olfactory system is an excellent model to study neuroplasticity due to constant turnover of olfactory sensory neurons (Byrd and Brunjes, 2001; Oehlmann et al., 2004) and the presence of stem cell niches in the olfactory organ and olfactory bulb (Byrd and Brunjes, 2001; Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006). Since synapses are constantly being formed and degraded between olfactory sensory neurons and olfactory bulb neurons, astrocytes within the olfactory bulb likely mediate this process, although zebrafish astrocytes in the olfactory bulb are relatively unexplored, even though similar glial cells have been explored in other brain regions of this fish (Grupp et al., 2010).

Immunohistochemical labeling using antibodies against the glial fibrillary acidic protein (GFAP), a common astrocyte and radial glia marker, shows long, organized processes in the telencephalon of adult zebrafish (Grupp et al., 2010). This labeling pattern is typical of radial glia and, given the lack of stellate-shaped labeling patterns, led many researchers to conclude that zebrafish lack astrocytes and retain radial glia that performs many of the tasks usually reserved for astrocytes. However, in the olfactory bulb of adult zebrafish, GFAP is expressed in processes that seem shorter and less organized than in the telencephalon and do not show the morphology of radial glial or traditional stellate astrocytes (Byrd and Brunjes, 1995). Given that descriptions of these cell types are vague, we propose a definition for the anti-GFAP-immunoreactive cells that fill the astrocyte role in zebrafish. In this study the term "astroglia" will refer to the cells in adult zebrafish that fill the astrocyte role and are likely the most similar in function to a mammalian astrocyte, inferred by their immunohistochemical labeling profile and morphology.

Glutamine synthetase (GS), an enzyme involved in glutamate metabolism, has been shown to be expressed by astrocytes (Norenberg and Martinez-Hernandez, 1979) and has been characterized in the telencephalon of zebrafish (Grupp et al., 2010) but not the olfactory bulb. We speculated that GS will be localized in astroglia in the olfactory bulb and using anti-GFAP and anti-GS antibodies may elucidate their morphology. Therefore, our study first examined astroglia in the olfactory bulb of adult zebrafish based on immunohistochemical reactivity and morphology.

Zebrafish have been shown to lack glial scarring after single, direct lesions to the telencephalon (März et al., 2011; Baumgart et al., 2012), but astroglial reactivity to repetitive injury of a peripheral component has not yet been explored. Furthermore, it is unknown if this damage persists, if astrogliosis will be chronic, and if a glial scar will form. Therefore, once we have identified astroglia in the olfactory bulb, the second aim of this study was to investigate astrogliosis in the olfactory bulb after a repetitive injury to the olfactory organ. We used a method previously characterized by our lab (Scheib et al., 2019) in which a wax plug is inserted into the nasal cavity to crush the olfactory organ every 12 h for 7 days. Briefly, this form of insult results in a loss of olfactory sensory neuron axons in the olfactory bulb, which may be detected by astroglia in the glomeruli.

Our hypothesis was that repetitive peripheral damage from repeated wax plug insertions causes astrogliosis in the olfactory bulb. We examined qualitatively and quantitatively the size and amount of astroglial branches to detect astrogliosis, and we investigated astroglial proliferation. Since previous studies of zebrafish astrogliosis have shown a lack of glial scarring, we also hypothesized that there will be no glial scar if the olfactory system is allowed to recover fully.

MATERIALS AND METHODS

Animals

Adult male and female zebrafish, *Danio rerio*, over 6 months of age, were obtained from local commercial sources. The fish were maintained in 15-gallon aquaria filled with aerated, conditioned water at 28°C and fed commercial flake food (Tetra) twice daily; each morning and afternoon. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (USA). All protocols on animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (project number 16-04-01). A sample size of 3–5 was used for every time point.

Repetitive Damage to the Olfactory Organ

The method for deafferentation involved the insertion of a wax plug into the rosette, as previously reported (Scheib et al., 2019). Zebrafish (n = 30) were anesthetized with 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) until unresponsive

to a tail pinch. Fish were placed on a chilled putty dish and covered with a chilled paper towel to support them and keep them anesthetized during the procedure. A small ball of medical-grade paraffin orthodontic wax mixed with a trace amount of Methylene Blue powder (for visualization) was inserted into the right naris; the left naris remained unplugged for use as an internal control for comparison. The plugs often fell out as the fish swam (averaged approximately 6 h), so plugs were checked every 12 h and reinserted if lost over the course of 4 h, 12 h, 1 day and 7 days survival times. To examine recovery potential, some fish had plugs removed after 7 days of repeated insertions and were left for 7 days with no plug before euthanasia. Untreated control animals were anesthetized prior to euthanasia on day 0 and were not subjected to wax plug insertions.

Tissue Processing

After survival times up to 7 days, untreated control fish and treated fish (a minimum of three fish per group for each survival time were used) were over anesthetized with 0.03% MS222 and perfused transcardially with PBS before immersion in 4% paraformaldehyde for 24 h at 4°C. Either dissected brains or decalcified whole heads were rinsed in PBS and mounted in a gelatin and sucrose mixture that was fixed in 4% paraformaldehyde for 24 h at 4°C. The gelatin block was cryoprotected through a gradient of sucrose solutions up to 30% sucrose. Blocks were then flash-frozen in 2-methyl butane, embedded in OCT (Tissue-Tek), and sectioned on a cryostat (Leica CM1860) at 10 μm for rosette analysis, 30 μm for whole bulb and glomerular analysis, or 50 μm for proliferation analysis. Sectioned brains were mounted on Colorfrost Plus (Fisherbrand) positively charged slides or gelatin-covered neutral slides (CEL & Associates Inc., Los Angeles, CA, USA) and vacuum sealed overnight. Olfactory organ morphologies were observed in tissue stained with typical hematoxylin and eosin protocols.

Immunohistochemistry

An antibody to keyhole limpet hemocyanin (KLH) was used to label olfactory sensory neuron axons and antibodies to GFAP or GS were used to label astroglia in the olfactory bulb. Mounted sections were rinsed in PBS and immersed in a blocking solution of 3% normal goat serum and 0.4% Triton X-100 in PBS for 1 h at room temperature. Sections were incubated for 2 h at room temperature with anti-GFAP (Dako Z0334 made in rabbit; 1:1,000 in blocking solution) or anti-GS (Millipore Sigma MAB302 made in the mouse; 1:1,000 in blocking solution). Slides were rinsed in PBS and incubated in Alexa Fluor 488 goat anti-rabbit or goat anti-mouse IgG (Invitrogen; 1:200 in blocking solution) for 1 h at room temperature or 24 h at 4°C. Following rinses in PBS, sections were incubated in 3% normal rabbit serum for 1 h at room temperature, rinsed in PBS, then incubated in 30 μg/ml Fab fragments (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature to block any anti-rabbit binding sites. Slides were then rinsed in PBS and incubated with the second primary antibody anti-KLH (Sigma H0892 made in rabbit; 1:1,000 in blocking solution) overnight at 4°C. Sections were rinsed in PBS and incubated in Alexa Fluor 563 goat anti-rabbit IgG (Invitrogen; 1:200 in blocking solution) for 1 h at room temperature. Some slides were rinsed and incubated in Hoescht dye (1:15,000) for 10 min at room temperature to view all nuclei. Slides were rinsed in PBS and coverslipped using a PVA-DABCO mounting solution.

No primary antibody controls were performed to ensure that the observed labeling was specific to the primary antibodies. Slides were incubated overnight at 4°C in blocking solution, rinsed in PBS, then incubated in AlexaFluor 488 goat anti-rabbit as above for 1 h at room temperature. Following PBS rinses, slides were incubated in AlexaFluor 563 goat anti-mouse as above for 1 h at room temperature. Slides were rinsed in PBS and coverslipped using a PVA-DABCO mounting medium.

Quantitative Analysis of Astroglial Branching and Hypertrophy

Olfactory bulbs were viewed on a confocal microscope (Nikon Ti Eclipse). Maximum intensity projections of Z stacks consisting of 11 optical slices at 2 μm through 50 μm tissue sections were taken of whole bulbs at low magnification and of a ventral medial glomerulus that was consistently found in all samples at high magnification using Nikon C2 Elements software. The full intensity image was viewed using ImageJ software, with the channels split individually into grayscale, and an estimate of the amount of labeling in treated and untreated tissues was obtained by comparing the optical density (OD) of the grayscale representation of antibody labeling. To obtain a mean gray value of anti-GFAP labeling of whole bulbs, the bulb was traced on the GFAP channel and measured. To account for background intensity, a similarly sized area was traced on an unstained area of the slide. To obtain a mean gray value of anti-GFAP labeling associated with the glomerulus, the glomerulus was traced on the KLH channel, the size and position of the trace were maintained when switched to the GFAP channel, and a mean gray value was taken within the trace. Background values for glomeruli were obtained on the GFAP channel by drawing a circle in an area without distinct labeling and with KLH labeling in the glomerulus. These data were converted to an OD using the following formula: OD = −log (intensity of the background/intensity of area of interest). Then, the ODs of a single Z stack projection from each bulb was compared using a percent difference formula: % Difference = (OD of experimental bulb − OD of internal control)/OD of internal control of the same fish. Both the raw OD data and percent difference data were compared using ANOVA with Tukey's test for multiple comparisons or repeated-measures ANOVA with Bonferroni *post hoc* test on GraphPad software. *P* values less than 0.05 were considered significant.

RESULTS

Astroglial Structures in the Olfactory Bulb of Adult Zebrafish

Antibodies against GFAP and GS were used to identify astroglial structures in the olfactory bulb (**Figure 1**). Both antibodies

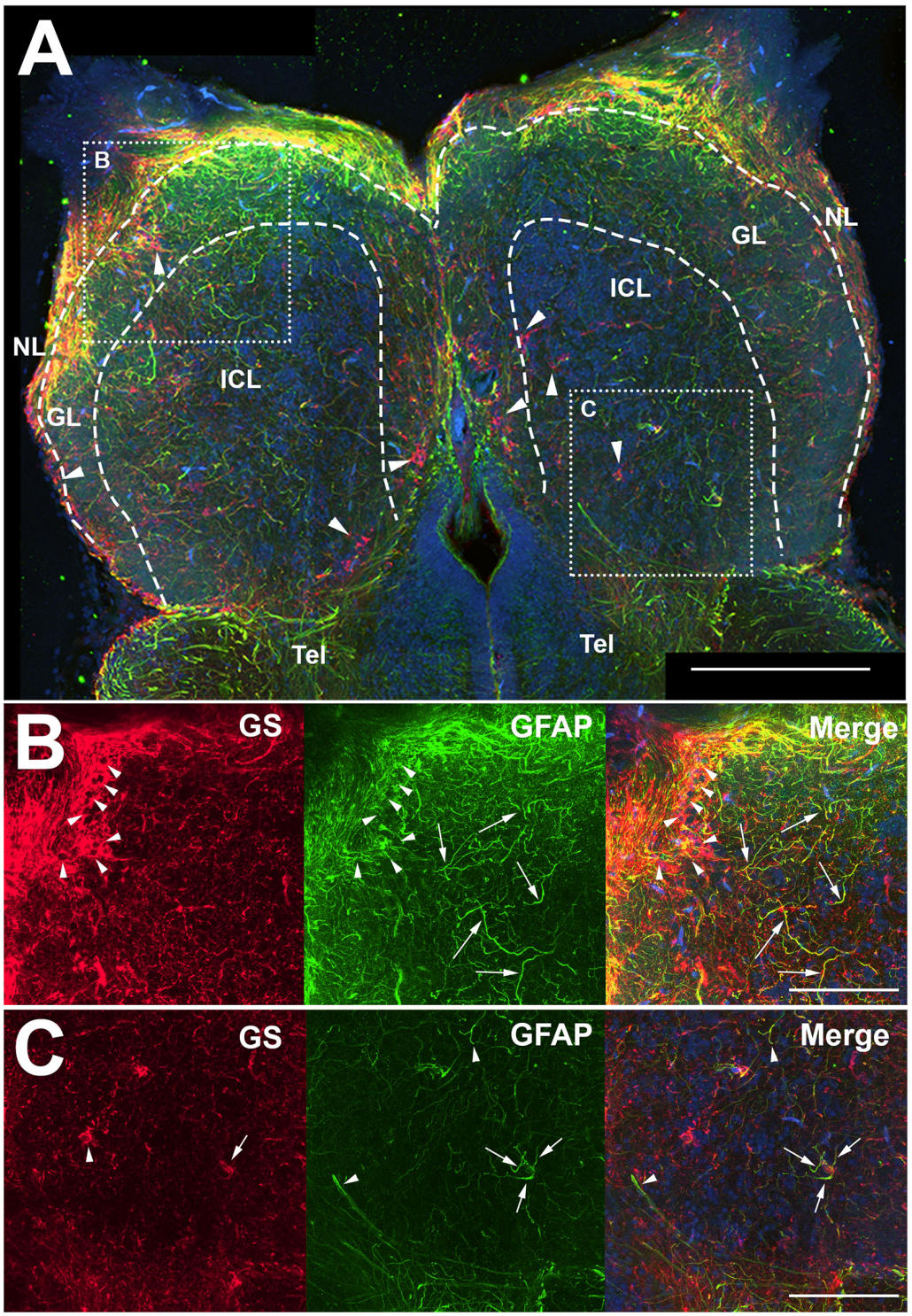


FIGURE 1 | Z-stack images of anti-glial fibrillary acidic protein (GFAP) and anti-glutamine synthetase (GS) immunoreactivity in the olfactory bulb of uninjured adult zebrafish. **(A)** There was a high degree of labeling for both antibodies in the nerve layer (NL) and glomerular layer (GL) of the olfactory bulb that varied in co-labeling. Anti-GS (red) labeling did not reveal clear structures with the exception of ring-like labeling seen in all layers of the bulb (arrowheads). There were numerous, distinct anti-GFAP (green) labeled processes in the GL and internal cell layer (ICL) that varied in size and length but had no obvious organization. Tel, Telencephalon. **(B)** Higher magnification of the rostral bulb region shown in **(A)** confirmed that ring-like structures (arrowheads) were labeled mostly with anti-GS and distinct processes (arrows) were labeled predominantly with anti-GFAP. **(C)** Higher magnification of the caudal bulb region revealed less labeling with both antibodies in this region, but anti-GS positive ring-like structures and anti-GFAP positive processes were apparent. Scale bar = 100 μm **(A)** or 20 μm **(B,C)**.

labeled structures throughout the uninjured, control olfactory bulb, and the nerve layer (NL) appeared to have a high degree of labeling for both proteins (**Figure 1A**). The glomerular layer (GL) had many, distinct anti-GFAP labeled processes that varied in co-labeling with anti-GS, and the internal cell layer (ICL) consisted primarily of diffuse anti-GFAP labeled processes. The anti-GFAP profiles did not appear to have any obvious organization but did appear to be denser in the GL than in the ICL. Anti-GS labeling revealed ring-like structures in all layers of the olfactory bulb that, upon higher magnification analysis, consisted of a Hoechst dye-labeled center (**Figure 1B**). Higher magnification also confirmed our observation that anti-GFAP clearly labeled processes that varied in co-labeling with anti-GS. Antibodies against GFAP did not reveal evidence of a classical, stellate astrocyte morphology as seen in mammals; instead we found only processes that lacked clear organization (**Figure 1C**). However, some anti-GFAP profiles appeared to terminate on capillaries, a feature that would be expected of astrocytes.

Only antibodies against GFAP clearly labeled cellular processes. Some of these processes appeared to terminate in endfeet on capillaries (**Figure 2**). This may indicate an astrocyte-specific physiological role in the regulation of the blood-brain barrier and/or neurovascular coupling (Newman and Volterra, 2004; MacVicar and Newman, 2015; Molofsky and Deneen, 2015; Liu et al., 2018). Since our study focused on morphological changes of astroglial branches, we determined that anti-GFAP antibodies were a superior marker over anti-GS for astroglia and astrogliosis in the adult zebrafish olfactory bulb.

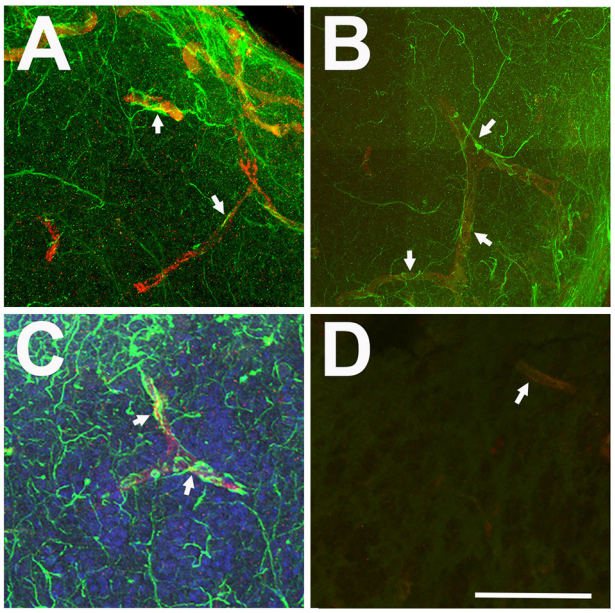


FIGURE 2 | Relationship between anti-GFAP labeling and capillaries viewed in Z-stack images. **(A–C)** Anti-GFAP processes (green) often appeared to terminate onto capillaries (arrows), identified by their tubular appearance and red auto-fluorescence. **(D)** Antibody controls where the primary antibody was eliminated show low levels of green background staining and red auto-fluorescent capillaries (arrow). Scale bar = 20 μm for all.

Repetitive Peripheral Damage Alters Anti-GFAP Labeling

To investigate whether astroglia responds to repetitive peripheral damage, zebrafish were subjected to wax plug insertions into the nasal cavity every 12 h for 7 days to destroy the olfactory organ, and their olfactory bulbs were labeled with anti-GFAP to identify astroglial processes (**Figure 3A**). The olfactory organ was altered dramatically with wax plug insertions, as shown previously (Scheib et al., 2019). Control rosettes displayed the typical pattern of sensory epithelium lining lamellae (**Figure 3B**), while 1 day (**Figure 3C**) and 7 days (**Figure 3D**) of wax plug insertions caused a progressive deterioration of the organ. With 1 week of recovery after cessation of wax plug insertions (**Figure 3E**), the olfactory organ returned to control morphology. The pattern of anti-GFAP labeling was similar in both olfactory bulbs in untreated control fish (**Figure 3F**). While there was no obvious increase in anti-GFAP labeling after 1 day of repetitive peripheral damage, there seemed to be some alteration in the labeling of processes in the GL of the affected bulb (**Figure 3G**). After 7 days of repetitive peripheral damage, affected olfactory bulbs appeared to have anti-GFAP labeling similar to control bulbs (**Figure 3H**). To estimate the amount of antibody labeling and determine if there were changes in labeling patterns, OD measurements were made of anti-GFAP labeling within the olfactory bulb. There was no significant difference between left olfactory bulbs of untreated and treated fish throughout the time course ($p = 0.1379$); therefore, these served as internal controls. Labeling was significantly higher in the affected bulb (0.2152 ± 0.0578) compared to the internal control bulb (0.0931 ± 0.0090) at 1 day, and the percent difference in labeling between the bulbs was significantly different (**Figure 3I**). At 7 days, labeling had returned to control levels and was not different from controls. Thus, the affected bulb had significantly more anti-GFAP labeling at 1 day, yet labeling returned to control levels by 7 days.

Since it appeared that the majority of the alterations in astroglial process density was in the GL, and glomeruli are areas with a high density of synapses where astroglia likely have influence, higher magnification analysis of astroglial processes within glomeruli was done to further explore our observations (**Figure 4**). Glomeruli were identified by their roughly spherical structure when labeled with antibodies against KLH and a ventral medial glomerulus was chosen for detailed analysis because it is easily identified by its stereotypical shape and location. There were several thin anti-GFAP-positive profiles within glomeruli that varied in size and length in untreated control bulbs (**Figures 4A,A′**). Four hours after the first wax plug insertion into the nasal cavity there was a distinct increase in the amount and overall size of astroglial branches within the affected glomeruli when compared to the untreated and internal control bulbs (**Figures 4B,B′**). This same effect was apparent at 12 h (**Figures 4C,C′**) and 1 day (**Figures 4D,D′**). While the right, treated bulb showed alterations in GFAP expression, the left, internal control bulb retained

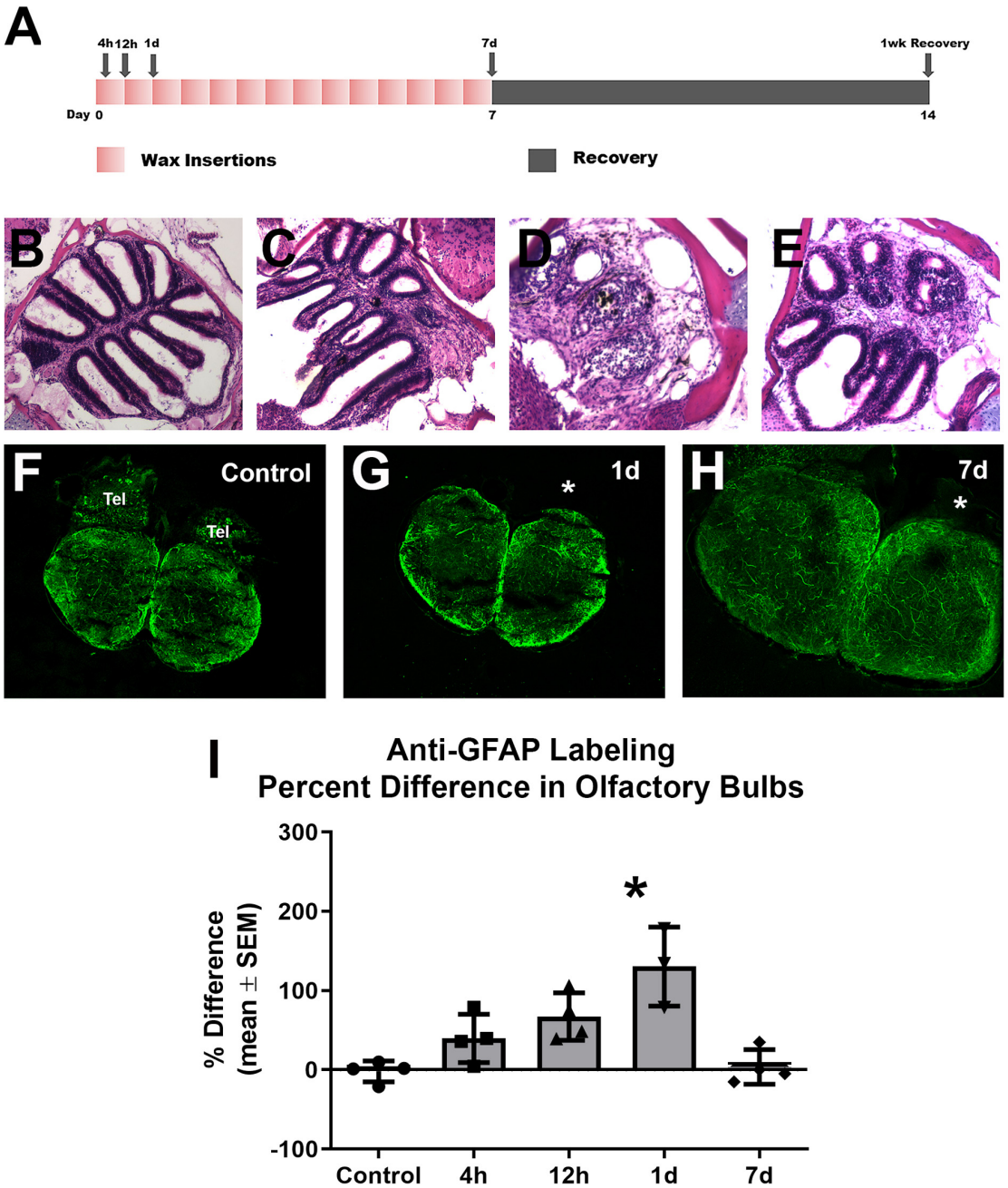
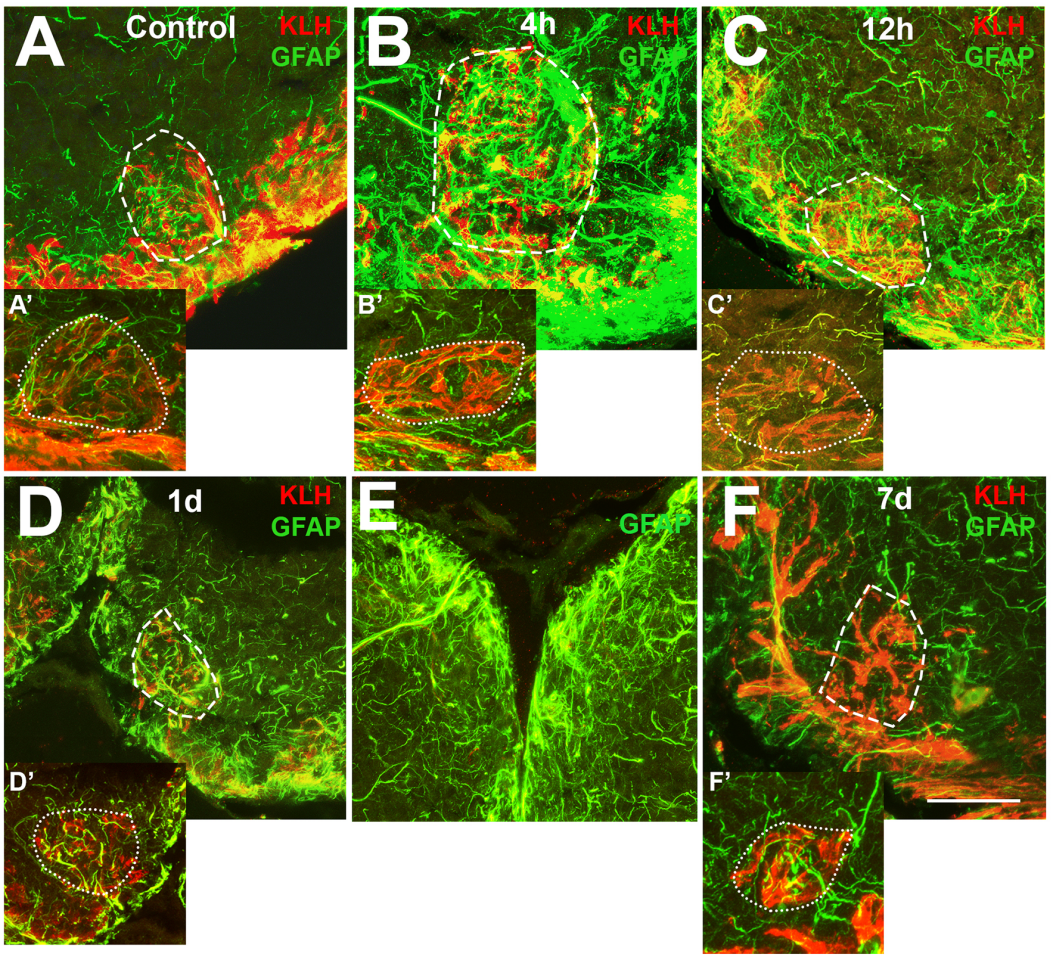


FIGURE 3 | Gross analysis with Z-stack images of anti-GFAP labeling in the olfactory bulb following repetitive peripheral damage. **(A)** Illustration of the treatment paradigm shows that fish receiving repeated wax plug insertions were analyzed at various survival times during wax treatment and after a recovery period. Images of olfactory organs show typical rosette morphology in control fish **(B)**, significant damage at 1 day **(C)** and 7 days **(D)** of wax plug insertions, and return to control morphology with 1 week of recovery **(E)** after removal of the plug. **(F)** Untreated control fish had typical anti-GFAP labeling in all layers of both olfactory bulbs. Tel = Telencephalon, $n = 4$. **(G)** After 1 day of repetitive peripheral damage to the olfactory organ, the affected bulb (asterisk) appeared to have slightly more anti-GFAP labeling than the internal control bulb, $n = 3$. **(H)** By 7 days, there was no noticeable difference in labeling between bulbs, and anti-GFAP labeling in the affected bulb (asterisk) appeared at control levels, $n = 4$. **(I)** A comparison of anti-GFAP labeling between bulbs showed a significant difference at 1 day of repetitive peripheral damage when compared to controls but no difference at 7 days of damage. $^*p < 0.05$.

control levels of anti-GFAP labeling (**Figure 4E**). However, astroglial processes appeared to return to typical morphology within glomeruli by 7 days (**Figures 4F,F′**) of repetitive peripheral injury.

To quantify this observation, OD measurements of anti-GFAP labeling within a specific glomerulus in the ventral medial cluster were compared. There was no significant difference between glomeruli in the left bulbs in untreated



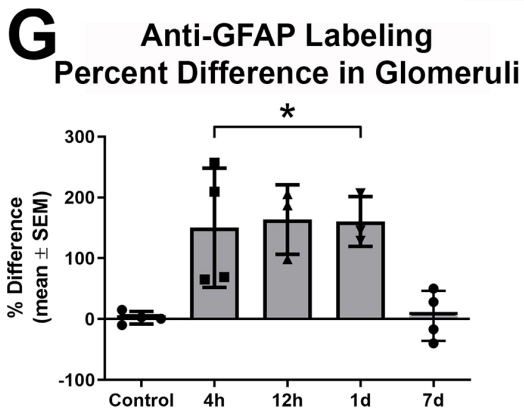


FIGURE 4 | Higher magnification analysis of Z-stacks of anti-GFAP (green) and anti-keyhole limpet hemocyanin (KLH; red) labeling during repetitive damage to the olfactory organ. **(A)** Anti-KLH allowed identification of a ventral medial glomerulus (dashed outline). Anti-GFAP labeled processes within the glomerulus in the right olfactory bulb (dashed outline) varied in size and length and had no clear organization in untreated control bulbs. **(A')** A glomerulus (dotted outline) in the same region of the left olfactory bulb of the same fish displayed similar morphology of anti-GFAP labeled processes, $n = 4$. **(B)** After 4 h of damage to the olfactory organ, anti-GFAP labeled processes appeared to be more numerous and thicker within the glomerulus (dashed outline) of the affected bulb when compared to the comparable glomerulus (dotted outline) of the internal control bulb **(B')**, $n = 4$. **(C)** By 12 h after damage to the periphery, there were numerous anti-GFAP labeled processes associated with the glomerulus (dashed outline) in the affected bulb that varied in length and thickness, while the glomerulus (dotted outline) in the internal control bulb **(C')** retained typical labeling, $n = 3$. **(D)** At 1 day after repeated damage to the olfactory organ, affected glomeruli (dashed outline) still appeared to have thicker and more numerous anti-GFAP labeled processes when compared to glomeruli (dotted outline) in the internal control bulb **(D')**, $n = 3$. **(E)** Side by side comparison of both bulbs at 1 day showed that the left, internal control bulb retained control levels of anti-GFAP labeling, while the right, treated bulb showed more and thicker labeled processes. **(F)** By 7 days after repetitive peripheral damage, anti-GFAP labeled processes in glomeruli (dashed outline) in the affected bulb

(*Continued*)

FIGURE 4 | Continued
appeared to have similar morphology to anti-GFAP labeled processes in glomeruli (dotted outline) of the internal control bulb **(F')**, $n = 4$. Scale bar = 20 μm for all. **(G)** The percent difference of anti-GFAP optical density (OD) measurements between treated and untreated glomeruli was significantly different at 4 h, 12 h, and 1 day of repetitive peripheral damage but was no longer significantly different after 7 days of repeated damage. $^*p < 0.05$.

and treated fish during the time course ($p = 0.0810$), so these served as internal controls. The mean ODs in glomeruli of affected bulbs at 4 h (0.2010 ± 0.0557), 12 h (0.2010 ± 0.0870), and 1 day (0.1761 ± 0.1305) were significantly higher when compared to the internal control bulbs (4 h = 0.0843 ± 0.0195, 12 h = 0.0735 ± 0.0201, 1 day = 0.0703 ± 0.0549). The percent difference between right and left glomeruli was significantly different at these time points when compared to controls (**Figure 4G**). The percent difference in labeling at 7 days was not different from controls. Thus, the affected glomeruli had more anti-GFAP labeling during 4 h, 12 h, and 1 day of repetitive peripheral damage, yet returned to control levels by 7 days.

Lack of Glial Scar

Lastly, to determine if there was any evidence of glial scar formation, zebrafish were allowed to recover for 1 week from 7 days of repetitive damage to the olfactory organ, and their olfactory bulbs were labeled with antibodies to GFAP and KLH (**Figure 5**). It was previously reported that the removal of wax plugs after 7 days of insertions allows recovery of the olfactory organ and reinnervation of the olfactory bulb within 1 week (Scheib et al., 2019). Anti-GFAP labeling appeared to be similar to untreated controls after 1 week of recovery in whole bulbs and in glomeruli (**Figures 5A,B′**).

Anti-GFAP labeling in the whole bulb and in ventral medial glomeruli was compared. Following the significant increase in labeling at 1 day of repeated damage reported above, there was no significant difference between the mean OD values of left bulbs of control fish and 1 week recovery fish ($p = 0.7735$) nor right bulb OD means ($p = 0.8793$). There was also no significant difference in the percent difference between bulbs at 1 week of recovery compared to controls (**Figure 5C**). No significant difference was found in the mean ODs between left glomeruli ($p = 0.9352$) nor right glomeruli ($p = 0.2553$) between controls and 1 week recovered fish (**Figure 5D**).

DISCUSSION

Astroglia in the Olfactory Bulb of Adult Zebrafish

Zebrafish lack evidence of classical stellate astrocytes, but they have glia that expresses the morphology and immunoreactivity of radial glia in most areas of the brain (Grupp et al., 2010). This observation, along with the fact that radial glia can directly differentiate into astrocytes (Malatesta et al., 2008), has led to the inference that radial glia performs the tasks of mammalian astrocytes in the zebrafish brain. However, since the identification and classification of zebrafish glia are incomplete, conflicts in the definitions for these cells have arisen. Typical radial glia does not perform astrocyte tasks, by definition, in mammalian systems, where glial cell types are much better understood. Thus, we classify "astroglia" in this report as the cells that are the most like mammalian astrocytes in the adult zebrafish olfactory bulb, identified by their morphology, immunohistochemical reactivity, and inferred physiological roles.

Anti-GFAP antibodies are commonly used to identify astrocytes and glial scars in adult mammals (Sofroniew, 2009, 2015). Here, we show the immunohistochemical profile of anti-GFAP labeling in the olfactory bulb of adult zebrafish, which lack clearly identifiable cell bodies and organization of glial branches, as previously reported (Byrd and Brunjes, 1995). This morphology is distinct from the organization of anti-GFAP labeling in the zebrafish telencephalon, which shows the morphology of radial glia, and appears to be unique to the olfactory bulb. Another consideration is that olfactory ensheathing cells, a unique glial cell type found in the olfactory nerve, also express GFAP (Barber and Dahl, 1987) and are expected in the olfactory bulb especially following damage (Barnett and Riddell, 2004; Nazareth et al., 2015). In an attempt to elucidate further astroglial morphologies in the olfactory bulb, antibodies against GS, a cytosolic enzyme in astrocytes (Norenberg and Martinez-Hernandez, 1979), were also used. We hypothesized that GS may be expressed specifically in astroglia in the zebrafish olfactory system and not in radial glia or olfactory ensheathing cells. Therefore, anti-GS antibodies were selected in an attempt to eliminate the labeling of anti-GFAP-immunoreactive radial glia and olfactory ensheathing cells from astroglia.

Anti-GS labeling revealed many ring-like structures seen in all layers of the olfactory bulb that are likely cell bodies since they were co-labeled with Hoechst dye at their centers. Since these profiles had some co-labeling with anti-GFAP, they are possibly astroglial cell bodies. However, there was inconclusive evidence to determine that these were, in fact, astroglial cell bodies and, since this study focused on astrogliosis where observations on the size and number of branches were needed, we selected anti-GFAP antibodies as our primary marker for astroglial processes and astrogliosis. Furthermore, since anti-GFAP labeled processes were prevalent in glomeruli, areas of a high density of synapses between olfactory sensory neuron axons and olfactory bulb neurons, and appeared to terminate on capillaries, we concluded that anti-GFAP antibodies were a more reliable marker for astroglia, because the location of immunoreactive processes suggests physiological roles, such as communication at synapses and with capillaries. Therefore, the remainder of this study focused on astroglial branches identified using anti-GFAP antibodies and proliferating glial cells identified using anti-GFAP and anti-PCNA antibodies.

Astrogliosis in the Presence of Repetitive Peripheral Damage

Adult zebrafish were subjected to wax plug insertions into the right nasal cavity to crush the olfactory organ every 12 h for 7 days, while the left side served as the internal control.

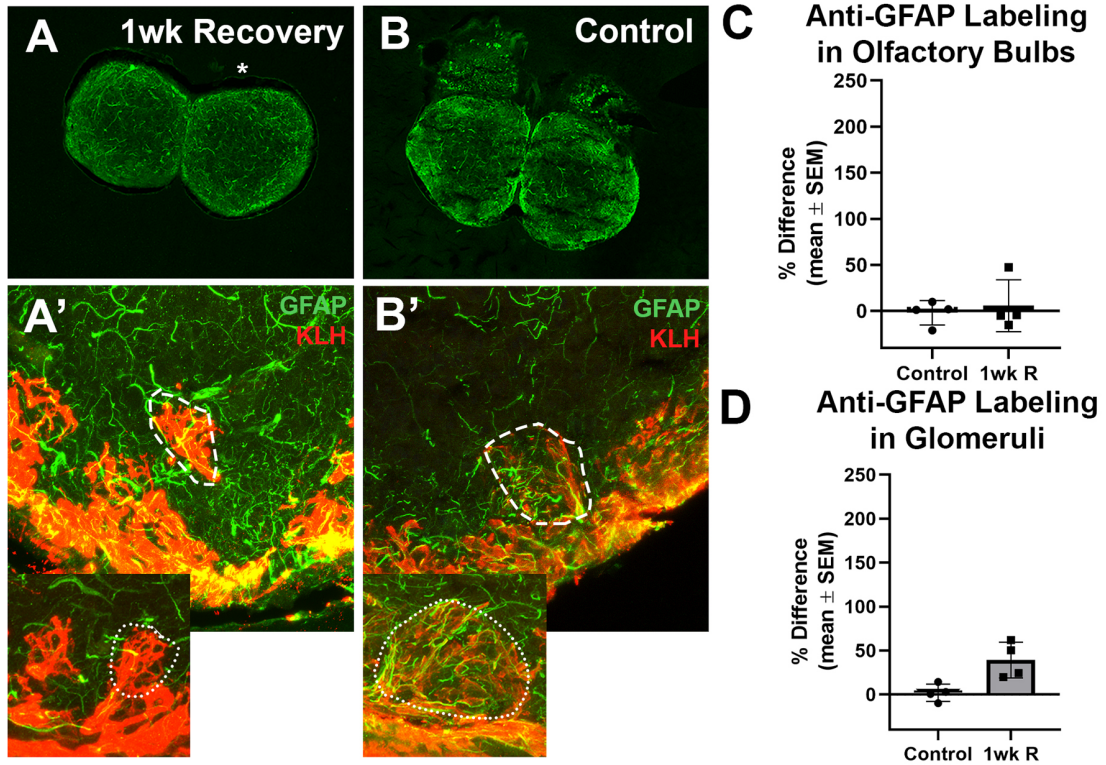


FIGURE 5 | The recovery of the olfactory bulb does not involve evidence of a glial scar. **(A)** After the olfactory system was allowed 1 week to recover from 7 days of repetitive peripheral damage, there was no apparent difference in the overall amount of anti-GFAP (green) and anti-KLH (red) labeling in the affected bulb (asterisk) when compared to the internal control bulb ($n = 4$). There was also no noticeable difference in the overall amount of anti-GFAP labeling compared to untreated control fish **(B)**. **(A')** Higher magnification Z-stack images revealed that anti-GFAP labeling (green) within anti-KLH (red) labeled glomeruli (dotted line) in the affected bulb after 1 week of recovery also appeared to be similar to that of glomeruli in the internal control bulb (dotted outline in inset) and in untreated control bulbs **(B')** on the right (dashed outline) and left (dotted outline in inset) sides. There was no significant difference in the percent difference of OD of anti-GFAP labeling in the olfactory bulb **(C)** or ventral medial glomeruli **(D)** of zebrafish that were allowed 1 week of recovery from 7 days of repetitive peripheral damage.

This technique results in a significant reduction in olfactory organ size and structure and reduces afferent innervation of the olfactory bulb (Scheib et al., 2019). Astroglial morphology and the amount of anti-GFAP labeling in the olfactory bulb were investigated. Qualitative and quantitative analyses revealed an increase in the size and number of branches in and around glomeruli during timepoints up to 1 day of repeated damage when compared to the internal control bulb and untreated control fish.

Comparisons of anti-GFAP labeling were made between affected and internal control sides of whole olfactory bulbs and also of individual glomeruli. There was no significant difference in OD among left sides, which served as external and internal controls throughout the time course; therefore, the left side was shown to be unaffected by insult to the right bulb. There was a significant increase in OD of labeling in the affected bulb at 1 day that was likely due to increased amount and size of astroglial branches, which is typical of astrogliosis (Sofroniew and Vinters, 2010). The wax plug caused deterioration of olfactory sensory neurons (Scheib et al., 2019) and, since their axons project to glomeruli in the bulb where astroglial processes were seen, astroglia likely detects this insult and respond with astrogliosis. Since the literature on zebrafish astrocytes, astroglia, and olfactory ensheathing cells is severely lacking, the identity of these anti-GFAP-immunoreactive proliferating profiles remains unclear.

Astrogliosis increased to a significant level by 1 day and attenuated morphologically by 7 days of damage. This is not typical of what would be expected of mammalian astrocytes in a chronic injury environment, which would have retained their astrogliosis morphology and formed glial scars (Ojo et al., 2016; Kulbe and Hall, 2017). This is the first study on astrogliosis during repetitive insults to the zebrafish olfactory system, and the apparent attenuation of astrogliosis is a novel finding.

Repetitive peripheral injury was insufficient to cause astroglial scarring in the olfactory bulb of zebrafish. Insults to other areas of the zebrafish nervous system have been reported previously to be insufficient to cause a glial scar formation (Kroehne et al., 2011; Baumgart et al., 2012; Noorimotlagh et al., 2017), suggesting unique differences in injury response between teleosts and mammals. There was no noticeable difference in the amount of anti-GFAP labeling after 1 week of recovery from repetitive peripheral damage between affected and unaffected bulbs, meaning there

was no evidence of a glial scar, adding to the evidence of dynamic zebrafish neuroplasticity. Newly proliferated astrocytes typically form glial scars in mammals (Wanner et al., 2013), therefore we next investigated astroglial proliferation using this model.

These data suggest that astrogliosis in the adult zebrafish olfactory system occurs after a repetitive peripheral injury that attenuates and leaves no residual glial scar. Future studies will examine if astroglial proliferation occurs in response to this damage. This novel finding suggests that astrogliosis differs in this zebrafish repetitive damage model compared to mammals (Kane et al., 2012; Petraglia et al., 2014; Kulbe and Hall, 2017), where repetitive insults results to the brain can cause chronic astrogliosis and the development of protein tangles and plaques. Since astrocytes are such dynamic cells in the CNS, perhaps chronic astrogliosis causes the secondary damage necessary to cause the development of these disease states. If so, understanding astroglia and astrogliosis in zebrafish might lead to novel medical treatments for humans sufferers of these diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservations, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Western Michigan University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JS and CB-J conceived and designed the study, analyzed and interpreted the data and contributed to manuscript revision, read and approved the submitted version, and agree to be accountable for the content of the work. JS collected data and wrote the first draft of the manuscript. CB-J obtained funding for the work.

FUNDING

This work was supported financially by Western Michigan University (WMU) funds (CB-J) and a WMU graduate student research grant (JS).

ACKNOWLEDGMENTS

We are grateful to the Department of Biological Sciences for use of the confocal microscope and to the Byrd lab members for helpful comments throughout this study.

REFERENCES

Adolf, B., Chapouton, P., Lam, C. S., Topp, S., Tannhauser, B., Strahle, U., et al. (2006). Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Dev. Biol.* 295, 278–293. doi: 10.1016/j.ydbio.2006.03.023

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., et al. (2016). Astrocyte scar formation aids CNS axon regeneration. *Nature* 532, 195–200. doi: 10.1038/nature17623

Baldwin, K. T., and Eroglu, C. (2017). Molecular mechanisms of astrocyte-induced synaptogenesis. *Curr. Opin. Neurobiol.* 45, 113–120. doi: 10.1016/j.conb.2017.05.006

Barber, P. C., and Dahl, D. (1987). Glial fibrillary acidic protein (GFAP)-like immunoreactivity in normal and transected rat olfactory nerve. *Exp. Brain Res.* 65, 681–685. doi: 10.1007/bf00235993

Barnett, S., and Riddell, J. (2004). Olfactory ensheathing cells (OECs) and the treatment of CNS injury: advantages and possible caveats. *J. Anat.* 204, 57–67. doi: 10.1111/j.1469-7580.2004.00257.x

Barros, L. F., and Deitmer, J. W. (2010). Glucose and lactate supply to the synapse. *Brain Res. Rev.* 63, 149–159. doi: 10.1016/j.brainresrev.2009.10.002

Baumgart, E. V., Barbosa, J. S., Bally-Cuif, L., Götz, M., and Ninkovic, J. (2012). Stab wound injury of the zebrafish telencephalon: a model for comparative analysis of reactive gliosis. *Glia* 60, 343–357. doi: 10.1002/glia.22269

Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R., and Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *J. Comp. Neurol.* 377, 577–595. doi: 10.1002/(sici)1096-9861(19970127)377:4<577::aid-cne8>3.0.co;2-#

Burda, J. E., Bernstein, A. M., and Sofroniew, M. V. (2016). Astrocyte roles in traumatic brain injury. *Exp. Neurol.* 275, 305–315. doi: 10.1016/j.expneurol.2015.03.020

Byrd, C. A., and Brunjes, P. C. (1995). Organization of the olfactory system in adult zebrafish. *J. Comp. Neurol.* 358, 247–259. doi: 10.1002/cne.903580207

Byrd, C. A., and Brunjes, P. C. (2001). Neurogenesis in the olfactory bulb of adult zebrafish. *Neuroscience* 105, 793–801. doi: 10.1016/s0306-4522(01)00215-9

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I., and Brand, M. (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. *Dev. Biol.* 295, 263–277. doi: 10.1016/j.ydbio.2006.03.040

Grupp, L., Wolburg, H., and Mack, A. F. (2010). Astroglial structures in the zebrafish brain. *J. Comp. Neurol.* 518, 4277–4287. doi: 10.1002/cne.22481

Kane, M. J., Angoa-Pérez, M., Briggs, D. I., Viano, D. C., Kreipke, C. W., and Kuhn, D. M. (2012). A mouse model of human repetitive mold traumatic brain injury. *J. Neurosci. Methods* 203, 41–49. doi: 10.1016/j.jneumeth.2011.09.003

Kroehne, V., Freudenreich, D., Hans, S., Kaslin, J., and Brand, M. (2011). Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors. *Development* 138, 4831–4841. doi: 10.1242/dev.072587

Kulbe, J. R., and Hall, E. D. (2017). Chronic traumatic encephalopathy-integration of canonical traumatic brain injury secondary injury mechanisms with tau pathology. *Prog. Neurobiol.* 158, 15–44. doi: 10.1016/j.pneurobio.2017.08.003

Liu, C.-Y., Yang, Y., Ju, W.-N., Wang, X., and Zhang, H.-L. (2018). Emerging roles of astrocytes in neuro-vascular unit and the tripartite synapse with emphasis on reactive gliosis in the context of Alzheimer's disease. *Front. Cell. Neurosci.* 12:193. doi: 10.3389/fncel.2018.00193

MacVicar, B. A., and Newman, E. A. (2015). Astrocyte regulation of blood flow in the brain. *CSH Perspect. Biol.* 7:a020388. doi: 10.1101/cshperspect.a020388

Malatesta, P., Appolloni, I., and Calzolari, F. (2008). Radial glia and neural stem cells. *Cell Tissue Res.* 331, 165–178. doi: 10.1007/s00441-007-0481-8

März, M., Schmidt, R., Rastegar, S., and Strähle, U. (2011). Regenerative response following stab injury in the adult zebrafish telencephalon. *Dev. Dynam.* 240, 2221–2231. doi: 10.1002/dvdy.22710

Molofsky, A. V., and Deneen, B. (2015). Astrocyte development: a guide for the perplexed. *Glia* 63, 1320–1329. doi: 10.1002/glia.22836

Nazareth, L., Lineburg, K. E., Chuah, M. I., Tello Velasquez, J., Chehrehasa, F., St John, J. A., et al. (2015). Olfactory ensheathing cells are the main phagocytic cells that remove axon debris during early development of the olfactory system. *J. Comp. Neurol.* 523, 479–494. doi: 10.1002/cne.23694

Newman, E. A., and Volterra, A. (2004). Glial control of synaptic function. *Glia* 47, 207–208. doi: 10.1002/glia.20085

Noorimotlagh, Z., Babaie, M., Safdarian, M., Ghadiri, T., and Rahimi-Movaghar, V. (2017). Mechanisms of spinal cord injury regeneration in zebrafish: a systematic review. *Iran. J. Basic Med. Sci.* 20, 1287–1296. doi: 10.22038/IJBMS.2017.9620

Norenberg, M. D., and Martinez-Hernandez, A. (1979). Fine structural localization of glutamine synthetase in astrocytes of rat brain. *Brain Res.* 161, 303–310. doi: 10.1016/0006-8993(79)90071-4

Oehlmann, V. D., Berger, S., Sterner, C., and Korsching, S. I. (2004). Zebrafish beta tubulin 1 expression is limited to the nervous system throughout development, and in the adult brain is restricted to a subset of proliferative regions. *Gene Exp. Patterns* 4, 191–198. doi: 10.1016/j.modgep.2003.09.001

Ojo, J. O., Mouzon, B., Algamal, M., Leary, P., Lynch, C., Abdullah, L., et al. (2016). Chronic repetitive mild traumatic brain injury results in reduced cerebral blood flow, axonal injury, gliosis, and increased T-tau and tau oligomers. *J. Neuropathol. Exp. Neurol.* 75, 636–655. doi: 10.1093/jnen/nlw035

Pekny, M., Wilhelmsson, U., and Pekna, M. (2014). The dual role of astrocyte activation and reactive gliosis. *Neurosci. Lett.* 565, 30–38. doi: 10.1016/j.neulet.2013.12.071

Pérez-Alvarez, A., and Araque, A. (2013). Astrocyte-neuron interaction at tripartite synapses. *Curr. Drug Targets* 14, 1220–1224. doi: 10.2174/13894501131499902O3

Petraglia, A., Plog, B., Dayawansa, S., Dashnaw, M., Czerniecka, K., Walker, C., et al. (2014). The pathophysiology underlying repetitive mild traumatic brain injury in a novel mouse model of chronic traumatic encephalopathy. *Surg. Neurol. Int.* 5:184. doi: 10.4103/2152-7806.147566

Scheib, J., Pozzuto, J. M., and Byrd-Jacobs, C. A. (2019). Reversible deafferentation of the zebrafish olfactory bulb with wax plug insertion. *J. Neurosci. Methods* 311, 47–56. doi: 10.1016/j.jneumeth.2018.10.014

Sofroniew, M. V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci.* 32, 638–647. doi: 10.1016/j.tins.2009.08.002

Sofroniew, M. V. (2015). Astrogliosis. *Cold Spring Harb. Perspect. Biol.* 7:a020420. doi: 10.1101/cshperspect.a020420

Sofroniew, M. V., and Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol.* 119, 7–35. doi: 10.1007/s00401-009-0619-8

Sullan, M. J., Asken, B. M., Jaffee, M. S., DeKosky, S. T., and Bauer, R. M. (2018). Glymphatic system disruption as a mediator of brain trauma and chronic traumatic encephalopathy. *Neurosci. Biobehav. Rev.* 84, 316–324. doi: 10.1016/j.neubiorev.2017.08.016

Takeda, A., Atobe, Y., Kadota, T., Goris, R. C., and Funakoshi, K. (2015). Axonal regeneration through the fibrous scar in lesioned goldfish spinal cord. *Neuroscience* 284, 134–152. doi: 10.1016/j.neuroscience.2014.09.066

Vitalo, A. G., Sîrbulescu, R. F., Ilieș, I., and Zupanc, G. K. H. (2016). Absence of gliosis in a teleost model of spinal cord regeneration. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 202, 445–456. doi: 10.1007/s00359-016-1089-9

Wanner, I. B., Anderson, M. A., Song, B., Levine, J., Fernandez, A., Gray-Thompson, Z., et al. (2013). Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells *via* STAT3-dependent mechanisms after spinal cord injury. *J. Neurosci.* 33, 12870–12886. doi: 10.1523/JNEUROSCI.2121-13.2013

Zupanc, G. K. H. (2008). Adult neurogenesis and neuronal regeneration in the brain of teleost fish. *J. Physiol. Paris* 102, 357–373. doi: 10.1016/j.jphysparis.2008.10.007

Zupanc, G. K., Hinsch, K., and Gage, F. H. (2005). Proliferation, migration, neuronal differentiation, and long-term survival of new cells in the adult zebrafish brain. *J. Comp. Neurol.* 488, 290–319. doi: 10.1002/cne.20571

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroanatomy

PERSPECTIVE
published: 18 March 2020
doi: 10.3389/fnana.2020.00010

Using Teleost Fish to Discern Developmental Signatures of Evolutionary Adaptation From Phenotypic Plasticity in Brain Structure

Zachary J. Hall[1] *and Vincent Tropepe*[2]*

[1]Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada, [2]Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada

Traditionally, the impact of evolution on the central nervous system has been studied by comparing the sizes of brain regions between species. However, more recent work has demonstrated that environmental factors, such as sensory experience, modulate brain region sizes intraspecifically, clouding the distinction between evolutionary and environmental sources of neuroanatomical variation in a sampled brain. Here, we review how teleost fish have played a central role in shaping this traditional understanding of brain structure evolution between species as well as the capacity for the environment to shape brain structure similarly within a species. By demonstrating that variation measured by brain region size varies similarly both inter- and intraspecifically, work on teleosts highlights the depth of the problem of studying brain evolution using neuroanatomy alone: even neurogenesis, the primary mechanism through which brain regions are thought to change size between species, also mediates experience-dependent changes within species. Here, we argue that teleost models also offer a solution to this overreliance on neuroanatomy in the study of brain evolution. With the advent of work on teleosts demonstrating interspecific evolutionary signatures in embryonic gene expression and the growing understanding of developmental neurogenesis as a multi-stepped process that may be differentially regulated between species, we argue that the tools are now in place to reframe how we compare brains between species. Future research can now transcend neuroanatomy to leverage the experimental utility of teleost fishes in order to gain deeper neurobiological insight to help us discern developmental signatures of evolutionary adaptation from phenotypic plasticity.

Keywords: brain, evolution, plasticity, teleost, neurogenesis

OPEN ACCESS

Edited by:
Livia D'Angelo,
University of Naples Federico II, Italy

Reviewed by:
Satoshi Ogawa,
Monash University Malaysia, Malaysia
Carla Lucini,
University of Naples Federico II, Italy

***Correspondence:**
Vincent Tropepe
v.tropepe@utoronto.ca

Received: *14 January 2020*
Accepted: *02 March 2020*
Published: *18 March 2020*

Citation:
Hall ZJ and Tropepe V (2020) Using Teleost Fish to Discern Developmental Signatures of Evolutionary Adaptation From Phenotypic Plasticity in Brain Structure. Front. Neuroanat. 14:10. doi: 10.3389/fnana.2020.00010

EVOLUTIONARY ADAPTION VS. PHENOTYPIC PLASTICITY IN NEUROANATOMY

Studies comparing the brains of different species enable us to elucidate the extent to which evolutionary forces shape brain structure and, by extension, the perceptual, cognitive, and behavioral functions the brain supports. Traditionally, brain evolution has been studied neuroanatomically, focusing on interspecific variation in either whole brain size or the size of

individual brain structures. When comparing the brains of two or more species in this fashion, we assume that similarities in neuroanatomy are a product of conserved neurodevelopmental processes across species and that differences arise as a product of evolution.

Conversely, studies comparing the brains of individuals of the same species enable us to elucidate the extent to which environmental forces shape brain structure and function. Such phenotypic plasticity is often studied neuroanatomically, in which changes in brain structure are associated with environmental factors such as nutrient availability and sensory experience along with genetic factors. When comparing the brains of two or more individuals in this fashion, we assume that similarities in neuroanatomy are a product of conserved neurodevelopmental processes within a species and that differences arise as a product of a rearing environment.

But what happens when neuroanatomical variation manifests similarly between and within species? If, for example, the olfactory bulb is enlarged in one species compared to another, is this enlargement a product of evolutionary forces or a product of differences in olfactory experiences between species during neurodevelopment? Without understanding the extremes of phenotypic plasticity in neuroanatomy within a species, can we affirm that observed interspecific variation in brain structure is primarily a product of evolution and not the different environments experienced by the individuals sampled representing each species? In this perspective article, we discuss the utility of teleost fish species as animal models in addressing evolutionary and environmental sources of neuroanatomical variation. First, we review past literature, focusing on important insights derived from the study of teleost comparative neuroanatomy, and more recently, neurodevelopmental plasticity in response to environmental factors within a species. We then highlight the difficulty in isolating the influence of evolution from the environment by studying only brain size, discussing neurogenesis as a common developmental mechanism that appears to underlie both inter- and intraspecific changes in neuroanatomy. Finally, we consider recent advances in identifying uniquely evolutionary sources of neuroanatomical variation isolated from phenotypic plasticity and propose additional future research directions that may help to further distinguish evolutionary and developmental forces shaping the brain.

TELEOSTS AS MODEL SPECIES IN COMPARATIVE NEUROANATOMY

In general, average brain size has increased during vertebrate evolution and, while much of this is due to changes in overall body size, some of this variation is due to evolutionary forces acting on brain development beyond allometric constraints (Striedter, 2005). Such comparative neuroanatomy has generated hypotheses positing that evolutionary forces promoting larger brains both preceded and were necessary for the subsequent evolution of complex cognitive processes including social learning (van Schaik and Burkart, 2011) and tool manufacture (Emery and Clayton, 2005). However, focusing on variation in whole brain size has been criticized for concealing changes that may be occurring within the brain's major constituent parts independently (Healy and Rowe, 2007). Accordingly, models of brain evolution have also considered how the sizes of distinct brain structures scale with overall brain size based on correlations with ontogeny, habitat complexity, and behavioral specialization (Finlay and Darlington, 1995; Barton and Harvey, 2000). We now appreciate that both mosaic evolution (evolution of brain regions relatively independent from one another) and developmental constraints (concerted evolution of brain regions) play fundamental roles in explaining the neuroanatomical variation that we observe in nature (Striedter, 2005).

The conceptual basis for models of brain evolution is built on a foundation of mostly descriptive neuroanatomical approaches (Northcutt, 2002). Due to their extensive phenotypic, behavioral, and ecological diversity (Streelman and Danley, 2003; Shumway, 2008), teleost fish have been used historically in studies associating brain structure to evolutionary adaptation. For instance, Huber et al. (1997) generated an extensive database of brain region morphology for 189 African cichlid species across three inland lakes. Using comparative analysis, they reported that species evolved to engage in agile prey capture behavior exhibit larger cerebella and optic tecta, a midbrain structure and primary recipient of retinal input, compared to species evolved to feed on relatively stationary mollusks and plants. The authors interpret this neuroanatomical difference as a product of evolutionary specialization to improve visual and motor capabilities in order to track and chase moving prey.

Complementary to this work, Kotrschal and Palzenberger (1992) found that bottom-feeding benthivore cyprinid species exhibit an evolutionary increase in the size of brain structures involved in processing chemosensory and olfactory input, consistent with relaxed evolutionary pressure on visual capabilities and increased importance for smell and taste while feeding along turbid lake bottoms. These investigations set the stage for the concept of brain ecotypes, in which brain morphology is specialized to improve sensory processing in the modality most critical for feeding success (Sylvester et al., 2010). Since this work, additional teleost studies have identified evolutionary patterns in brain morphology associated with habitat complexity, social organization (Pollen et al., 2007), sexual selection, and parental care across species (Gonzalez-Voyer and Kolm, 2010). Whereas almost all comparative studies in teleost neuroanatomy include whole brain size in these analyses, they also include additional analysis of brain components, and typically explain whole brain size findings as a product of changes in specific brain structures. For example, monoparental female care in cichlids is associated with the evolution of a larger whole brain (Gonzalez-Voyer and Kolm, 2010). However, brain component analysis reveals that, whereas most brain components studied are larger in these species, cerebellar and hypothalamic volumes decrease. Collectively, teleost fishes have been shown to be powerful models in comparative neuroanatomy, identifying patterns in both concerted and mosaic brain evolution associated with evolutionary forces across species. Common to all of these studies is the assumption that evolutionary pressure to improve

certain types of sensory processing will drive changes in brain structure size and that such neuroanatomical measurements accurately capture species differences in the brain. However, research on phenotypic plasticity in teleost brains challenges this assumption, demonstrating that the environment similarly impacts brain structure within a species.

TELEOSTS AS MODEL SPECIES IN STUDYING PHENOTYPIC PLASTICITY IN NEUROANATOMY

A common criticism of comparative approaches to neuroanatomy is that single species must often be represented by individual measurements collected from few brains. For example, the work of Huber et al. (1997) discussed above included 189 cichlid species represented by 216 brains, indicating that most species data was derived from a single, adult brain from a museum collection. By reducing species to single measurements we overlook potential intraspecific variation in brain structure. This oversight can either over- or underrepresent the inferred evolutionary contributions to differences in neuroanatomy between species. Because comparative work in teleosts focuses on the size of individual brain structures, is there evidence of phenotypic plasticity in these same measurements within a species?

Some of the first evidence demonstrating the capacity of the environment to shape the teleost brain structure came from comparisons between wild-caught and lab-reared fish populations. Salmon reared in a hatchery exhibit reduced olfactory bulb and telencephalon size compared to age-matched wild salmon from the same genetic cohort (Kihslinger et al., 2006). The first generation of female guppies reared in the laboratory from wild parents exhibited reduced telencephala and optic tecta compared to wild-caught fish (Burns et al., 2009). Because the laboratory environment is traditionally considered to lack much of the sensory stimuli animals would encounter in the wild, these findings generated hypotheses suggesting that neuroanatomical development in fish is influenced by sensorimotor experiences, particularly those with ethological value to the species studied (Gonda et al., 2011). Consistent with these hypotheses, both male guppies collected from regions of high predation and laboratory-reared male guppies exposed to olfactory and visual predator cues during development have larger brains as adults compared to unexposed males (Reddon et al., 2018). Thus, phenotypic plasticity may be an important factor in explaining neuroanatomical variation when comparing brains.

Another approach to studying intraspecific variation in teleost neuroanatomy has been to compare fish populations of the same species inhabiting different environments. These studies have revealed habitat-dependent correlations with brain size similar to studies both comparing lab- and wild-bred populations and correlating habitat and brain structure across species comparatively. For example, whole-brain size is larger in sunfish that occupy a littoral shoreline habitat vs. those that live in a pelagic habitat (Axelrod et al., 2018), and marine populations of nine-spined sticklebacks had larger olfactory bulbs and telencephala relative to pond populations (Gonda et al., 2009). A limitation to comparing populations in this manner is that genetic differences among different populations of the same species can translate into different brain morphologies (Ishikawa et al., 1999), complicating the assertion that environmental factors alone explain these differences in the brain. However, we believe it is critical to note that intraspecific variation in neuroanatomy identified both between populations and also in lab- and wild-reared individuals is similar in form and even, in some cases, magnitude to those described between species in comparative teleost work.

Perhaps, then, size alone is insufficient to separate inter- and intraspecific variation in brain structure. Instead, one might ask whether differences in brain structure between and within species are achieved *via* the same developmental mechanisms. Mechanistic work examining evolution and phenotypic plasticity in the teleost brain highlights the depth of the problem in relying on mature neuroanatomy alone in comparative work: neurogenesis, the production, and incorporation of new brain cells appears to be a common mechanism generating variation in neuroanatomy both between species, *via* evolutionary changes in neurogenic brain development, and within species, as a form of sensory experience-dependent neuroplasticity.

NEUROGENESIS AS A COMMON MECHANISM OF EVOLUTIONARY AND ENVIRONMENTAL VARIATION IN BRAIN STRUCTURE

How do teleost brain regions get bigger in some species? In the vertebrates, the most discussed model of the evolutionary growth of brain structures is the "late equals large" model (Finlay et al., 2001). This model argues that vertebrate brains grow following a similar developmental sequence in which new neurons are generated and incorporated into different brain structures at different times. For a particular brain region to grow larger, the period of neurogenesis in which new neurons are added to that structure is protracted. This model has been used most popularly to explain the expansion of the cerebral cortex in humans (McKinney, 2002; Finlay and Brodsky, 2006). Whereas this model has been criticized for simplifying patterns of brain structure evolution (Barton and Harvey, 2000), opposing models similarly argue that regulating the timing and length of neurogenic periods underlies evolutionary changes in brain structure (reviewed in Montgomery et al., 2016). In teleosts, changes in the timing of neuron production within distinct brain regions are also thought to be the primary means through which differential brain growth between species occurs (Sylvester et al., 2011). If neurogenesis is the primary mechanism through which brain regions change size between species, then what role does neurogenesis play, if any, in influencing brain structure within species?

Traditionally, the influence of the environment and specific sensory experience on brain development has been assumed to manifest as synaptic plasticity in pre-existing neurons (Knudsen,

2004; Hensch, 2005). In part, this belief appears to stem from the assumption that, at least in mammals, neurogenesis is an embryonic process that is largely complete by birth, before an animal is exposed to the external environment. However, since the discovery of adult neurogenesis in the mammalian brain (Altman and Das, 1965), a growing body of work demonstrates that neurogenic processes continue to shape the brain well beyond embryogenesis (Feliciano et al., 2015). Furthermore, teleosts exhibit extensive neurogenesis in the brain throughout life compared to mammals (Zupanc and Horschke, 1995; Lindsey and Tropepe, 2006), indicating that neurogenesis may be a life-long neurobiological substrate for brain growth in fish.

One of the first studies to document experience-dependent modulation of developmental neurogenesis compared the telencephala of salmon reared in environments differing in water flow velocity and physical structure (Lema et al., 2005). The authors reported that the rearing environment affected neural progenitor cell proliferation rates in the telencephalon throughout development. Whereas the authors did not find a difference in telencephalon size associated with these changes in neurogenesis, this may be due to their environmental manipulation which enriched water flow in one condition and physical environment in another, preventing conclusions on the importance of a single type of sensory experience on telencephalon development.

Since this finding, work on adult zebrafish has identified visual, olfactory, and social experience-dependent modulation of neurogenesis (Lindsey and Tropepe, 2014; Lindsey et al., 2014). A limitation to these results in the context of our discussion here, however, is that analyses included only adult fish, which exhibit the lowest rates of neurogenesis compared to earlier in life. Thus, adult changes in neurogenesis would likely not translate into changes in brain size. Encouraged by these results, we tested whether similar sensory experience-dependent neurogenic modulation would occur postembryonically in zebrafish when neurogenesis persists at a much higher rate and is the primary driving force promoting brain growth (Cerveny et al., 2012; Furlan et al., 2017). We found that visual and sensorimotor experience regulated the neurogenic growth of the optic tectum and telencephalon in zebrafish, respectively (Hall and Tropepe, 2018a,b). Specifically, we found that rearing zebrafish larvae in a low-intensity light reduced the number of newly generated neurons that incorporate into the optic tectum. Anatomically, this reduced neuronal incorporation in the tectum reduced the size of the tectal neuropil, which is in part innervated by apical projections from the new neurons tracked in this study, in as few as 10 days of development (Hall and Tropepe, 2018a). In our second study, we found that restricting a zebrafish larva's movement significantly reduced the proliferation of neural precursors in the dorsal telencephalon, reducing the size of the telencephalon in as few as 6 days (Hall and Tropepe, 2018b). Our work demonstrates not only the capacity for sensory experience to modulate neuroanatomy *via* neurogenesis, but also shows that experience shapes tectal and telencephalic anatomy intraspecifically, two brain regions reported exhibiting evolutionary specialization in size across teleost species (van Staaden et al., 1994; Huber et al., 1997).

Collectively, the work above identifies a critical issue with discerning evolutionary and environmental sources of brain size variation: variation in brain structure appears to manifest similarly between and within species at the level of both changes in brain size and the neurogenic developmental processes preceding them.

DECIPHERING PHYLOGENETIC ADAPTATION VS. PHENOTYPIC PLASTICITY

The preceding discussion highlights the utility of teleost models for studying how evolutionary and environmental forces shape the brain. By limiting analyses to brain region size and rates of neurogenesis, however, we have been unable to discern the contributions of evolution and environment to neuroanatomy. One solution to revealing evolutionary signatures in the teleost brain has been to look even earlier in development, prior to changes in brain morphology and earlier developmental neurogenesis.

Research on mapping gene expression along the anterior-posterior and dorsal-ventral brain axes in related cichlid species has revealed species-specific differences in the timing of expression of these genes that subsequently developed into species differences in brain morphology (Sylvester et al., 2010). Cichlid species that develop brains supporting greater visual capacities for agile prey capture exhibit embryonic gene expression patterns that led to the relative growth of brain structures involved in processing vision, including the thalamus and optic tectum. Conversely, cichlid species developing brains supporting greater olfactory capabilities for benthic feeding exhibit embryonic gene expression patterns that led to the relative growth of ventral and anterior brain regions, including the subpallium and olfactory bulb. By interfering with the expression patterns of one of these genes, *Wnt*, using doses of lithium chloride, Sylvester et al. (2010) perturbed embryonic gene expression in such a way that an "olfactory-based" cichlid species would develop a brain anatomically reminiscent of a "visual-based" cichlid (Sylvester et al., 2011). This and other work showing how opposing *Hedgehog* and *Wingless* signaling pathways can regulate species difference in the structure of the fish telencephalon (Sylvester et al., 2013) have provided a new perspective in comparative neuroanatomy, identifying novel mechanisms through which evolution has shaped brain development manifesting as gene expression regulation in embryonic development, prior to (or coincident with) the onset of neurogenesis, changes in brain region size, and the processing of sensory experience.

We believe the next useful step is to connect these embryonic patterns of gene expression to prior work identifying species differences in mature neuroanatomy by refocusing on intermediary neurogenic developmental processes. Unlike prior work, however, we believe studying interspecific differences in developmental neurogenesis must recognize that neurogenesis itself is a multi-stepped process involving neural stem cells producing intermediate progenitor cells producing neurons,

which may or may not survive long enough to integrate into neural circuitry (Lindsey et al., 2018). Accordingly, when a brain region is larger in an individual or species, this can be achieved through a multitude of different neurogenic mechanisms, such as changes in cell proliferation, fate, and survival, which in turn may generate mature brain structures with vastly different neuronal compositions. For example, we found that visual experience modulates optic tectum size by affecting the survival of all tested neuronal phenotypes equally (Hall and Tropepe, 2018a), suggesting that tectal size is proportionally scaled by visual experience. Does a similar proportional scaling occur across teleost species or do the tecta of different teleost species contain different proportions of neurons serving different functions?

Characterizing developmental neurogenesis in teleosts has revealed that different brain regions incorporate new neurons generated by distinct neural stem cell populations. For example, the dorsal telencephalon incorporates new neurons generated by radial glial neural stem cells whereas the ventral telencephalon primarily incorporates new neurons generated by neuroepithelial stem cells (Wullimann, 2009; Lindsey et al., 2018). We found that, at least intraspecifically, these neural stem cell niches can be regulated independently of one another (Hall and Tropepe, 2018a,b) and persist into adulthood (Lindsey et al., 2012). Such neuroanatomical modularity in developmental neurogenesis may be a novel avenue in which interspecific differences in brain structure may manifest through proposed models of mosaic brain evolution discussed above.

In a recent display of the power in connecting embryonic gene expression with subsequent changes in neurogenesis, Cárdenas et al. (2018) found that interspecific differences in embryonic *Robo* gene expression influence whether or not intermediate progenitor cells either abstain from dividing and differentiate into neurons or first divide before differentiating, ultimately doubling the number of neurons produced by a neural stem cell population. The authors suggest that such *Robo*-dependent increases in progenitor proliferation underlie the extreme forebrain expansion necessary to evolve a cerebral cortex in mammals (Kriegstein et al., 2006) compared to other vertebrates. Whereas analogous forebrain progenitors in zebrafish are assumed not to divide and instead strictly differentiate into neurons (Furlan et al., 2017), whether these neural progenitors exhibit differences in proliferative behavior across fish species during development as a mechanism underlying evolutionary specialization in telencephalon size may be a promising future research avenue.

CONCLUSION

With the advent of comparative neurobiological work at the levels of both embryonic gene expression and mature neuroanatomy and the growing understanding of developmental neurogenic processes in teleosts, we are poised to weave molecular embryonic, developmental, and neuroanatomical techniques to modernize our understanding of vertebrate brain evolution. By adopting integrative approaches through which early patterns of gene expression are translated into neurogenic growth processes in development that ultimately culminate in mature brain structures, we will develop a novel understanding of how evolutionary signatures in species-specific embryonic gene expression develop into evolutionary signatures in brain structure and function. As discussed above, teleosts have made fundamental contributions towards unveiling the problem of isolating evolutionary and environmental contributions in the brain by relying on neuroanatomy alone; however, teleosts have also provided some of the only insight illuminating a path towards new integrative approaches to overcome this problem. Teleosts remain one of the most, if not the most, accessible clades to collect multiple species and study their development in the lab or in the wild, enabling a complete gene-to-development-to-neuroanatomy approach across species and leading the way for a new understanding of evolved differences in the central nervous system.

AUTHOR CONTRIBUTIONS

ZH and VT conceived and wrote the manuscript.

FUNDING

This work was supported by the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

Altman, J., and Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* 124, 319–335. doi: 10.1002/cne.901240303

Axelrod, C. J., Laberge, F., and Robinson, B. W. (2018). Intraspecific brain size variation between coexisting sunfish ecotypes. *Proc. Biol. Sci.* 285:20181971. doi: 10.1098/rspb.2018.1971

Barton, R. A., and Harvey, P. H. (2000). Mosaic evolution of brain structure in mammals. *Nature* 405, 1055–1057. doi: 10.1038/35016580

Burns, J. G., Saravanan, A., and Rodd, F. H. (2009). Rearing environment affects the brain size of guppies: lab-reared guppies have smaller brains than wild-caught guppies. *Ethology* 115, 122–133. doi: 10.1111/j.1439-0310.2008.01585.x

Cárdenas, A., Villalba, A., de Juan Romero, C., Picó, E., Kyrousi, C., Tzika, A. C., et al. (2018). Evolution of cortical neurogenesis in amniotes controlled by robo signaling levels. *Cell* 174, 590–606. doi: 10.1016/j.cell.2018.06.007

Cerveny, K. L., Varga, M., and Wilson, S. W. (2012). Continued growth and circuit building in the anamniote visual system. *Dev. Neurobiol.* 72, 328–345. doi: 10.1002/dneu.20917

Emery, N. J., and Clayton, N. S. (2005). Evolution of the avian brain and intelligence. *Curr. Biol.* 15, R946–R950. doi: 10.1016/j.cub.2005.11.029

Feliciano, D. M., Bordey, A., and Bonfanti, L. (2015). Noncanonical sites of adult neurogenesis in the mammalian brain. *Cold Spring Harb. Perspect. Biol.* 7:a018846. doi: 10.1101/cshperspect.a018846

Finlay, B. L., and Brodsky, P. (2006). "Cortical evolution and development conserved programs producing predictable, disproportionate cortical growth and systematic proliferation of cortical areas," in *Evolution of Nervous Systems*, eds J. Kaas and L. Krubitzer (London: Elsevier), 73–96.

Finlay, B., and Darlington, R. (1995). Linked regularities in the development and evolution of mammalian brains. *Science* 268, 1578–1584. doi: 10.1126/science.7777856

Finlay, B. L., Darlington, R. B., and Nicastro, N. (2001). Developmental structure in brain evolution. *Behav. Brain Sci.* 24, 263–278. doi: 10.1017/s0140525x01003958

Furlan, G., Cuccioli, V., Vuillemin, N., Dirian, L., Muntasell, A. J., Coolen, M., et al. (2017). Life-long neurogenic activity of individual neural stem cells and continuous growth establish an outside-in architecture in the teleost pallium. *Curr. Biol.* 27, 3288–3301. doi: 10.1016/j.cub.2017.09.052

Gonda, A., Herczeg, G., and Merilä, J. (2009). Habitat-dependent and-independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. *Proc. Biol. Sci.* 276, 2085–2092. doi: 10.1098/rspb.2009.0026

Gonda, A., Herczeg, G., and Merilä, J. (2011). Population variation in brain size of nine-spined sticklebacks (*Pungitius pungitius*)—Local adaptation or environmentally induced variation? *J. Evol. Biol.* 11:75. doi: 10.1186/1471-2148-11-75

Gonzalez-Voyer, A., and Kolm, N. (2010). Sex, ecology and the brain: evolutionary correlates of brain structure volumes in tanganyikan cichlids. *PLoS One* 5:e14355. doi: 10.1371/journal.pone.0014355

Hall, Z. J., and Tropepe, V. (2018a). Visual experience facilitates BDNF-dependent adaptive recruitment of new neurons in the postembryonic optic tectum. *J. Neurosci.* 38, 2000–2014. doi: 10.1523/jneurosci.1962-17.2018

Hall, Z. J., and Tropepe, V. (2018b). Movement maintains forebrain neurogenesis *via* peripheral neural feedback in larval zebrafish. *Elife* 7:e31045. doi: 10.7554/elife.31045

Healy, S. D., and Rowe, C. (2007). A critique of comparative studies of brain size. *Proc. Biol. Sci.* 274, 453–464. doi: 10.1098/rspb.2006.3748

Hensch, T. K. (2005). Critical period plasticity in local cortical circuits. *Nat. Rev. Neurosci.* 6, 877–888. doi: 10.1038/nrn1787

Huber, R., van Staaden, M. J., Kaufman, L. S., and Liem, K. F. (1997). Microhabitat use, trophic patterns, and the evolution of brain structure in African cichlids. *Brain Behav. Evol.* 50, 167–182. doi: 10.1159/000113330

Ishikawa, Y., Yoshimoto, M., Yamamoto, N., and Ito, H. (1999). Different brain morphologies from different genotypes in a single teleost species, the medaka (*Oryzias latipes*). *Brain Behav. Evol.* 53, 2–9. doi: 10.1159/000006577

Kihslinger, R. L., Lema, S. C., and Nevitt, G. A. (2006). Environmental rearing conditions produce forebrain differences in wild Chinook salmon *Oncorhynchus tshawytscha*. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 145, 145–151. doi: 10.1016/j.cbpa.2006.06.041

Knudsen, E. I. (2004). Sensitive periods in the development of the brain and behavior. *J. Cogn. Neurosci.* 16, 1412–1425. doi: 10.1162/0898929042304796

Kotrschal, K., and Palzenberger, M. (1992). Neuroecology of cyprinids: comparative, quantitative histology reveals diverse brain patterns. *Environ. Biol. Fish.* 33, 135–152. doi: 10.1007/BF00002560

Kriegstein, A., Noctor, S., and Martínez-Cerdeño, V. (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat. Rev. Neurosci.* 7, 883–890. doi: 10.1038/nrn2008

Lema, S. C., Hodges, M. J., Marchetti, M. P., and Nevitt, G. A. (2005). Proliferation zones in the salmon telencephalon and evidence for environmental influence on proliferation rate. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 141, 327–335. doi: 10.1016/j.cbpb.2005.06.003

Lindsey, B. W., Darabie, A., and Tropepe, V. (2012). The cellular composition of neurogenic periventricular zones in the adult zebrafish forebrain. *J. Comp. Neurol.* 520, 2275–2316. doi: 10.1002/cne.23065

Lindsey, B. W., Di Donato, S., Kaslin, J., and Tropepe, V. (2014). Sensory-specific modulation of adult neurogenesis in sensory structures is associated with the type of stem cell present in the neurogenic niche of the zebrafish brain. *Eur. J. Neurosci.* 40, 3591–3607. doi: 10.1111/ejn.12729

Lindsey, B. W., Hall, Z. J., Heuzé, A., Joly, J. S., Tropepe, V., and Kaslin, J. (2018). The role of neuro-epithelial-like and radial-glial stem and progenitor cells in development, plasticity and repair. *Prog. Neurobiol.* 170, 99–114. doi: 10.1016/j.pneurobio.2018.06.004

Lindsey, B. W., and Tropepe, V. (2006). A comparative framework for understanding the biological principles of adult neurogenesis. *Prog. Neurobiol.* 80, 281–307. doi: 10.1016/j.pneurobio.2006.11.007

Lindsey, B. W., and Tropepe, V. (2014). Changes in the social environment induce neurogenic plasticity predominantly in niches residing in sensory structures of the zebrafish brain independently of cortisol levels. *Dev. Neurobiol.* 74, 1053–1077. doi: 10.1002/dneu.22183

McKinney, M. L. (2002). "Brain evolution by stretching," in *Human Evolution Through Developmental Change*, eds N. Minugh-Purvis and K. J. McNamara (Baltimore, MD: John Hopkins Press), 173–189.

Montgomery, S. H., Mundy, N. I., and Barton, R. A. (2016). Brain evolution and development: adaptation, allometry and constraint. *Proc. Biol. Sci.* 283:20160433. doi: 10.1098/rspb.2016.0433

Northcutt, R. G. (2002). Understanding vertebrate brain evolution. *Integr. Comp. Biol.* 42, 743–756. doi: 10.1093/icb/42.4.743

Pollen, A. A., Dobberfuhl, A. P., Scace, J., Igulu, M. M., Renn, S. C. P., Shumway, C. A., et al. (2007). Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. *Brain Behav. Evol.* 70, 21–39. doi: 10.1159/000101067

Reddon, A. R., Chouinard-Thuly, L., Leris, I., and Reader, S. M. (2018). Wild and laboratory exposure to cues of predation risk increases relative brain mass in male guppies. *Funct. Ecol.* 32, 1847–1856. doi: 10.1111/1365-2435.13128

Shumway, C. A. (2008). Habitat complexity, brain, and behavior. *Brain Behav. Evol.* 72, 123–134. doi: 10.1159/000151472

Streelman, J. T., and Danley, P. D. (2003). The stages of vertebrate evolutionary radiation. *Trends Ecol. Evol.* 18, 126–131. doi: 10.1016/s0169-5347(02)00036-8

Striedter, G. F. (2005). *Principles of Brain Evolution.* Sunderland, MA: Sinauer Associates.

Sylvester, J. B., Pottin, K., and Streelman, J. T. (2011). Integrated brain diversification along the early neuraxes. *Brain Behav. Evol.* 78, 237–247. doi: 10.1159/000329840

Sylvester, J. B., Rich, C. A., Loh, Y. H. E., van Staaden, M. J., Fraser, G. J., and Streelman, J. T. (2010). Brain diversity evolves *via* differences in patterning. *Proc. Natl. Acad. Sci. U S A* 107, 9718–9723. doi: 10.1073/pnas.1000395107

Sylvester, J. B., Rich, C. A., Yi, C., Peres, J. N., Houart, C., and Streelman, J. T. (2013). Competing signals drive telencephalon diversity. *Nat. Commun.* 4:1745. doi: 10.1038/ncomms2753

van Schaik, C. P., and Burkart, J. M. (2011). Social learning and evolution: the cultural intelligence hypothesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 366, 1008–1016. doi: 10.1098/rstb.2010.0304

van Staaden, M. J., Huber, P., Kaufman, L. S., and Liem, K. F. (1994). Brain and body size, general patterns and evolutionary trends. *Zoology* 98, 165–178.

Wullimann, M. F. (2009). Secondary neurogenesis and telencephalic organization in zebrafish and mice: a brief review. *Integr. Zool.* 4, 123–133. doi: 10.1111/j.1749-4877.2008.00140.x

Zupanc, G. K., and Horschke, I. (1995). Proliferation zones in the brain of adult gymnotiform fish: a quantitative mapping study. *J. Comp. Neurol.* 353, 213–233. doi: 10.1002/cne.903530205

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroanatomy

REVIEW
published: 26 March 2020
doi: 10.3389/fnana.2020.00011

Brain and Behavioral Asymmetry: A Lesson From Fish

Maria Elena Miletto Petrazzini[1], Valeria Anna Sovrano[2,3], Giorgio Vallortigara[2] and Andrea Messina[2]**

[1] School of Biological and Chemical Sciences, Queen Mary University of London, London, United Kingdom, [2] Center for Mind/Brain Sciences, University of Trento, Rovereto, Italy, [3] Department of Psychology and Cognitive Science, University of Trento, Rovereto, Italy

It is widely acknowledged that the left and right hemispheres of human brains display both anatomical and functional asymmetries. For more than a century, brain and behavioral lateralization have been considered a uniquely human feature linked to language and handedness. However, over the past decades this idea has been challenged by an increasing number of studies describing structural asymmetries and lateralized behaviors in non-human species extending from primates to fish. Evidence suggesting that a similar pattern of brain lateralization occurs in all vertebrates, humans included, has allowed the emergence of different model systems to investigate the development of brain asymmetries and their impact on behavior. Among animal models, fish have contributed much to the research on lateralization as several fish species exhibit lateralized behaviors. For instance, behavioral studies have shown that the advantages of having an asymmetric brain, such as the ability of simultaneously processing different information and perform parallel tasks compensate the potential costs associated with poor integration of information between the two hemispheres thus helping to better understand the possible evolutionary significance of lateralization. However, these studies inferred how the two sides of the brains are differentially specialized by measuring the differences in the behavioral responses but did not allow to directly investigate the relation between anatomical and functional asymmetries. With respect to this issue, in recent years zebrafish has become a powerful model to address lateralization at different level of complexity, from genes to neural circuitry and behavior. The possibility of combining genetic manipulation of brain asymmetries with cutting-edge *in vivo* imaging technique and behavioral tests makes the zebrafish a valuable model to investigate the phylogeny and ontogeny of brain lateralization and its relevance for normal brain function and behavior.

Keywords: behavioral lateralization, brain asymmetry, genetics, fish, zebrafish, drivers of lateralization

OPEN ACCESS

Edited by:
Paolo De Girolamo, University of Naples Federico II, Italy

Reviewed by:
Nina Patzke, Hokkaido University, Japan
Sebastian Ocklenburg, Ruhr University Bochum, Germany

****Correspondence:***
Maria Elena Miletto Petrazzini
mariaelena.milettopetrazzini@gmail.com
Andrea Messina
andrea.messina@unitn.it

Received: *30 December 2019*
Accepted: *05 March 2020*
Published: *26 March 2020*

Citation:
Miletto Petrazzini ME, Sovrano VA, Vallortigara G and Messina A (2020) Brain and Behavioral Asymmetry: A Lesson From Fish. Front. Neuroanat. 14:11. doi: 10.3389/fnana.2020.00011

INTRODUCTION

Brain lateralization is defined as the different functional specialization of the left and right sides of the brain. It was first described in the 19th century by M. Dax and P. Broca who showed that lesions in specific areas in the left hemisphere but not in the right one, were associated with deficits in producing language thus suggesting left hemisphere dominance for

speech. For more than a century brain lateralization has been considered a human peculiarity linked to handedness, and complex cognitive functions, such as language (McManus, 1999; MacNeilage et al., 2009).

This belief has been challenged in the 1970s by a series of independent discoveries. Severing the left hypoglossal nerve impaired singing in songbirds, whereas severing the right nerve did not (Nottebohm, 1971). Unilateral hemispheric lesions in rats differently affected their exploratory behavior (Denenberg et al., 1978). Pharmacological treatment in the left hemisphere in chicks disrupted their visual discrimination abilities (Rogers and Anson, 1979). Since these first discoveries, the study of brain lateralization in non-human animals has become a burgeoning field of research and evidence of functional lateralization has been reported in species from all vertebrate classes (reviewed in see Vallortigara et al., 2011; Frasnelli et al., 2012; Ströckens et al., 2013; Ocklenburg et al., 2013; Rogers and Vallortigara, 2015; Vallortigara and Versace, 2017), thus demonstrating that it is a general feature of the animal brains (Rogers and Vallortigara, 2017). In particular, research on vertebrates has described a general pattern of lateralization among species, with the right hemisphere specialized in controlling social behavior, responding to novel and unexpected stimuli (e.g., predators) and processing global information whereas the left hemisphere is specialized to categorize stimuli, regulate routine behavior in familiar circumstances and focus attention to targets (Rogers et al., 2013; Rogers, 2014). For instance, a right-eye bias (left hemisphere dominance) for prey catching has been described in chicks (Mench and Andrew, 1986) pigeons (Güntürkün and Kesch, 1987; Güntürkün et al., 2000) and toads (Vallortigara et al., 1998; Robins and Rogers, 2004) and a left-eye bias (right dominance) in escape response to predators has been observed in dunnarts (Lippolis et al., 2005), horses (Austin and Rogers, 2007), lizards (Bonati et al., 2010, 2013), and toads (Lippolis et al., 2002).

Although investigation of brain lateralization in fish started more recently, data collected over the past 20 years have contributed much to the field (Vallortigara and Bisazza, 2002; Bisazza and Brown, 2011; Duboc et al., 2015). An advantage of using fish is due to the fact the eyes are laterally placed and the optic nerves decussate at the optic chiasm so that visual stimuli perceived with the right eye are predominantly processed by the left side of the brain and vice versa. As a consequence, it is possible to measure lateralized behavior in response to unilaterally presented stimuli and draw inferences about the functional specializations of the two hemispheres. Therefore, the observation of behavior represents a powerful non-invasive tool to assess the degree and direction of their brain lateralization.

Here we will provide a general overview of brain lateralization in fish. In particular, we will first present some examples of lateralized behaviors observed in the wild and in the laboratory highlighting the importance of these studies to comprehend the environmental impact on the development of asymmetrical biases and to understand the advantages and disadvantages of lateralized brains. We will then focus on the genetic mechanisms involved in the development of brain asymmetries discussing the relevance of zebrafish (*Danio rerio*) as a powerful animal model to link genetic, functional and behavioral asymmetries.

Behavioral and Perceptual Asymmetries

There is considerable evidence of asymmetries in motor responses and sensory perception in fish (reviewed in Stancher et al., 2018; **Table 1**). An example of motor lateralization (i.e., behavioral bias at one of the two sides of the body) is represented by the fast escape response following threatening stimuli, more commonly known as C-start response. This response consists of a unilateral muscle contraction, which causes C-shape body bending, followed by a flip of the tail that allows the fish to flee from danger. This motor bias is triggered by the Mauthner cells, a pair of giant reticulospinal neurons that elicit muscle contraction and suppresses simultaneous activity of the opposite neuron thus allowing short response latencies (i.e., less than 20 ms; Domenici and Blake, 1997; Eaton et al., 2001; Korn and Faber, 2005). It has been showed that the right and left Mauthner neurons differ in size in the goldfish (*Carassius auratus*): individuals with the right larger neuron preferentially turn to the left side and vice versa thus suggesting that neuroanatomical asymmetry regulates the asymmetry of the C-start response (Moulton and Barron, 1967; Mikhailova et al., 2005). It has been suggested that asymmetry arises as a trade-off between direction and speed of escape responses (Vallortigara, 2000). Escape performance is fundamental for individual survival and strongly lateralized shiner perch (*Cymatogaster aggregata*) showed higher escape reactivity and superior ability to escape from predator attacks compared to non-lateralized fish (Dadda et al., 2010b). However, considerable variation in the direction of the fast start response has been observed. For instance, Heuts (1999) showed a population right-bias in C-start direction in zebrafish and goldfish while Lippolis et al. (2009) described a leftward population bias in a non-teleost fish, the Australian lungfish (*Neoceratodus forsteri*). By contrast, Bisazza et al. (1997a) and Izvekov and Nepomnyashchikh (2010) observed a bimodal distribution in the killifish (*Jenynsia multidentate*) and in the roach (*Rutilus rutilus)* with a similar number of individuals escaping to the left or to the right. Furthermore, a reversal in turning bias, from right to left, was observed in both juvenile and adult goldbelly topminnows (*Girardinus falcatus*) upon repeated presentations of a potential predator (Cantalupo et al., 1995) suggesting that the familiarity with the situation could lead the fish to perceive the stimulus as innocuous (since it never attacked the subjects) with shift toward control by the left side of the brain.

Note that in these studies it is difficult to discern the pure motor component from the behavioral bias that can be induced by visual lateralization. For instance, it is known that western mosquitofish (*Gambusia holbrooki*) and goldbelly topminnows preferentially use the right eye to monitor a predator when observed in the detour test, in which the fish had to swim along a runway until it faced a barrier behind which the predator was located, thus exhibiting a leftward turning bias (Bisazza et al., 1997b, 1998). However, the rightward turning preference described in four out of five species of minnows (Osteichthyes: Cyprinidae) observed in a T-shaped arena in the absence of any visual stimulus provided evidence of true motor

TABLE 1 | Types of behavioral lateralization investigated and species in which lateralization has been observed or not.

Types of behavioral lateralization	Species	Occurrence of behavioral lateralization	References
Motor asymmetry			
Fast escape response	Goldfish (*Carassius auratus*)	Yes	Moulton and Barron, 1967; Mikhailova et al., 2005
	Shiner perch (*Cymatogaster aggregata*)	Yes	Dadda et al., 2010b
	Zebrafish *(Danio rerio)*	Yes	Heuts, 1999
	Australian lungfish (*Neoceratodus forsteri)*	Yes	Lippolis et al., 2009
	Killifish *(Jenynsia multidentata)*	Yes	Bisazza et al., 1997a
	Roach (*Rutilus rutilus*)	Yes	Izvekov and Nepomnyashchikh, 2010
	Goldbelly topminnows *(Girardinus falcatus)*	Yes	Cantalupo et al., 1995
	Giant danio (*Devario aequipinnatus*)	Yes	Stennett and Strauss, 2010
	Scissortail rasbora *(Rasbora trilineata)*	No	Stennett and Strauss, 2010
	Zebrafish *(Danio rerio)*	Yes	Stennett and Strauss, 2010
	White Cloud Mountain minnow (*Tanichthys albonubes*)	Yes	Stennett and Strauss, 2010
	Fathead minnow (*Pimephales promelas*)	Yes	Stennett and Strauss, 2010
Rotational swimming	Roach (*Rutilus rutilus*)	Yes	Izvekov and Nepomnyashchikh, 2010
	Mosquitofish *(Gambusia hoolbrooki)*	Yes	Bisazza and Vallortigara, 1996
	Sterlet sturgeon (*Acipenser ruthenus*)	Yes	Izvekov et al., 2014
	Roach (*Rutilus rutilus*)	Yes	Izvekov et al., 2012
Coiled posture	North eastern Pacific hagfish (*Eptatretus stoutii*)	Yes	Miyashita and Palmer, 2014
Perceptual asymmetry			
Foraging behavior	Zebrafish *(Danio rerio)*	Yes	Miklosi and Andrew, 1999; Hata and Hori, 2011
	Australian lungfish (*Neoceratodus forsteri*)	Yes	Lippolis et al., 2009
	Scale-eating cichlids (*Perissodus microlepis*)	Yes	Hori, 1993; Hori et al., 2007; Stewart and Albertson, 2010; Van Dooren et al., 2010; Lee et al., 2012; Takeuchi et al., 2012
	Cichlid *(Neolamprologus moori)*	Yes	Hori et al., 2007
	Freshwater goby (*Rhinogobius flumineus*)	Yes	Seki et al., 2000
	Japanese medaka (*Oryzias latipes*)	Yes	Hata et al., 2012
	Tanganyikan cichlid *(Julidochromis ornatus)*	Yes	Hata et al., 2012
	Scale-eating characiform (*Exodon paradoxus*)	Yes	Hata et al., 2011
Social behavior	Mosquitofish (*Gambusia hoolbrooki*)		
	Females	Yes	Bisazza et al., 1998, 1999; Sovrano et al., 1999, 2001; De Santi et al., 2001
	Males	No	Bisazza et al., 1998; Sovrano et al., 1999
	Goldbelly topminnow (*Girardinus falcatus*)		
	Females	Yes	Bisazza et al., 1998
	Males	No	
	Convict cichlid (*Amatitlania nigrofas*)		
	Females	Yes	Moscicki et al., 2011
	Males	No	
	Breeding cichlid (*Neolamprologus pulcher*)	Yes	Reddon and Balshine, 2010
	Zebrafish *(Danio rerio)*	Yes	Sovrano et al., 2001; Sovrano and Andrew, 2006
	Redtail splitfin (*Xenotoca eiseni*)		
	Females	Yes	Sovrano et al., 1999
	Males	No	Sovrano et al., 1999
	Angelfish *(Pterophyllum scalare)*	Yes	Sovrano et al., 1999
	Eurasian minnow (*Phoxinus phoxinus*)	Yes	Sovrano et al., 1999

(Continued)

TABLE 1 | Continued

Types of behavioral lateralization	Species	Occurrence of behavioral lateralization	References
	Blue gourami (*Trichogaster trichopterus*)	Yes	Sovrano et al., 1999
	Sarasins minnow (*Xenopoecilus sarasinorum*)	Yes	Sovrano et al., 2001; Sovrano, 2004
	Elephantnose fish (*Gnathonemus petersii*)	Yes	Sovrano et al., 2001
	Soldierfish *(Myripristis pralinia)*	Yes	Roux et al., 2016
Mating behavior	Mosquitofish *(Gambusia hoolbrooki)*	Yes	Bisazza et al., 1998
	Goldbelly topminnow *(Girardinus falcatus)*	Yes	Bisazza et al., 1998
	Guppy *(Poecilia reticulata)*	Yes	Kaarthigeyan and Dharmaretnam, 2005
Agonistic behavior	Siamese fighting fish (*Betta splendens*)	Yes	Cantalupo et al., 1996; Bisazza and De Santi, 2003; Clotfelter and Kuperberg, 2007; Takeuchi et al., 2010; Forsatkar et al., 2015; HedayatiRad et al., 2017
	Mosquitofish *(Gambusia hoolbrooki)*	Yes	Bisazza and De Santi, 2003
	Redtail splitfin *(Xenotoca eiseni)*	Yes	Bisazza and De Santi, 2003

asymmetries rather than behavioral lateralization induced by eye-use preference (Stennett and Strauss, 2010).

Rotational bias represents another example of motor asymmetry. When a fish is inserted in a circular environment it usually swims along its wall in either a clockwise or a counterclockwise direction even in the absence of any visual cue. Rotational biases have been found both in teleost species (Bisazza and Vallortigara, 1996; Izvekov and Nepomnyashchikh, 2010) and in chondrostean fish (Izvekov et al., 2014). Although the preferential direction of turning may be due to a specific eye preference to monitor the inner space (visual bias), this bias was also observed in the roach under infrared light (Izvekov et al., 2012) thus excluding visual lateralization as possible explanation of the asymmetrical activity. Despite studies on the Class Agnata (jawless fish) are very limited, motor lateralization has been described in the north eastern Pacific hagfish (*Eptatretus stoutii*). These eel-like, boneless, jawless, and sightless fish regularly rest in a tightly coiled posture but the clockwise or the counterclockwise coiling occurs equally often in the population (Miyashita and Palmer, 2014). The discovery of this behavioral bias in these fishes that are believed to be the most ancient group of living vertebrates (Ströckens et al., 2013) suggests that motor biases may represent the first evolutionary step for lateralization in vertebrates. Note, however, that studies on the lancelet, *Branchiostoma* (also known as amphioxus) provide key evidence for early asymmetry in chordate evolution. The mouth is on the left side of the body in larvae, but not in adults, meaning that the neural circuitry necessary to detect the prey are likely located on the left side of the brain. Despite the mouth is innervated by a nerve plexus that is on the left side of the larval brain, this connection is maintained also in the adults even if the mouth becomes frontal and symmetrical (Jeffries and Lewis, 1978). These data may explain the specialization of the left hemisphere to control feeding responses in vertebrates.

As mentioned, asymmetric behavioral responses can be attributed to lateralized processing of perceptual information (e.g., specific eye preferences to observe different classes of stimuli). For what concerns fish, research on brain lateralization has mainly focused on visual laterality rather than other sensory modalities (but see for an exception on fin use Bisazza et al., 2001a).

Behavioral preferences to attack a particular side of a prey and biases in foraging responses have been widely described in a variety of species. In the last decade, researchers showed an increased interest in studying the lateralization of foraging behavior from a behavioral, anatomical and genetic standpoint. For instance, zebrafish preferentially use the right eye when approach a target to bite (Miklosi and Andrew, 1999) and the Australian lungfish, which is considered to be the closest extant ancestor of tetrapods (Schultze, 1986), has been found to exhibit a rightwards bending of the body while feeding (Lippolis et al., 2009), in line with previous studies showing a left hemisphere dominance in controlling feeding behavior in several vertebrate species (see Andrew, 2002b for a review).

Among fish, scale-eating cichlids of genus *Perissodus* have become an attractive and useful model to study lateralization as they represent a striking example of interaction between morphological and behavioral asymmetries. These fishes exhibit jaw asymmetries that are dimorphic: individuals that open their mouth rightward preferentially attack the left side of their prey to tear off scales whereas fish that open the mouth leftward attack the right side (Hori, 1993; Takeuchi et al., 2012). This mouth-opening asymmetry has been described in other species (zebrafish, Hata and Hori, 2011; the cichlid *Neolamprologus moori,* Hori et al., 2007; the freshwater goby *Rhinogobius flumineus*, Seki et al., 2000; the Japanese medaka *Oryzias latipes* and the Tanganyikan cichlid *Julidochromis ornatus*, Hata et al., 2012; and a scale-eating characiform, *Exodon paradoxus*, Hata et al., 2011) and it has been advanced to be genetically determined by a one-locus two-allele Mendelian system, with the lefty dominant over the righty suggesting a common genetic basis in this morphological asymmetry among these species

(Hori, 1993; Hori et al., 2007; Stewart and Albertson, 2010). However, a recent study on the scale-eating cichlid fish *Perissodus microlepis* has shown a strong behavioral bias even in laboratory-reared juveniles with relatively symmetrical mouth raising the hypothesis that mouth asymmetry is not a prerequisite for lateralized behavior but rather the preference to attack one side or the other may be expressed at an early age and may facilitate the development of the morphological asymmetry (Van Dooren et al., 2010; Lee et al., 2012). Future investigations are now required to better understand the relation between asymmetries in morphology and behavior, the mechanisms underlying the development of left-right axis and whether phenotypic plasticity contributes to shape the morphology in other species.

There is a large number of studies suggesting a right hemisphere dominance associated with social behavior in bird, mammals and amphibians. In fact, chicks show a left eye advantage in discriminating a familiar from an unfamiliar conspecific (Vallortigara and Andrew, 1991, 1994; Vallortigara, 1992) face recognition is mainly processed in the right hemisphere in primates (Hamilton and Vermeire, 1988) and sheeps (Kendrick, 2006; Versace et al., 2007) and five species of anuran amphibians preferentially use the left eye when looking at their own mirror image (Bisazza et al., 2002). Mirrors have been used to investigate visual lateralization in fish as well. Bisazza et al. (1999) studied cooperative predator inspection in female mosquitofish by inserting a mirror parallel to the tank at the end of which a predator was visible. In this way, the fish could see its own reflection when swimming along the mirror thus perceiving the presence of a cooperative partner. Mosquitofish were found to approach the predator more closely when the mirror was placed on the left side rather than on the right one, indicating a preferential use of the left eye when looking a conspecific. Consistent results were obtained using the mirror test, in which the mosquitofish were inserted in a tank with mirror walls, as the fish spent more time shoaling with the virtual companion when it was perceived on the left side (De Santi et al., 2001). The same left-eye preference has been observed in species belonging to different orders (Osteoglossiformes, Cypriniformes, Cyprinodontiformes, Beloniformes; Sovrano et al., 1999, 2001; Sovrano, 2004; Sovrano and Andrew, 2006) and also in females of a non-shoaling fish, the convict cichlid (*Amatitlania nigrofas*), but not in males. The authors suggested that despite the adults of this species do not form shoals, social experience early in development (during parental care) may have had lasting effects on lateralization in response to social stimuli (Moscicki et al., 2011). Interestingly, Reddon and Balshine (2010) described an opposite pattern in a highly social, cooperatively breeding cichlid fish (*Neolamprologus pulcher*) as males exhibited a right population preference to view their mirror image while females showed no significant population preference. It has been argued that the sex difference in eye use can be accounted to differences in social and sex motivation when viewing conspecifics. For instance, female mosquitofish and goldbelly topminnow exhibited a consistent rightward bias to detour a barrier to reach same sex conspecifics, whereas no preference was observed in males (Bisazza et al., 1998). Male mosquitofish did not show any eye preference during mirror-image inspection either (Sovrano et al., 1999). Note that females of both species are more social than males, which do not normally show social behavior and this may explain the absence of a side bias in response to social stimuli (Sovrano et al., 1999). Despite the absence of a behavioral bias in the detour task in presence of social companions (conspecifics of the same sex) in males topminnow and mosquitofish, a significant population bias has been observed when fish were presented with sexual stimuli (conspecific of the opposite sex) (Bisazza et al., 1998) whereas females showed a right-eye population biases for looking at the opposite-sex only when sexually deprived. Similarly, male-deprived female guppies (*Poecilia reticulata*) showed a stronger leftward turning bias in the detour (meaning right eye use) when viewing orange colored males than drab (Kaarthigeyan and Dharmaretnam, 2005).

Furthermore, lateralized perception of conspecifics may change as a function of familiarity. Juvenile soldierfish (*Myripristis pralinia*) with ablation of the left telencephalic hemisphere no longer displayed a preference toward conspecific versus heterospecifics fish but maintained this ability after the ablation in the right side of the brain thus showing that the left hemisphere was responsible for visual recognition of conspecifics (Roux et al., 2016). Right/left asymmetries to distinguish, respectively, familiar and unfamiliar conspecifics have been documented in different species (reviewed in Rosa Salva et al., 2012). Despite the direction of the lateralization in the soldierfish was reversed, the results provide further evidence of differential specialization of the two hemispheres in processing visual stimuli.

There is evidence that aggressive responses are mainly processed by the right hemisphere in many vertebrates (Rogers, 2002). For instance, gelada baboon (Casperd and Dunbar, 1996), chicks (Vallortigara et al., 2001), lizards (Hews and Worthington, 2001), and toads (Vallortigara et al., 1998) are more likely to attack a rival male on their left side than on their right side. In contrast to the previous examples, individual, but not population, lateralization in eye use during aggressive interactions (e.g., body posture) has been observed in male Siamese fighting fish (*Betta splendens*) when looking at their own reflection in a mirror (Cantalupo et al., 1996; Clotfelter and Kuperberg, 2007). Interestingly, the left or right preference was correlated with the morphological asymmetry in head incline; lefties (left-curved body) and righties (right-curved body) showed left- and right-biased eye use during aggressive displays, respectively (Takeuchi et al., 2010). However, Bisazza and De Santi (2003) described right population-level lateralization in mosquitofish, Siamese fighting fish and redtail splitfin (*Xenotoca eiseni*), suggesting that the difference observed with respect to the fighting fish might be due to different experimental conditions. The same right bias has been recently reported by Forsatkar et al. (2015) in nest-holding males fighting fish although the stages of reproduction and the paternal care affected the eye-preference with a shift from the right-eye to the left-eye after spawning. Similarly, exposure to an antidepressant drug (fluoxetine) reduced aggressive behavior and caused a change from a right to a left-eye use in this species even if the underlying mechanisms are still unknown (HedayatiRad et al., 2017).

All these data taken together indicate that eye preference when viewing conspecifics may stem from the natural history of the species but may also vary depending on the motivational state of the individuals that affects how certain information can be processed based on the context. It is clear that lateralization can be a highly flexible and complex phenomenon among species, within species and within individuals.

Factors Involved in the Development of Brain Lateralization

It is widely acknowledged that genetic factors are involved in the establishment of lateralization. However, it is also clear that other environmental and physiological factors may play a crucial role in the development of brain asymmetry (reviewed in Deng and Rogers, 2002; Rogers, 2010, 2014). A well-known example is handedness in humans: although a genetic component has been reported for hand preference (Paracchini and Scerri, 2017), this behavioral bias can be modified as observed in different cultures where left-handers were pushed to "conform to normality," that is right-handedness. This suggests that lateralization is a trait that results from the interplay between genes and environment (Cowell and Denenberg, 2002). There is compelling evidence in animals, that several environmental factors other than genetic mechanisms, modulate lateralization, such as light, hormones, rearing environment, pollution and stress (**Table 2**).

Genetic Mechanisms

Clear evidence of heritability of lateralization was provided by Bisazza et al. (2000b) using artificial selection experiments in goldbelly topminnows. Males and females were initially tested on a detour task for their eye-preference to inspect a predator. Only males and females with similar high laterality scores were mated together and, then, their progeny was tested in the same test. Offspring exhibited the same behavioral biases observed in their parents (e.g., the progeny of the right-eye fish, used the right eye to monitor the predator) showing that lateralization was inherited both in strength (i.e., an individual can be more or less lateralized) and direction (left or fight). Furthermore, subsequent studies demonstrated that belonging to these lines selected on the basis of their eye preference to monitor a threating stimulus was predictive of behavioral lateralization in other tasks (e.g., eye used by males in sexual behavior or agonistic attacks), suggesting that these fish may have a mirror-reversed organization of cerebral specializations (Bisazza et al., 2001b, 2005; Dadda et al., 2007, 2009, 2012). We will then show how sophisticated molecular and genetic techniques have been used in zebrafish to address the role of genes in the establishment of brain asymmetry.

Hormones

Steroid hormones have been suggested to affect brain lateralization in humans and non-human animals (Beking et al., 2017). However, data collected in humans are ambiguous and the effect of hormones on the development of lateralization is still heavily disputed suggesting that animal models could be useful to allow experimental manipulation not feasible in humans. For instance, the injection of testosterone and corticosterone *in ovo* altered the development of the asymmetry of thalamofugal visual pathway in chicks (Schwarz and Rogers, 1992; Rogers and Deng, 2005). Reddon and Hurd (2008) observed that convict cichlids males (*Archocentrus nigrofasciatus*) were more lateralized when looking at an aversive stimulus whereas females where more lateralized when looking at a stimulus associated to a positive reinforcement thus suggesting a potential effect of hormones on visually guided behavioral lateralization. Recently, Schaafsma and Groothuis (2011) directly investigated, in fish, the impact of postnatal testosterone on the eye preference when inspecting a predator. Results showed a right-eye population bias to monitor the predator only in fish treated with testosterone, but not in control fish. Furthermore, males were more responsive to the treatment providing first evidence of an involvement of hormones also in fish lateralization. However, the relation between lateralization and steroid hormones is still unclear and future studies may be of help to better understand the role of hormones in modulating brain and behavioral asymmetries.

Light Stimulation

Exposure to light represents one of the best described examples of environmental factors affecting brain lateralization. Light stimulation plays a crucial role in the asymmetrical development of the visual pathway in the domestic chick. Infact, chick position within the eggs determines which eye receives light stimulation through the shell (Rogers, 1990, 1997). Chicks with either the left or the right eye covered develop a reversed pattern of asymmetry whereas chicks from eggs incubated in the dark do not exhibit any asymmetry (Rogers, 2008). This neuroanatomical asymmetry is reflected on the behavior as chicks hatched from eggs with their left eye occluded used the right eye (left hemisphere) to distinguish food from pebbles and the left eye (right hemisphere) to monitor a predator; the behavioral asymmetry is reversed in chick hatched from eggs with the right eye occluded (Rogers, 2008, 2014; Chiandetti and Vallortigara, 2009, 2019; Vallortigara et al., 2011; Chiandetti et al., 2013, 2017). The amount of environmental light received during the development influences lateralization in fish too. Pre-natal effect of light exposure has been observed in live-bearing fish by exposing females goldbelly topminnow to either high or low intensity of light during pregnancy. Only progeny from the light group developed behavioral asymmetries in a visual and motor task (Dadda and Bisazza, 2012). Budaev and Andrew (2009) found that light vs. dark incubation of zebrafish embryos determined eye preference for avoiding a predator. Embryos exposed to light kept at greater distance when a potential predator was seen with the left rather than right eye whereas this behavioral asymmetry was reduced in dark-incubated zebrafish. However, light exposure had a differential effect during the first few days of development as absence of light on day 1 reversed eye-preference but the shift was reduced in absence of light on day 2 or 3 indicating a sensitive period for the effect of light. Although the authors suggested that early light stimulation may affect expression of genes involved in the asymmetric development of the habenulae, subsequent studies showed that darkness delays neurogenesis in the habenular nuclei but does not eliminate asymmetric gene expression (De Borsetti et al., 2011). Recently, Sovrano et al. (2016) showed that only zebrafish exposed to

TABLE 2 | Environmental factors that influence the development of lateralization.

Environmental factor	Species	Impact on lateralization	References
Light stimulation	Goldbelly topminnow (*Girardinus falcatus*)	Yes	Dadda and Bisazza, 2012
	Zebrafish (*Danio rerio)*	Yes	Budaev and Andrew, 2009; Sovrano et al., 2016
Pollution Elevated-CO2	Yellowtail demoiselle, (*Neopomacentrus azysron*)	Yes	Domenici et al., 2012; Nilsson et al., 2012
	Clownfish *(Amphiprion percula)*	Yes	Nilsson et al., 2012
	Spiny damselfish (*Acanthochromis polyacanthus*)	Yes	Jarrold and Munday, 2018
	Three-spined stickleback (*Gasterosteus aculeatus*)	Yes	Jutfelt et al., 2013
	Sand smelt (*Atherina presbyter*)	Yes	Lopes et al., 2016
	Zebrafish *(Danio rerio)*	Yes	Vossen et al., 2016
	Two-spotted gobies (*Gobiusculus flavescens*)	Yes	Sundin and Jutfelt, 2018
	Copper rockfish (*Sebastes caurinus*)	Yes	Hamilton et al., 2017
	Goldsinny wrasse (*Ctenolabrus rupestris*)	No	Sundin and Jutfelt, 2015
	Atlantic cod (*Gadus morhua*)	No	Jutfelt and Hedgärde, 2015
	Blue rockfish (*Sebastes mystinus*)	No	Hamilton et al., 2017
	Damselfish, (*Pomacentrus wardi)*	Yes	Domenici et al., 2014
Warming	Damselfish, (*Pomacentrus wardi)*	Yes	Domenici et al., 2014
Anthropogenic noise	European eels (*Anguilla anguilla*)	Yes	Simpson et al., 2015
Chemical pollutants	Surgeonfish *(Acanthurus triostegus)*	Yes	Besson et al., 2017
Hypoxia	Staghorn sculpin *(Leptocottus armatus)*	Yes	Lucon-Xiccato et al., 2014
Rearing environment	Rainbowfish *(Melanotaenia duboulayi)*	Yes	Bibost et al., 2013
	Guppy *(Poecilia reticulata)*	Yes	Broder and Angeloni, 2014; Dadda and Bisazza, 2016
	Poeciliid *(Brachraphis episcopi)*	Yes	Brown et al., 2004, 2007
	Whitetail damsels (*Pomacentrus chrysurus*)	Yes	Ferrari et al., 2015
	Yellow-and-blueback fusiliers (*Caesio teres*)	Yes	Chivers et al., 2016
Stressor factors	Yellowtail demoiselle, (*Neopomacentrus azysron*)	Yes	Domenici et al., 2012
	Three-spined stickleback (*Gasterosteus aculeatus*)	Yes	Jutfelt et al., 2013
	Small-spotted catsharks (*Scyliorhinus canicular*)	Yes	Green and Jutfelt, 2014
	Arctic charr (*Salvelinus alpinus*)	Yes	Backstroöm et al., 2015
	Poeciliid *(Brachraphis episcopi)*	Yes	Brown et al., 2007
	Goldbelly topminnow *(Girardinus falcatus)*	Yes	Dadda et al., 2007

natural light/dark (LD) cycle, developed a left-eye preference in the mirror test but not zebrafish exposed to different wavelengths of light suggesting an effect of lighting condition on development of social recognition.

Pollution

In the last decade, a new environmental factor has been added to the list of agents modulating the development and expression of lateralization: pollution.

Ocean acidification is caused by increased concentration of CO_2 dissolved into the water due to the rise in anthropogenic-related atmospheric CO_2. Growing evidence now indicates that elevated-CO_2 concentrations can alter behavior and sensory abilities of larval and juvenile fishes (e.g., fish are attracted by chemical that they usually avoid) and also affect lateralized behavior (Munday et al., 2009; Simpson et al., 2011; Cattano et al., 2018; Esbaugh, 2018). Several studies have shown that exposure to elevated-CO2 causes loss of lateralization both in coral reef fish (yellowtail demoiselle, *Neopomacentrus azysron*; clownfish, *Amphiprion percula*; spiny damselfish, *Acanthochromis polyacanthus*) and in temperate fish (three-spined stickleback, *Gasterosteus aculeatus*; sand smelt, *Atherina presbyter,* zebrafish; two-spotted gobies, *Gobiusculus flavescens*) potentially having negative consequences for survival in natural habitats by increasing vulnerability to predators and affecting social cohesion (Domenici et al., 2012; Nilsson et al., 2012; Jutfelt et al., 2013; Lopes et al., 2016; Vossen et al., 2016; Jarrold and Munday, 2018; Sundin and Jutfelt, 2018). Loss of behavioral lateralization induced by elevated CO2 is restored by treatment with an antagonist of the GABA-A receptor, suggesting that high level of CO2 interferes with neurotransmitter function (Nilsson et al., 2012; Lai et al., 2015; Lopes et al., 2016). Taken together, these studies indicate that ocean acidification could represent a problem that affects fish on a global scale.

However, there are species-specific differences in tolerance to increased level of CO_2. No effect of CO_2 on behavioral lateralization has been reported in goldsinny wrasse (*Ctenolabrus rupestris*) (Sundin and Jutfelt, 2015), juvenile Atlantic cod (*Gadus morhua*) (Jutfelt and Hedgärde, 2015) and in Blue rockfish (*Sebastes mystinus*) although changes in behavioral lateralization have been described in the phylogenetically closely related species the Copper rockfish (*S. caurinus*) (Hamilton et al., 2017). Differences in response to CO_2 may be due to increased

adaptive response in some species compared to others. Future studies are now required to understand whether and to what extent species have the capacity to adapt to elevated CO_2 to make good predictions about the ecological consequences of ocean acidification.

Other stressors that disrupt lateralization are ocean warming (Domenici et al., 2014), anthropogenic noise in aquatic environments (e.g., commercial shipping and recreational boating) (Simpson et al., 2015), chemical pollutants added to water (i.e., pesticide) (Besson et al., 2017) and hypoxia that is exacerbated by human activities (e.g., agriculture and discharge of raw sewage) that increases coastal eutrophication (Lucon-Xiccato et al., 2014). It is clear from these studies that some environmental factors affecting lateralization are due anthropic activities and that management and policy decisions are needed to reduce their negative effects on fish behavior that can, in turn have severe implications for community structure and ecosystem function.

Rearing Environment

Early visual experience has been found to influence behavioral lateralization. Bibost et al. (2013) investigated the role of environmental complexity by rearing rainbowfish (*Melanotaenia duboulayi*) in enriched and impoverished conditions and found that males from impoverished habitats were more lateralized than males from enriched environment in their schooling behavior. Females, instead, showed the opposite pattern. Recently, Dadda and Bisazza (2016) showed that newborn guppies raised in an asymmetric environment exhibited eye preference in the mirror test congruent with the direction of asymmetric stimulation suggesting that early different exposure to left/right visual information affects the development of brain asymmetries. Several studies have documented that predation pressure represents another key factor determining the degree of lateralization. The poeciliid *Brachraphis episcopi* reared in high predation environments showed a different pattern of visual lateralization compared to fish from low predation areas (e.g., right eye use to monitor a predator compared to non-visual lateralization; Brown et al., 2004, 2007) and guppies exposed to olfactory predator cues were more highly lateralized than conspecifics reared in absence of threatening cues (Broder and Angeloni, 2014). In line with previous findings, juvenile whitetail damsels (*Pomacentrus chrysurus*) exposed to alarm cues (i.e., injured conspecific cues that elicited an antipredator response) displayed increased behavioral lateralization compared to low-risk condition fish (Ferrari et al., 2015). Chivers et al. (2016) also found that predation pressure affected the strength of lateralization in juvenile yellow-and-blueback fusiliers (*Caesio teres*) and hypothesized that predation stress induced phenotypic plasticity of lateralization which is likely to be adaptive as higher degree of asymmetries increases fitness and survival in environments with high predation risk.

Despite the field of research on environmental mechanisms affecting lateralization is expanding, only the interaction between genetic and environmental factors may provide a clear picture of the relative contribution of these drivers in determining brain asymmetries.

Stress

Stressor factors could influence brain lateralization in fishes (Brown et al., 2007; Dadda et al., 2007; Jutfelt et al., 2013; Domenici et al., 2014; Green and Jutfelt, 2014; Backstroöm et al., 2015) as in other animal species (Ocklenburg et al., 2016). Data from literature showed that increased levels of carbon dioxide were associated to decreased behavioral lateralization in the yellowtail demoiselle (Domenici et al., 2012) and in the three-spined stickleback (Jutfelt et al., 2013) whereas an opposite effect was reported in small-spotted catsharks (*Scyliorhinus canicular;* Green and Jutfelt, 2014). Among the other stressor factors, social interaction (Arctic charr, *Salvelinus alpinus*: Backstroöm et al., 2015), predation pressure (*Brachraphis episcopi*: Brown et al., 2007) and the introduction in a new environment (goldbelly topminnows: Dadda et al., 2007) could also affect behavioral lateralization in different fish species. Beside individual variations in response to stressful environments, the relationship between stress-reactivity and laterality has also been investigated. For instance, it has been shown that the degree of laterality in Port Jackson sharks (*Heterodontus portusjacksoni*) was correlated with stress and stronger lateralized individuals were more reactive (Byrnes et al., 2016), whereas this correlation has not been observed in zebrafish (Fontana et al., 2019). However, research on the relation between laterality and stress is still in the early stages and further investigation is essential to understand the role of steroid hormones (i.e., glucocorticoids) in modulating functional hemispheric asymmetries.

Pros and Cons of Asymmetric Brains

The ubiquitous nature of lateralization suggests that it confers advantages on the individuals. For instance, the specialization of each hemisphere in controlling different functions is supposed to prevent the simultaneous activation of incompatible responses leading to more efficient information processing and rapid responses (Andrew, 1991; Vallortigara, 2000). Furthermore, it allows sparing neural tissue by avoiding duplication of functions in the two hemispheres and increases neural capacity (Levy, 1977; Vallortigara and Rogers, 2005). Another benefit related to lateralization consists of the capacity to simultaneously process multiple types of stimuli. As a consequence, lateralized individuals may carry out different tasks in parallel thus coping better with situations involving divided attention (Rogers, 2000; Vallortigara and Rogers, 2005). This hypothesis was first tested in chicks (Rogers, 2014) and then has been confirmed in fish by comparing lateralized and non-lateralized topminnows while performing two simultaneous tasks: predator vigilance and prey capture. Lateralized individuals were faster at capturing prey in the presence of a predator as they monitored it with one eye and used the opposite eye for catching prey, whereas non-lateralized fish continuously switched from one eye to the other for both functions (Dadda and Bisazza, 2006a). Similarly, lateralized female topminnows foraged more efficiently than non-lateralized females when they had to attend to a harassing male at the same time (Dadda and Bisazza, 2006b).

Evidence for the hypothesis that lateralization is linked to enhanced performances comes from studies showing higher

cohesion and coordination in schools of lateralized topminnows than in schools of non-lateralized fishes. Furthermore, lateralized individuals were located in the center of the school, a position normally safer and energetically less expensive, whereas non-lateralized fish were at the periphery (Bisazza and Dadda, 2005). Recently, Bibost and Brown (2013) observed in two rainbowfish species (*Melanotaenia duboulayi* and *Melanotaenia nigrans*) that individuals occupied positions in the schools based on the eye they preferentially used to monitor conspecifics: fish with a left-eye bias adopted a position on the right side of the school, whereas fish with a right-eye bias were located on the left side of the school. These data confirm that lateralization influences schooling behavior allowing individuals to process visual information more quickly in the appropriate hemisphere. Furthermore, lateralized topminnows have been found to reorient themselves better than non-lateralized conspecifics using both geometric and non-geometric cues thus showing improved spatial navigation skills (Sovrano et al., 2005).

Cerebral lateralization conveys a selective advantage also by increasing learning abilities. Rainbowfish selected on the basis of the eye used to monitor their mirror image, where trained to associate a red light with a food reward using a classical conditioning paradigm. Despite the authors did not include non-lateralized individuals, they found that left-lateralized fish learned the task faster compared to right-lateralized in line with the idea that cognitive abilities may be influenced by the degree of laterality (Bibost and Brown, 2014). Dadda et al. (2015) found that strongly lateralized guppies selected in the mirror test, out-performed non-lateralized guppies in two numerical tasks: both when they were trained to discriminate between arrays containing a different number of geometric figures and when they were observed in a spontaneous choice task for their preference to join the larger of two shoals. No difference between fish with left or right-eye use was found. Consistent results were obtained in the shoal choice task when guppies where selected for high or low lateralization using the detour test in presence of a predator rather than the mirror test (Gatto et al., 2019). Numerical abilities are linked to brain lateralization also in the threespine stickleback as fish tested in a shoal choice task in monocular condition (i.e., with one eye covered) performed better than fish observed in binocular condition (i.e., no eyes covered) but only when presented with certain numerical contrasts. One possible explanation of the better performance in the monocular fish may be the absence of conflicting responses from the two hemispheres that allowed more effective information processing when making a choice (Mehlis-Rick et al., 2018).

Despite clear advantages, lateralization can also provide costs to the fitness of organisms. In natural environments the position of predators, prey and competitors is unpredictable as they can appear on both sides of an individual. Hence, lateralized organisms can be more vulnerable to attacks or miss feeding opportunity if the stimuli are not perceived in the "preferred" visual hemifield (Rogers et al., 2004). The hypothesis that lateralization can hinder performance has been tested in tasks that requires interhemispheric communication by comparing lateralized and non-lateralized topminnows in two tests: a bisection-like test and a shoal choice test (Dadda et al., 2009). In the former experiment, fish were trained to select the middle door in a row of nine in order to join their social companions. Non-lateralized individuals performed better as they chose more often the central door, whereas lateralized individuals systematically chose the door on the left or right of the correct one. In the second experiment, the fish were presented with two shoals of conspecific differing in quality (number and size of fishes) placed in a way that each of them was visible in a different visual hemifield. Non-lateralized fish chose the high-quality shoal but lateralized fish selected the shoal on the side of the eye dominant for analyzing social stimuli. In both cases, the suboptimal choices observed have been attributed to the lack of integration of information between the hemispheres, as if visual information remained confined to the hemisphere that initially received it.

The advantages provided by lateralization explain why asymmetries can occur at "individual-level" (e.g., each individual exhibits its own directional bias but left and right asymmetries are equally distributed within the population) but not why lateralization often occurs at a population-level (i.e., the majority of individuals within the population exhibits the same directional bias) (Vallortigara, 2006a). A problem arises as lateralization at the population level can make individual behavior more predictable: a predator can exploit the fact that prey show a preferential escape direction and can attack on the other unexpected side to increase its success. Similarly, predators specialized in a lateralized attack might increase their capture success but prey can learn how to avoid them. In this scenario, it would be better to have a 50:50 distribution of right and left lateralized individuals within a population. For instance, group hunting sailfish show individual-level lateralization when attacking shoals of sardines but the collective predictability is minimized by random group assortment and attack alternation so that each individual only sporadically performs multiple attacks (Herbert-Read et al., 2016). It is possible then, that group hunting may represent a condition that favors the evolution of individual-level lateralization (Kurvers et al., 2017). Foraging behavior in scale-eating cichlid fish represents another example in which selective pressures favored equal distribution of behavioral bias as the proportion of left and right lateralized individuals oscillates around a 50:50 ratio (Lee et al., 2012).

Ghirlanda and Vallortigara (2004) developed a theoretical model showing that in the context of pray-predator interactions and competitive-cooperative interactions, population-level lateralization may have arisen as an evolutionary stable strategy (ESS) when it is more advantageous for individually lateralized organisms to align their biases to the direction of other asymmetrical organisms to coordinate their behavior with them (Vallortigara and Rogers, 2005; Ghirlanda et al., 2009). According to this model, the alignment of the direction of lateralized biases in a population may have evolved when the individuals experienced greater benefits when performing the same behavioral tactic (Vallortigara, 2006b). Hence, "social" species would be expected to be lateralized at the population level whereas "solitary" species at the individual level only. Empirical evidence in support of this hypothesis comes from a study by Bisazza et al. (2000a) showing that behavioral biases at

population level were more frequent in social fish species than in solitary ones.

It is clear that escaping in the same direction reduces the chance of each individual within the groups to be caught by the predator because of the "dilution effect" (i.e., it is more difficult for the predator to target a specific individual). However, as mentioned before, predators can learn how to anticipate the behavior of the pray. As a consequence, it would be better for some individuals to escape in the opposite unexpected direction to increase their chance of survival. Therefore, a combination of opposite selective forces (i.e., the need for coordination and the need for unpredictability) seems to play a crucial role in guiding the alignment of the direction of asymmetries. The successful strategy for group-living pray would consist in joining the majority to gain protection with a minority that increases its chance to survive by surprising the predator. But how can we explain the existence of majority and minority biases with respect to laterality? It has been suggested that lateralization at population level may be under effect of frequency-dependent selection, a process in which the advantage of one phenotype (e.g., right biased individuals) depends on its frequency in relation to the other phenotype (e.g., left biased individuals) and the advantage would disappear when the minority increases in number (Connor and Hartl, 2004). Frequency-dependent selection does in fact emerge spontaneously as an ESS in Ghirlanda and Vallortigara (2004)'s model. Loffing (2017) showed that left-handers are more successful in competitive sports that reflect some elements of fighting (e.g., boxing, fencing) and proposed that left-handedness in humans is maintained by frequency-dependent mechanisms. In case of fish, despite two-thirds of mosquitofish preferentially use the right eye to monitor the predator, the remaining third used the left eye (De Santi et al., 2001), confirming advantages associated with the existence of the minority type of lateralization.

In conclusion, it is possible to argue that the advantages associated with lateralization counteract the possible disadvantages and that the trade-off between costs and benefit would account for the presence of a certain proportion of non-lateralized individuals in animal populations and for the maintenance of population-level lateral biases (Bisazza et al., 1997a, 2000a; Güntürkün et al., 2000; Brown et al., 2007; Takeuchi and Hori, 2008; Frasnelli and Vallortigara, 2018; Vallortigara, 2019; Vallortigara and Rogers, 2020).

Brain Asymmetry in Zebrafish: Insight From Habenular Nuclei

In the last 20 years, zebrafish has become an excellent model to study the central nervous system (CNS) lateralization due to many advantages it offers in term of body transparency, small size, rapid embryonic development and genetic manipulation (Kalueff et al., 2014; Stewart et al., 2014). As a consequence, zebrafish represents a powerful tool to look inside the developmental and functional basis of brain asymmetry following a comprehensive bottom-up approach (from genes to behavior) and vice versa (from behavior to genes) (Duboc et al., 2015).

The most pronounced structural asymmetry in zebrafish brain was found in the epithalamus. The epithalamus is the dorsal part of the vertebrate diencephalon and displays a marked structural and functional left-right asymmetry that is conserved in a large number of vertebrates (Concha and Wilson, 2001; Bianco and Wilson, 2009; Aizawa et al., 2011). For example, fish and mammalian habenulae are considered to be homologous structures as they are subdivided into a lateral and a medial domain in both taxa (Amo et al., 2010), but they are anticlockwise rotated by 90° compared to each other (Güntürkün and Ocklenburg, 2017). In detail, zebrafish epithalamus contains an unpaired pineal complex, medially positioned, and two bilateral habenular nuclei (Bianco and Wilson, 2009; Aizawa et al., 2011; Güntürkün and Ocklenburg, 2017). It has been established that there is asymmetric termination of forebrain neurons in the habenulae and that there are left/right asymmetries in the efferent connectivity of the habenular nuclei with the interpeduncular nucleus (IPN) in the midbrain, suggesting a conserved connecting system between forebrain and ventral midbrain across vertebrates (Bianco and Wilson, 2009; Miyasaka et al., 2009; Aizawa et al., 2011; Beretta et al., 2012; Roussigné et al., 2012). Furthermore, connectional asymmetries in zebrafish epithalamus are recognizable at the level of pineal complex. The pineal complex is composed by two main structures: a pineal and a parapineal organ (Concha and Wilson, 2001). The pineal is a photosensitive gland, medially positioned, involved in the release of melatonin and in the circadian clock and it does not generate any symmetric/asymmetric connection with the lateral habenular complex. On the contrary, the parapineal complex is the second example of asymmetry in zebrafish epithalamus since it is located on the left side respect to the pineal gland and projects only to the lateral subnucleus of the left dorsal habenula (Concha et al., 2000; Gamse et al., 2005).

Molecular Mechanisms Regulating the Development of Epithalamic Asymmetry in Zebrafish

The molecular events that regulate the development of asymmetric structures in the dorsal forebrain and, in particular, in the epithalamus of vertebrates are still partially unknown. Data collected in zebrafish showed an involvement of four major pathways in the establishment of epithalamus asymmetry: Nodal, Fibroblast Growth Factors (FGFs), Notch and Wnt/beta catenin (Hüsken and Carl, 2013; **Figure 1**).

During embryonic development of zebrafish, the epithalamus evolves as a bilateral symmetric structure that is subdivided in a dorsal and ventral domain that become asymmetric when the component of Nodal signaling pathway arrived from the dorsal and lateral mesoderm (Concha et al., 2003; **Figure 1B**). Different studies reported that mutant zebrafish lines lacking of notochord express the nodal-related gene *cyclops* (*cyc*, also called *ndr2*) bilaterally in the dorsal diencephalon, suggesting an involvement of the dorsal mesoderm in the development and maintenance of zebrafish epithalamus asymmetry (Rebagliati et al., 1998; Sampath et al., 1998; Bisgrove et al., 2000). The nodal-related genes (*squint* and *cyclops*, in particular) start to

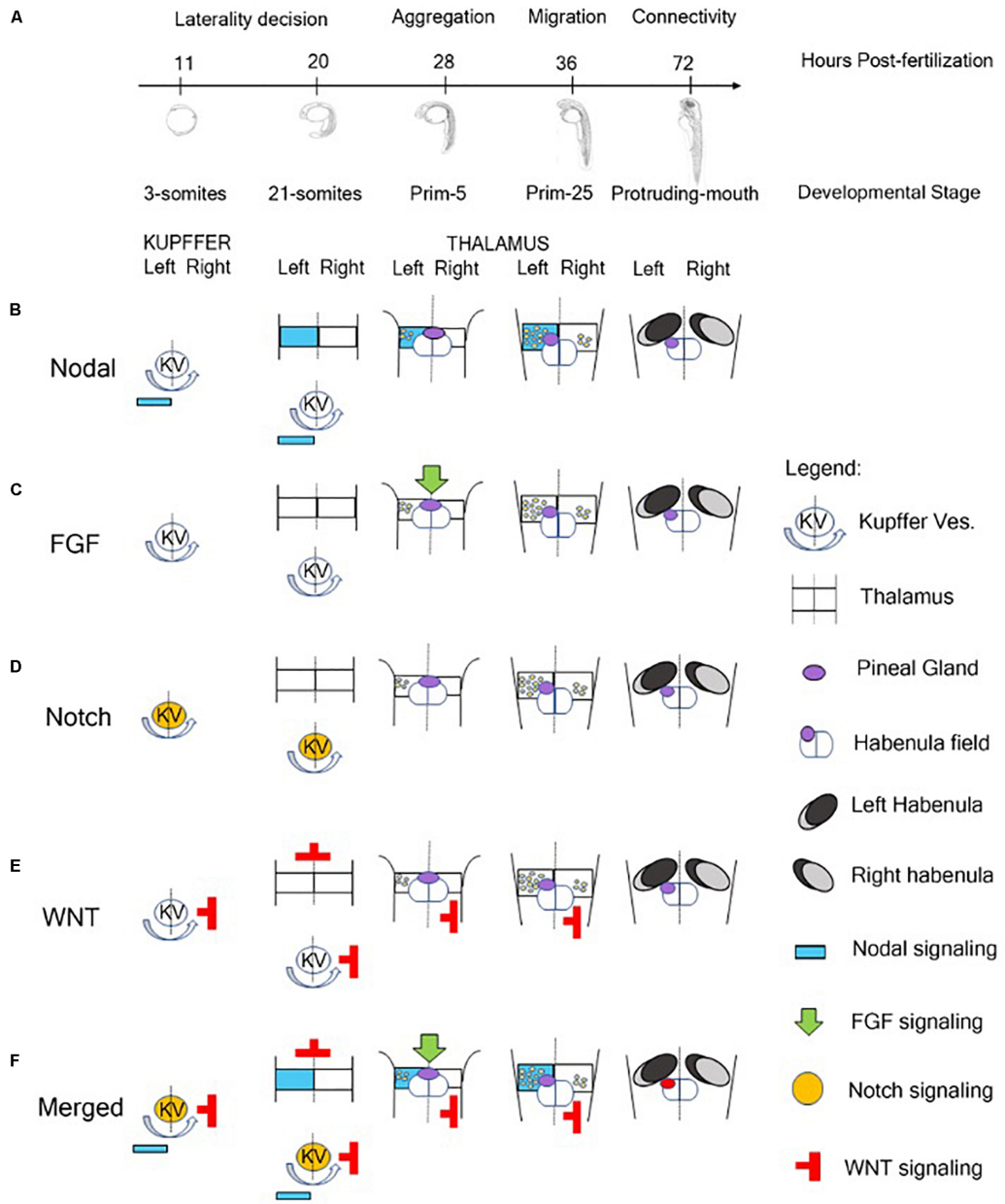


FIGURE 1 | Role of signaling pathway in the Generation of Neuroanatomical Asymmetry in zebrafish. **(A)** Timeline of developmental stages involved in the epithalamic lateralization in zebrafish. **(B)** Nodal signaling influences left-right asymmetry starting from 3-somites stage in which Kupffer's Vesicle contributes to the positioning of Nodal-related genes on the left side of zebrafish embryo (Raya et al., 2003). At 28 hpf, with the aggregation of the symmetric parapineal cells on the midline of epithalamus, the forming pineal complex becomes asymmetric with the migration of parapineal cells in the left side of the brain where Nodal-related genes contribute to the differentiation of left-sided habenular nuclei (Concha et al., 2000; Long et al., 2003; Carl et al., 2007; Inbal et al., 2007; Snelson and Gamse, 2009; Roussigné et al., 2012; Duboc et al., 2015). During later development, Nodal signaling is also involved in the generation of connectivity of epithalamic structures (Hüsken et al., 2014). **(C)** At 28 hpf, FGF signaling plays a role in breaking the symmetry of the brain contributing to the positioning of Nodal-related genes on the left side of the embryo (Neugebauer and Yost, 2014). **(D)** Notch pathway is involved in the control of cilia length of Kupffer's Vesicle responsible for breaking the initial symmetry generating a directional fluid flow from the Kuppfer's Vesicle to the left side of the zebrafish embryo and to positioning Nodal signaling molecules on the left side (Hashimoto et al., 2004; Gourronc et al., 2007; Hojo et al., 2007). **(E)** The Wnt/beta-catenin cascade acts in the lateral mesodermal plate before the induction of Nodal pathway components contributing to the establishment of left-right asymmetry of the brain in three different developmental stages of zebrafish: late gastrulation, somitogenesis and during epithalamic development (Carl et al., 2007; Hüsken and Carl, 2013). **(F)** In brief, Notch signaling influences the direction of fluid flow originated by ciliated cells of Kupffer's vesicle and contributes to the positioning of Nodal-related genes on the left side of zebrafish embryo and of Nodal inhibitors and WNT signaling molecules on the right. At later stage, FGF signaling breaks the symmetry of the epithalamic structures and, in synergy with Nodal pathway, plays a role in the establishment of brain asymmetry in zebrafish embryo contributing to the migration of parapineal cells on the left side and to the generation of asymmetric habenular nuclei.

be expressed during zebrafish gastrulation in the dorsal and lateral mesoderm driving the ventral neuroectoderm to acquire floorplate identity (Erter et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). Furthermore, Liang et al. (2000) established that these mesodermal signals could be required to position and preserve the left-sided gene expression in forebrain and, in particular, in the diencephalon. These authors localized the co-expression of *cyclops*, *antividin* (*atv*, a *lefty1*-related gene) and *pitx2* (a nodal-related transcription factor) in the left dorsal side of diencephalon in the region in which the medial invagination, forming the pineal complex, originates. Using an RNA-mediated rescue approach, they were also able to recover pineal complex structures in adult fish generated starting from mutant embryos lacking the left-sided expression of *cyc*, *atv,* or *pitx2*. Moreover, they reported that the pineal complex of these fishes was frequently displaced on the right of the epithalamus midline, proposing that the Nodal pathway was essential during zebrafish early embryogenesis to position the parapineal domain and resulting organs on the left side of zebrafish brain midline. These data suggested that signaling pathways regulating visceral laterality were also able to produce anatomical asymmetry of zebrafish forebrain (Liang et al., 2000).

Other important evidences of an involvement of Nodal signaling in the generation of asymmetric epithalamic structures derived from the earliest stages of habenular development in zebrafish. Roussigné et al. (2009) focused their attention on a habenular progenitor marker, named *cxcr4b* (*C-X-C chemokine receptors 4b*), which is expressed in the habenular progenitors prior to the leftward migration of parapineal cells. The removal of left/right bias, induced by Nodal signaling, was able to generate symmetric habenular nuclei promoting the idea of a role of this pathway as a guide for the development of brain asymmetry, rather than only for directing laterality. These data were also supported by evidence that SB431542, a chemical inhibitor of Nodal pathway, was able to alter epithalamus asymmetry in favor of the generation of symmetric or mild asymmetric structures compared to untreated controls. These results confirmed previous studies showing that the knock-down of Southpaw (another early mesodermal nodal-related gene) in zebrafish embryos resulted in a severe downregulation of left-sided expression of *cyclops*, *pitx2*, *lefty1*, and *lefty2* in the dorsal epithalamus (Long et al., 2003; Barth et al., 2005; Roussigné et al., 2009). Taken together, these data suggest that, in zebrafish, Nodal signaling derived from the dorsal and lateral mesoderm is responsible for the expression of nodal-related genes (*ndr2* or *cyclops*) on the left side of epithalamus orchestrating the leftward migration of parapineal cells and, consequently, the generation of the asymmetric structures in the brain through the transcription of feedback inhibitor *lefty1* and the homeodomain transcription factor *pitx2c* (Concha et al., 2000; Long et al., 2003; Carl et al., 2007; Inbal et al., 2007; Snelson and Gamse, 2009; Roussigné et al., 2012; Duboc et al., 2015).

Although the Nodal pathway plays a pivotal role in the generation of forebrain asymmetry, FGF signaling represents the initial driving force (**Figure 1C**). Regan et al. (2009) showed that the leftward migration of parapineal complex was driven by FGF8. In fact, zebrafish FGF8 mutant embryos, also called *acerebellar*, or FGF8 morphants are not able to develop epithalamic and habenular asymmetry, because parapineal cells fail to migrate resulting in a symmetric structure (Reifers et al., 1998; Draper et al., 2001; Regan et al., 2009). These data were also supported by experiments of chemical inhibition of FGF receptors. In fact, the temporally inhibition of FGF signaling through the drug SU5402, disrupted parapineal migration blocking parapineal cells closure to the midline and the implantation of FGF8-soaked beads rescued the migration defect toward the implantation site (Regan et al., 2009). Neugebauer and Yost (2014) reported that FGF signaling plays a role in breaking the symmetry of the brain controlling the expression of two transcription factors called *six3b* and *six7*. These genes are involved in the transcriptional repression of *lefty1*, one of the nodal left-sided targets. In detail, the knockdown of both *six3b* and *six7* leads to a bilateral expression of *lefty1* in the zebrafish dorsal epithalamus, while the overexpression of these genes represses *lefty1* in both sides of the embryo (Inbal et al., 2007). Other important evidence reported from these authors showed that FGF exerts a role also in the generation of brain asymmetry interacting with Nodal pathways (Neugebauer and Yost, 2014). Finally, the blocking of FGF signaling disrupts midline organization (Neugebauer and Yost, 2014). Overall, these data show a clear contribution of FGF to the establishment of epithalamic asymmetry, but the Nodal pathway and not FGF signaling is essential for the direction of asymmetry (Güntürkün and Ocklenburg, 2017).

Although FGF signaling is responsible to guide Nodal pathway in breaking symmetry of epithalamic structures of zebrafish brain, a critical role of the successful Nodal–mediated left-right asymmetry induction is played by Notch pathway. Notch pathway is involved in the control of cilia length and, in particular, of the cilia of an epitelium that originates from the dorsal forerunner cells at the end of zebrafish gastrulation and organizes a fluid-filled organ, called Kupffer's Vesicle (Melby et al., 1996; Essner et al., 2005; Takeuchi et al., 2007; Lopes et al., 2010; **Figure 1D**). These cilia are responsible for breaking the initial symmetry generating a directional fluid flow from the Kuppfer's Vesicle to the left side of the zebrafish and medaka embryo and to position Nodal signaling molecules on the left side. Furthermore, this flow positions *Charon*, an antagonist of *nodal* belonging to the *cerberus-like* family and under the transcriptional control of Notch signaling, on the right side (Hashimoto et al., 2004; Gourronc et al., 2007; Hojo et al., 2007). These data were also supported by earlier studies conducted by Raya et al. (2003) that showed that the bilateral injection of Notch mRNA caused the bilateral expression of *ndr2* and *pitx2*, normally expressed only on the left side, reporting for the first time a fundamental relation between Notch and Nodal signaling in the generation of asymmetry in zebrafish embryos. The involvement of Kuppfer's Vesicle in the positioning of Nodal-relates leftward markers were also confirmed by experiment with *mother-of-snow-white* (*msw*) fish, a maternal-effect gene that control Kuppfer's Vesicle morphogenesis and that is able to influence brain asymmetry and lateralized behaviors (Domenichini et al., 2011).

The last pathway involved in the establishment of the brain asymmetry in zebrafish is the Wnt pathway. During development, the Wnt/beta-catenin cascade acts in the lateral mesodermal plate before the induction of Nodal pathway components. The major role of this pathway in the establishment of left-right asymmetry of the brain is related to three different developmental stages of zebrafish: late gastrulation, somitogenesis and during epithalamic development (Carl et al., 2007; Hüsken and Carl, 2013; **Figure 1E**). Carl et al. (2007) reported that mutations of *axin/masterblind,* a wnt inhibitor expressed at the end of gastrulation in the forming anterior neural plate, or the transient wnt inhibition with lithium chloride leads to zebrafish embryos that showed a loss of the asymmetrical distribution of Nodal-related genes in the brain, but not in the lateral mesoderm, suggesting a role of Wnt pathway in the establishment of left-right asymmetry in the brain. The mechanism through which Wnt pathway influences Nodal signaling is still partially unclear but the hypothesis converges on *six3* gene that is downstream Wnt signaling at the end of gastrulation in the anterior neural plate and works as repressor of Nodal left-sided target genes in the forming neural tube (Carl et al., 2002; Lagutin et al., 2003; Inbal et al., 2007; Sagasti, 2007; Hüsken and Carl, 2013). During somitogenesis, a second involvement of Wnt signaling contributes to the development of Kupffer's Vesicle mediating the activation of the ciliogenic transcription factor *foxj1a* and contributing to the positioning of Nodal related genes on the left side of the embryos reinforcing the action played by Notch signaling (Caron et al., 2012; Hüsken and Carl, 2013). Finally, during epithalamic and habenular development, Wnt signaling mediates the activation of the transcription factor *tcf7l2* that is expressed in the dorsal habenular nuclei (left and right) and mediates the ability of dorsal habenular neurons to respond appropriately to signals deriving from the environment into they are born in a left-right manner (Hüsken et al., 2014).

Zebrafish as Tool to Study Brain Asymmetry

Although zebrafish has contributed to establish and clarify several developmental processes that generate asymmetric structures in vertebrate brain, this species has also made it possible to adopt different strategies to study and control the generation of such asymmetries. Over the years, in fact, researchers have developed different experimental protocols in order to establish brain asymmetry exploiting chemical, environmental (non-genetic), surgical and genetic factors.

We have already previously mentioned chemical compounds able to influence the generation of asymmetrical brain structures in fish, some of which are antagonist of the major molecular pathway that contribute to the embryonic development of vertebrates. SB431542, a specific inhibitor of TGF-beta type I receptors, is able to downregulate the expression of left-sided Nodal-related factors (*pitx2* and *lefty1)* generating in the dorsal epithalamus symmetric habenular nuclei instead of asymmetric structures (Roussigné et al., 2009). SU5402, a drug that inhibits FGF receptors, acts blocking the migration of parapineal cells from the midline to the left side of the epithalamus generating a symmetric distribution of *lefty1* and symmetric habenulae (Regan et al., 2009). IWR-1 is a stabilizer of *axin* that mediates the degradation of beta-catenin (*wnt* effector) generating a double-left habenular phenotype in the zebrafish larvae (Dreosti et al., 2014).

Modulation of brain and behavioral asymmetry in zebrafish embryos can be also induced by change in the environment: light stimulation and temperature. As described for other vertebrates, also in zebrafish asymmetry is modulated by light (Andrew et al., 2009b; Budaev and Andrew, 2009). Zebrafish embryos grown in the dark during the first day of development showed a reversed lateralized behavioral pattern, suggesting a contribution of the light in the development of brain asymmetry with, possibly, an involvement of habenular asymmetry in this process (Andrew et al., 2009b; Budaev and Andrew, 2009). Another environmental factor that can influence zebrafish brain laterality is temperature. Data reported a randomization of habenular nuclei direction followed by a loss of lateralization in the ability to respond to sensory stimuli (visual and olfactory) in zebrafish embryos that were grown at 22°C for 3–4 h at the tailbud stage instead of 28°C (Roussigné et al., 2009; Dreosti et al., 2014).

Experiments of surgical ablation of parapineal using two-photon laser microscopy in reporter zebrafish lines (e.g., FoxD3:GFP lines) were optimized to study the involvement of parapineal cells in the establishment of left-right asymmetry of the zebrafish brain. This surgical procedure gives rise to zebrafish embryos with a symmetric double-right phenotype that contributes to establish the influence of epithalamic cells in the asymmetric distribution of Nodal-related left-sided genes and the generation of asymmetrical habenular nuclei (Concha et al., 2003; Aizawa et al., 2005; Gamse et al., 2005; Bianco et al., 2008) and to clarify the involvement of habenular nuclei in response to visual (left) or olfactory (right) stimuli (Dreosti et al., 2014).

The transparency of embryos and larvae, and the possibility of an easy manipulation and accessibility to transgenesis of the embryos represent the most important advantages of using zebrafish in biomedical research and neuroscience. In the last decade, new genetically encoded optical tools and fluorescent sensors have been generated to monitor neural development and neuron activity with a very high space-time resolution (Keller and Ahrens, 2015). In fact, zebrafish represents a good compromise between system complexity and practical simplicity. In order to study the development and function of left-right asymmetry in the brain, genes expressed in the subnuclei of different regions of the zebrafish brain were identified and used as markers and transgenes. For example, Gamse et al. (2003, 2005) defined six molecular distinct domains in the zebrafish larval habenula using a combinatorial expression of *potassium channel tetramerization domain containing genes* (*kctd12.1*, *kctd12.2,* and *kctd8*). A combined approach that implies the use of a transgenic line [Tg(*brn3a–hsp70:GFP*)] and an expression marker (*kctd12.1*) helped to clarify the boundaries between the medial (*brn3a*) and lateral (*kctd12.1*) habenula (Aizawa et al., 2005), the neurotransmitters map of the asymmetric dorsal habenular nuclei (deCarvalho et al., 2014),

and the extension of axons and asymmetric connections of the habenular compartments toward zebrafish telencephalic hemispheres and ventral midbrain (Beretta et al., 2017). Another important tool to study brain asymmetry and laterality was represented by Tg(foxD3:GFP) fishes that express the *green fluorescent protein (GFP)* under the control of the *foxd3* promoter, a marker of pineal and parapineal precursors and neurons. This transgenic line has been widely used to study epithalamic asymmetry (Bianco et al., 2008; Garric et al., 2014; Hüsken et al., 2014; Khuansuwan et al., 2016), involved signaling pathways (Concha et al., 2000, 2003; Gamse et al., 2003; Carl et al., 2007; Roussigné et al., 2009; Regan et al., 2009; Clanton et al., 2013), epithalamic asymmetric connections (Aizawa et al., 2005; Gamse et al., 2005; Bianco et al., 2008; Miyasaka et al., 2009; Krishnan et al., 2014; Turner et al., 2016), and the relationships between brain and behavioral asymmetries (Agetsuma et al., 2010; Dreosti et al., 2014; Krishnan et al., 2014; Facchin et al., 2015). Finally, Lekk et al. (2019) has recently generated a CRISPR/Cas9 transgenic line in which the knock-out of *sox1a* give rise to the first genetic right isomerism of the habenula (Lekk et al., 2019).

Lesson From Other Fish and Evidence of Telencephalic Lateralization (Large Scale Fish)

Brain asymmetry has also been reported in different species of fishes. In 2009, Reddon and colleagues showed continuous variation of habenular asymmetry that correlated with growth rate in the cichlid fish *Geophagus brasiliensis,* with leftward bias in low growing fishes and larger right habenula in the faster growing individuals, also finding a positive correlation between the habenular structures and behavioral asymmetries. Similar results were obtained in another cichlid fish, *Amatitlania nigrofasciata* (Gutiérrez-Ibáñez et al., 2011). Moreover, genetic variations affecting brain asymmetry were also reported in the adult Chinook salmon (*Onchorhynchus tshawytscha*) (Wiper et al., 2017). But the most well-documented case of a relationship between behavioral lateralization and morphological asymmetry in fish is represented by *Peridossus microlepis,* a cichlid fish that is endemic of Lake Tanganyika in Africa (Hori, 1993). This species is characterized by individuals that attack their prey on the flank with a side preference associated with a morphological asymmetry of the mouth that seems to be genetically encoded (Lee et al., 2015; Raffini et al., 2017). Lee et al. (2015) used a genome-wide RNA sequencing approach and showed that different regions of the brain (such as optic tectum, telencephalon, hypothalamus, and cerebellum) displayed a different molecular signature and that some of the genes expressed in the paired brain regions (e.g., telencephalon and optic tectum) were differentially expressed between the two hemispheres suggesting that specific asymmetries in genes expression could be associated with asymmetric behavior.

Taken together, these data open the possibility to include innovative powerful tools, such as genome-wide RNA sequencing approaches, to further investigate the correlation between brain and behavioral asymmetries in fish to in order to link ecological traits to genetics and extend the results to other vertebrates.

The Zebrafish as a Model to Investigate the Relationship Between Structural and Functional Brain Asymmetries

There is considerable evidence that zebrafish exhibit several lateralized behaviors. Adults observed in a detour task showed a left-eye bias to view an empty space or familiar environment, but they used the right eye to view a novel complex environment. Similarly, zebrafish were found to prefer to use the left eye to view a familiar social species and the right eye to view a not familiar and potentially competitive species such as the fighting fish (Miklosi et al., 1997), suggesting that the right eye is preferentially used to look at stimuli that elicit strong reactions. Adults also exhibited a preference for using the right eye when they had to approach a target to bite (Miklosi and Andrew, 1999). When larval zebrafish entered a novel lit environment after gradually dimming the light in their own compartment, they showed a strong tendency to turn to the left (Watkins et al., 2004). However, when the light was rapidly turned off, they preferentially turned to the right, showing a locomotor behavior similar to a startle-response (Burgess and Granato, 2007). Zebrafish larvae also favored the left eye for viewing their own reflection although differences in behavior have been observed in different strains (Sovrano and Andrew, 2006). Moreover, larvae had an initial preference to use the left eye to look at a novel object and then they shifted to the right eye, presumably when the object became familiar. The right-eye bias was maintained even when the fish were presented with the same object after 2 h, thus providing evidence of long-term memory (Andersson et al., 2015). Interestingly, in larval zebrafish, the general preference for the use of the left eye during inspection of its own mirror image is punctuated by a series of very short duration events and with precise cyclicality (about 160 s), during which the right eye (left hemisphere) is used instead (Andrew et al., 2009a). Similar phenomena have been observed in the processes of consolidation in memory in higher vertebrates, which are hypothesized to be related to processes that take place in the nervous system of "recording" of memory traces located in the right and left hemispheres (Andrew, 2002a). Andrew et al. (2009a) has also documented the existence of anomalies in the duration and periodicity of the events of use of the right eye also in mutant zebrafish strains characterized by inversion of parapineal asymmetries.

In the last two decades much effort has been devoted to investigate the relationship between brain and behavioral asymmetries. One advantage of using zebrafish in this research field is that it offers experimental manipulations that cannot be used in humans for ethical and practical reasons. Different strategies have been adopted to modify the L-R epithalamic asymmetry. For instance, Barth et al. (2005) used larvae from a genetic line known as *frequent-situs-inversus* (*fsi*) in which the parapineal was located to the right side of the pineal organ in about 25% of individuals (rather than 3% as reported in wild-type) and this neuroanatomical symmetry was concordant with

visceral reversal of gut and heart. Fish with left (Lpp) and right parapineal (Rpp) observed in different assays showed reversed behavioral asymmetries in the mirror test and in the biting test. In the first test, Lpp larvae used the left eye to view their mirror image, whereas Rpp larvae used the right eye. In the biting test, Lpp adults looked at the stimulus to bite with the right eye and the Rpp used more often the left eye. Despite, there was no difference in turning behavior when larvae entered a novel environment between the two groups, their latency to emerge differed and it was higher in Lpp than in Rpp. Taken together, these results suggest that there might be a causal relationship between epithalamic asymmetries, some lateralized behaviors and behaviors related to fear/anxiety. Change in the frequency of reversed asymmetry in the epithalamus can also been obtained as a result of artificial selection for the eye used in the mirror test. Zebrafish selected for five generations for right-eye use showed a significant increase of reversed asymmetry whereas selection for left-eye led to a decrease of asymmetry (Facchin et al., 2009a). In a subsequent study, larvae from the line selected for the right-eye use were sorted for the left or right position of the parapineal using the foxD3:GFP marker and were then observed when adults in a series of laterality tests (i.e., eye used in predator inspection, rotational preference, and turning direction in the dark) (Dadda et al., 2010a). Opposite lateralized behaviors were observed between the Lpp and Rpp. Furthermore, differences in some personality traits were found as fish with Rpp were bolder in certain contexts, as reported by Barth et al. (2005). Along similar lines, Domenichini et al. (2011) found that Lpp and Rpp adults showed a reversed pattern for the eye used in the detour task to scrutinize a predator and their own mirror image but no difference was observed when they were presented with a neutral stimulus (i.e., a plant).

However, Facchin et al. (2009b) provided contrasting results when compared larvae with reversal of epithalamic asymmetry, induced by injection of *southpaw* antisense morpholino, with control larvae with typical L-R pattern. No difference was found in the mirror test and in the C-start response following an acoustic/vibrational stimulus or after presentation of a lateralized stimulus. Despite behavioral responses were similar, larvae with right parapineal showed a significant delay in the onset of navigation and reduced swimming speed. Consistent with these findings in larvae, adults with reversed L-R brain asymmetry were discovered to manifest different behaviors indicative of anxiety: Rpp spent more time in the bottom section of a novel tank, showed reduced explorative behavior in the mirror test, increased latency in exiting from a confined box and higher cortisol levels compared to Lpp (Facchin et al., 2015). The scenario that emerges from these studies is far from simple as the disruption of directionality in the zebrafish epithalamus clearly seems to affect some, but not all lateralized behaviors and also plays a role in regulating stress response. It is worth noting that discrepancies among studies may be ascribed to different strains used, different methods adopted and different ways of analyzing data making it clear the need of standardized protocols to enhance reproducibility.

Finally, manipulations of brain asymmetry also affect sensory responses to light and odor. Imaging of the neural activity of dorsal habenula neurons (dHb) showed that light mainly activated the neurons in the left dHb, whereas odor mainly activated the neurons in the right dHb. This pattern of sensory processing was found to be opposite in fish with reversed L-R asymmetry. Furthermore, loss of asymmetry in fish with double -left- or double-right-sided brains caused loss of response to both stimuli suggesting that the alteration of brain lateralization could be causative of cognitive disfunctions rather than their consequences (Dreosti et al., 2014).

CONCLUSION

It is clear that brain lateralization is a widespread and well-conserved phenomenon in vertebrates (see Vallortigara and Rogers, 2020). Research on fish has proved to be valuable in understanding its biological function and the evolutionary significance. Whether brain lateralization is a homologous trait inherited by a common ancestor in vertebrates or if it has emerged more than once as result of convergent evolution has not yet been determined.

Boorman and Shimeld (2002) suggested that structural asymmetry has probably evolved numerous times in animals, but its frequent occurrence may reflect conserved molecular mechanisms. Since all members of Bilateria (i.e., animals with bilateral symmetry) share directional asymmetries, it is plausible to hypothesize, by parsimony, that these traits are homologous. If we focus our attention on the vertebrates, research on fish can help to answer this question. Fish are the most ancient vertebrates (first fossils date back to ∼500 million years ago, Shu et al., 1999), represent half of the vertebrate species on the planet (Diana, 2003) and have adapted to live in almost every aquatic niche. Consequently, they represent a useful tool to investigate the role of phylogeny and ecology in the development of brain lateralization given the complexity of their social and physical environment and the diversity of the existing species. The ubiquity of morphological asymmetry associated to functional asymmetry in fishes may indicate a monophyletic origin and may have been present in the ancestors of vertebrates. Furthermore, evidence of asymmetry in coelacanths and lungfish which share a common ancestor with terrestrial tetrapods (i.e., amphibians, reptiles, birds, and mammals), support the idea that they inherited this trait from fish (Hori et al., 2017).

In humans, an increasing number of studies has noticed an association between atypical pattern of cerebral asymmetry and cancer (Sandson et al., 1992; Klar, 2011), immune reactivity (Neveu, 2002), autism (Escalante-Mead et al., 2003; Herbert et al., 2005), schizophrenia (Klar, 1999; Ribolsi et al., 2009), and dyslexia (Heim and Keil, 2004; Wijers et al., 2005).

Despite rapid and continuous progress has been made in neuroimaging, neurostimulation and genetic techniques used to investigate lateralization in humans, it remains difficult, for ethical and practical reasons, to assess the role of the environmental stimulation and of the extent of genes contribution to the development of brain asymmetry. Among animal models, the zebrafish has rapidly become a

powerful species to investigate lateralization at different level of complexity, from genes, to structural and functional asymmetry, providing insights into the establishment of brain lateralization and the molecular processes involved. The combination of behavioral analysis, imaging and cutting-edge molecular genetic techniques represents a unique approach to investigate gene-by-environment interaction effects, how genetically encoded asymmetry may chance across the lifespan and how anatomical asymmetries are linked to behavior. Research on fish and, in particular on zebrafish, is of paramount importance to increase our comprehension of the biological relevance of brain lateralization and to understand how defects in brain asymmetry contribute to neurological disorders and pathologies in humans and other animals.

AUTHOR CONTRIBUTIONS

MM, AM, GV, and VS conceived and designed organization of the manuscript. All authors contributed to the manuscript writing.

FUNDING

Our research was financed by the University of Trento, a Human Frontiers Research Grant (HFSP Research Grant RGP0008/2017), the European Research Council Grant (ERC Grant 833504-SPANUMBRA) and the European Union's Horizon 2020 Research and Innovation Programme (Marie Skłodowska-Curie Action 750200).

REFERENCES

Agetsuma, M., Aizawa, H., Aoki, T., Nakayama, R., Takahoko, M., Goto, M., et al. (2010). The habenula is crucial for experience-dependent modification of fear responses in zebrafish. *Nat. Neurosci.* 13, 1354–1356. doi: 10.1038/nn.2654

Aizawa, H., Amo, R., and Okamoto, H. (2011). Phylogeny and ontogeny of the habenular structure. *Front. Neurosci.* 5:138. doi: 10.3389/fnins.2011.00138

Aizawa, H., Bianco, I. H., Hamaoka, T., Miyashita, T., Uemura, O., Concha, M. L., et al. (2005). Laterotopic representation of left-right information onto the dorso-ventral axis of a zebrafish midbrain target nucleus. *Curr. Biol.* 15, 238–243. doi: 10.1016/j.cub.2005.01.014

Amo, R., Aizawa, H., Takahoko, M., Kobayashi, M., Takahashi, R., Aoki, T., et al. (2010). Identification of the zebrafish ventral habenula as a homolog of the mammalian lateral habenula. *J. Neurosci.* 30, 1566–1574. doi: 10.1523/JNEUROSCI.3690-09.2010

Andersson, M. Å., Ek, F., and Olsson, R. (2015). Using visual lateralization to model learning and memory in zebrafish larvae. *Sci. Rep.* 5:8667. doi: 10.1038/srep08667

Andrew, R. J. (1991). *The Use of the Chick as a Model*. Oxford: Oxford University Press.

Andrew, R. J. (2002a). "Memory formation and brain lateralization," in *Comparative Vertebrate Lateralization*, eds L. J. Rogers, and R. J. Andrew, (Cambridge: Cambridge University Press), 582–633.

Andrew, R. J. (2002b). "The earliest origins and subsequent evolution of lateralisation," in *Comparative Brain Lateralisation*, eds L. J. Rogers, and R. J. Andrew, (Cambridge: Cambridge University Press), 70–93.

Andrew, R. J., Dharmaretnam, M., Györi, B., Miklüsi, A., Watkins, J. A. S., and Sovrano, V. A. (2009a). Precise endogenous control of involvement of right and left visual structures in assessment by zebrafish. *Behav. Brain Res.* 196, 99–105. doi: 10.1016/j.bbr.2008.07.034

Andrew, R. J., Osorio, D., and Budaev, S. (2009b). Light during embryonic development modulates patterns of lateralization strongly and similarly in both zebrafish and chick. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364, 983–989. doi: 10.1098/rstb.2008.0241

Austin, N. P., and Rogers, L. R. (2007). Asymmetry of flight and escape turning responses in horses. *Laterality* 12, 464–474. doi: 10.1080/13576500701495307

Backstroöm, T., Heynen, M., Braönnaös, E., Nilsson, J., Winberg, S., and Magnhagen, C. (2015). Social stress effects on pigmentation and monoamines in Arctic charr. *Behav. Brain Res.* 291, 103–107. doi: 10.1016/j.bbr.2015.05.011

Barth, K. A., Miklosi, A., Watkins, J., Bianco, I. H., Wilson, S. W., and Andrew, R. J. (2005). fsi zebrafish show concordant reversal of laterality of viscera, neuroanatomy, and a subset of behavioural responses. *Curr. Biol.* 15, 844–850. doi: 10.1016/j.cub.2005.03.047

Beking, T., Geuze, R. H., and Groothuis, T. G. G. (2017). "Investigating effects of steroid hormones on lateralization of brain and behaviour," in *Lateralized Brain Functions: Methods in Human and Non-Human Species*, eds L. J. Rogers, and G. Vallortigara, (Berlin: Springer), 633–666.

Beretta, C. A., Dross, N., Guglielmi, L., Bankhead, P., Soulika, M., Gutierrez-Triana, J. A., et al. (2017). Early commissural diencephalic neurons control habenular axon extension and targeting. *Curr. Biol.* 27, 270–278. doi: 10.1016/j.cub.2016.11.038

Beretta, C. A., Dross, N., Guiterrez-Triana, J. A., Ryu, S., and Carl, M. (2012). Habenula circuit development: past, present, and future. *Front. Neurosci.* 6:51. doi: 10.3389/fnins.2012.00051

Besson, M., Gache, C., Bertucci, F., Brooker, R. M., Roux, N., Jacob, H., et al. (2017). Exposure to agricultural pesticide impairs visual lateralization in a larval coral reef fish. *Sci. Rep.* 7:9165. doi: 10.1038/s41598-017-09381-0

Bianco, I. H., Carl, M., Russell, C., Clarke, J. D., and Wilson, S. W. (2008). Brain asymmetry is encoded at the level of axon terminal morphology. *Neural Dev.* 3:9. doi: 10.1186/1749-8104-3-9

Bianco, I. H., and Wilson, S. W. (2009). The habenular nuclei: a conserved asymmetric relay station in the vertebrate brain. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364, 1005–1020. doi: 10.1098/rstb.2008.0213

Bibost, A.-L., and Brown, C. (2013). Laterality influences schooling position in rainbowfish, *Melanotaenia* spp. *PLoS One* 8:e80907. doi: 10.1371/journal.pone.0080907

Bibost, A.-L., and Brown, C. (2014). Laterality influences cognitive performance in rainbowfish *Melanotaenia duboulayi*. *Anim. Cogn.* 17, 1045–1051. doi: 10.1007/s10071-014-0734-3

Bibost, A.-L., Kydd, E., and Brown, C. (2013). "The effect of sex and early environment on the lateralization of the rainbowfish *Melanotaenia duboulayi*," in *Lateralization in Vertebrates*, eds D. Csermely, and L. Regolin, (Berlin: Springer-Verlag), 9–24.

Bisazza, A., and Brown, C. (2011). "Lateralization of cognitive functions in fish," in *Cognition and Behavior*, eds C. Brown, K. Laland, and J. Krause, (Oxford: Wiley-Blackwell), 298–324.

Bisazza, A., Cantalupo, C., Capocchiano, M., and Vallortigara, G. (2000a). Population lateralisation and social behaviour: a study with 16 species of fish. *Laterality* 5, 269–284. doi: 10.1080/713754381

Bisazza, A., Cantalupo, C., and Vallortigara, G. (1997a). Lateral asymmetries during escape behaviour in a species of teleost fish (*Jenynsia lineata*). *Physiol. Behav.* 61, 31–35. doi: 10.1016/S0031-9384(96)00308-3

Bisazza, A., and Dadda, M. (2005). Enhanced schooling performance in lateralized fishes. *Proc. Biol. Sci.* 272, 1677–1681. doi: 10.1098/rspb.2005.3145

Bisazza, A., Dadda, M., and Cantalupo, C. (2005). Further evidence for mirror-reversed laterality in lines of fish selected for leftward or rightward turning when facing a predator model. *Behav. Brain Res.* 156, 165–171. doi: 10.1016/j.bbr.2004.05.022

Bisazza, A., and De Santi, A. (2003). Lateralization of aggression in fish. *Behav. Brain Res.* 141, 131–136. doi: 10.1016/S0166-4328(02)00344-3

Bisazza, A., De Santi, A., Bonso, S., and Sovrano, V. A. (2002). Frogs and toads in front of a mirror: lateralisation of response to social stimuli in tadpoles of five anuran species. *Behav. Brain Res.* 134, 417–424. doi: 10.1016/S0166-4328(02)00055-4

Bisazza, A., De Santi, A., and Vallortigara, G. (1999). Laterality and cooperation: mosquitofish move closer to a predator when the companion is on their left side. *Anim. Behav.* 57, 1145–1149. doi: 10.1006/anbe.1998.1075

Bisazza, A., Facchin, L., Pignatti, R., and Vallortigara, G. (1998). Lateralization of detour behaviour in poeciliid fish: the effect of species, gender and sexual motivation. *Behav. Brain Res.* 91, 157–164. doi: 10.1016/S0166-4328(97)00114-9

Bisazza, A., Facchin, L., and Vallortigara, G. (2000b). Heritability of lateralization in fish: concordance of right-left asymmetry between parents and offspring. *Neuropsychologia* 38, 907–912. doi: 10.1016/S0028-3932(00)00018-X

Bisazza, A., Lippolis, G., and Vallortigara, G. (2001a). Lateralization of ventral fins use during object exploration in the blue gourami (*Trichogaster trichopterus*). *Physiol. Behav.* 72, 575–578. doi: 10.1016/S0031-9384(01)00417-6

Bisazza, A., Pignatti, R., and Vallortigara, G. (1997b). Detour tests reveal task- and stimulus-specific neural lateralization in mosquitofish (*Gambusia holbrooki*). *Behav. Brain Res.* 89, 237–242. doi: 10.1016/S0166-4328(97)00061-2

Bisazza, A., Sovrano, V. A., and Vallortigara, G. (2001b). Consistency among different tasks of left-right asymmetries in lines of fish originally selected for opposite direction of lateralization in a detour task. *Neuropsychologia* 39, 1077–1085. doi: 10.1016/S0028-3932(01)00034-3

Bisazza, A., and Vallortigara, G. (1996). Rotational bias in mosquitofish (*Gambusia hoolbrooki*): the role of lateralization and sun-compass navigation. *Laterality* 1, 161–175. doi: 10.1080/713754231

Bisgrove, B. W., Essner, J. J., and Yost, H. J. (2000). Multiple pathways in the midline regulate concordant brain, heart and gut left-right asymmetry. *Development* 127, 3567–3579.

Bonati, B., Csermely, D., López, P., and Martín, J. (2010). Lateralization in the escape behaviour of the common wall lizard (*Podarcis muralis*). *Behav. Brain Res.* 207, 1–6. doi: 10.1016/j.bbr.2009.09.002

Bonati, B., Csermely, D., and Sovrano, V. A. (2013). Looking at a predator with the left or right eye: asymmetry of response in lizards. *Laterality* 17, 1–11. doi: 10.1080/1357650X.2012.673623

Boorman, C. J., and Shimeld, S. M. (2002). The evolution of left-right asymmetry in chordates. *Bioessays* 24, 1004–1011. doi: 10.1002/bies.10171

Broder, E. D., and Angeloni, L. M. (2014). Predator-induced phenotypic plasticity of laterality. *Anim. Behav.* 98, 125–130. doi: 10.1016/j.anbehav.2014.09.033

Brown, C., Gardner, C., and Braithwaite, V. A. (2004). Population variation in lateralised eye use in the poeciliid *Brachyraphis episcopi*. *Proc. Biol. Sci.* 271, S455–S457. doi: 10.1098/rsbl.2004.0222

Brown, C., Western, J., and Braithwaite, V. A. (2007). The influence of early experience on, and inheritance of cerebral lateralization. *Anim. Behav.* 74, 231–238. doi: 10.1016/j.anbehav.2006.08.014

Budaev, S., and Andrew, R. J. (2009). Patterns of early embryonic light exposure determine behavioural asymmetries in zebrafish: a habenular hypothesis. *Behav. Brain Res.* 200, 91–94. doi: 10.1016/j.bbr.2008.12.030

Burgess, H. A., and Granato, M. (2007). Modulation of locomotor activity in larval zebrafish during light adaptation. *J. Exp. Biol.* 210, 2526–2539. doi: 10.1242/jeb.003939

Byrnes, E. E., Vila Pouca, C., and Brown, C. (2016). Laterality strength is linked to stress reactivity in Port Jackson sharks (*Heterodontus portusjacksoni*). *Behav. Brain Res.* 305, 239–246. doi: 10.1016/j.bbr.2016.02.033

Cantalupo, C., Bisazza, A., and Vallortigara, G. (1995). Lateralization of predator-evasion response in a teleost fish (*Girardinus falcatus*). *Neuropsychologia* 33, 1637–1646. doi: 10.1016/0028-3932(95)00043-7

Cantalupo, C., Bisazza, A., and Vallortigara, G. (1996). Lateralization of displays during aggressive and courtship behaviour in the siamese fighting fish (*Betta splendens*). *Physiol. Behav.* 60, 249–252. doi: 10.1016/0031-9384(96)00015-7

Carl, M., Bianco, I. H., Bajoghli, B., Aghaallaei, N., Czerny, T., and Wilson, S. W. (2007). Wnt/Axin1/beta-catenin signaling regulates asymmetric nodal activation, elaboration, and concordance of CNS asymmetries. *Neuron* 55, 393–405. doi: 10.1016/j.neuron.2007.07.007

Carl, M., Loosli, F., and Wittbrodt, J. (2002). Six3 inactivation reveals its essential role for the formation and patterning of the vertebrate eye. *Development* 129, 4057–4063.

Caron, A., Xu, X., and Lin, X. (2012). Wnt/β-catenin signaling directly regulates Foxj1 expression and ciliogenesis in zebrafish Kupffer's vesicle. *Development* 139, 514–524. doi: 10.1242/dev.071746

Casperd, L. M., and Dunbar, R. I. M. (1996). Asymmetries in the visual processing of emotional cues during agonistic interactions by Gelada baboons. *Behav. Proc.* 37, 57–65. doi: 10.1016/0376-6357(95)00075-5

Cattano, C., Claudet, J., Domenici, P., and Milazzo, M. (2018). Living in a high CO2 world: a global meta-analysis shows multiple trait-mediated fish responses to ocean acidification. *Ecol. Monogr.* 88, 320–335. doi: 10.1002/ecm.1297

Chiandetti, C., Galliussi, J., Andrew, R. J., and Vallortigara, G. (2013). Early-light embryonic stimulation suggests a second route, via gene activation, to cerebral lateralization in vertebrates. *Sci. Rep.* 3:2701. doi: 10.1038/srep02701

Chiandetti, C., Lemaire, B. S., Versace, E., and Vallortigara, G. (2017). Early- and late-light embryonic stimulation modulates similarly chicks' ability to filter out distractors. *Symmetry* 9:84. doi: 10.3390/sym9060084

Chiandetti, C., and Vallortigara, G. (2009). Effects of embryonic light stimulation on the ability to discriminate left from right in the domestic chick. *Behav. Brain Res.* 198, 204–246. doi: 10.1016/j.bbr.2008.11.018

Chiandetti, C., and Vallortigara, G. (2019). Distinct effect of early and late embryonic light-stimulation on chicks' lateralization. *Neuroscience* 414, 1–7. doi: 10.1016/j.neuroscience.2019.06.036

Chivers, D. P., McCormick, M. I., Allan, B. J. M., Mitchell, M. D., Gon?alves, E. J., Bryshun, R., et al. (2016). At odds with the group: changes in lateralization and escape performance reveal conformity and conflict in fish schools. *Proc. Biol. Sci.* 283:20161127. doi: 10.1098/rspb.2016.1127

Clanton, J. A., Hope, K. D., and Gamse, J. T. (2013). Fgf signaling governs cell fate in the zebrafish pineal complex. *Development* 140, 323–332. doi: 10.1242/dev.083709

Clotfelter, E. D., and Kuperberg, E. S. (2007). Cerebral lateralization and its relationship to phylogeny and aggression in anabantoid fishes. *Brain Behav. Evol.* 69, 169–175. doi: 10.1159/000096985

Concha, M. L., Burdine, R. D., Russell, C., Schier, A. F., and Wilson, S. W. (2000). A nodal signaling pathway regulates the laterality of neuroanatomical asymmetries in the zebrafish forebrain. *Neuron* 28, 399–409. doi: 10.1016/s0896-6273(00)00120-3

Concha, M. L., Russell, C., Regan, J. C., Tawk, M., Sidi, S., Gilmour, D. T., et al. (2003). Local tissue interactions across the dorsal midline of the forebrain establish CNS laterality. *Neuron* 39, 423–438. doi: 10.1016/s0896-6273(03)00437-9

Concha, M. L., and Wilson, S. W. (2001). Asymmetry in the epithalamus of vertebrates. *J. Anat.* 199, 63–84. doi: 10.1046/j.1469-7580.2001.19910063.x

Connor, J. K., and Hartl, D. H. (2004). *A Primer of Ecological Genetics*. Sunderland, MA: Sinauer Associates.

Cowell, P. E., and Denenberg, V. H. (2002). "Development of laterality and the role of the corpus callosum in rodents and humans," in *Comparative Vertebrate Lateralization*, eds L. J. Rogers, and R. J. Andrew, (New York, NY: Cambridge University Press), 274–305.

Dadda, M., Agrillo, C., Bisazza, A., and Brown, C. (2015). Laterality enhances numerical skills in the guppy, *Poecilia reticulata*. *Front. Behav. Neurosci.* 9:285. doi: 10.3389/fnbeh.2015.00285

Dadda, M., and Bisazza, A. (2006a). Does brain asymmetry allow efficient performance of simultaneous tasks? *Anim. Behav.* 72, 523–529. doi: 10.1016/j.anbehav.2005.10.019

Dadda, M., and Bisazza, A. (2006b). Lateralized female topminnows can forage and attend to a harassing male simultaneously. *Behav. Ecol.* 17, 358–363. doi: 10.1093/beheco/arj040

Dadda, M., and Bisazza, A. (2012). Prenatal light exposure affects development of behavioural lateralization in a livebearing fish. *Behav. Proc.* 91, 115–118. doi: 10.1016/j.beproc.2012.06.008

Dadda, M., and Bisazza, A. (2016). Early visual experience influences behavioral lateralization in the guppy. *Anim. Cogn.* 19, 949–958. doi: 10.1007/s10071-016-0995-0

Dadda, M., Domenichini, A., Piffer, L., Argenton, F., and Bisazza, A. (2010a). Early differences in epithalamic left-right asymmetry influence lateralization and personality of adult zebrafish. *Behav. Brain Res.* 206, 208–215. doi: 10.1016/j.bbr.2009.09.019

Dadda, M., Koolhaas, W. H., and Domenici, P. (2010b). Behavioral asymmetry affects escape performance in a teleost fish. *Biol. Lett.* 6, 414–417. doi: 10.1098/rsbl.2009.0904

Dadda, M., Nepomnyashchikh, V. A., Izvekov, E. I., and Bisazza, A. (2012). Individual-level consistency of different laterality measures in the goldbelly topminnow. *Behav. Neurosci.* 126, 845–849. doi: 10.1037/a0030319

Dadda, M., Zandonà, E., Agrillo, C., and Bisazza, A. (2009). The costs of hemispheric specialization in a fish. *Proc. Biol. Sci.* 276, 4399–4407. doi: 10.1098/rspb.2009.1406

Dadda, M., Zandonà, E., and Bisazza, A. (2007). Emotional responsiveness in fish from lines artificially selected for a high or low degree of laterality. *Physiol. Behav.* 92, 764–772. doi: 10.1016/j.physbeh.2007.06.001

De Borsetti, N. H., Dean, B. J., Bain, E. J., Clanton, J. A., Taylor, R. W., and Gamse, J. T. (2011). Light and melatonin schedule neuronal differentiation in the habenular nuclei. *Dev. Biol.* 358, 251–261. doi: 10.1016/j.ydbio.2011.07.038

De Santi, A., Sovrano, V. A., Bisazza, A., and Vallortigara, G. (2001). Mosquitofish display differential left- and right-eye use during mirror-image scrutiny and predator-inspection responses. *Anim. Behav.* 61, 305–310. doi: 10.1006/anbe.2000.1566

deCarvalho, T. N., Subedi, A., Rock, J., Harfe, B. D., Thisse, C., Thisse, B., et al. (2014). Neurotransmitter map of the asymmetric dorsal habenular nuclei of zebrafish. *Genesis* 52, 636–655. doi: 10.1002/dvg.22785

Denenberg, V. H., Garbanati, J., Sherman, G., Yutzey, D. A., and Kaplan, R. (1978). Infantile stimulation induces brain lateralization in rats. *Science* 201, 1150–1152. doi: 10.1126/science.684436

Deng, C., and Rogers, L. J. (2002). "Factors affecting the development of lateralization in chicks," in *Comparative Vertebrate Lateralization*, eds L. J. Rogers, and R. J. Andrew, (Cambridge: Cambridge University Press), 206–246.

Diana, J. S. (2003). *Biology and Ecology of Fishes*, 2nd Edn. Traverse, MI: Cooper Publishing Group.

Domenichini, A., Dadda, M., Facchin, L., Bisazza, A., and Argenton, F. (2011). Isolation and genetic characterization of mother-of-snow-white, a maternal effect allele affecting laterality and lateralized behaviors in zebrafish. *PLoS One* 6:e25972. doi: 10.1371/journal.pone.0025972

Domenici, P., Allan, B., McCormick, M. I., and Munday, P. L. (2012). Elevated carbon dioxide affects behavioural lateralization in a coral reef fish. *Biol. Lett.* 8, 78–81. doi: 10.1098/rsbl.2011.0591

Domenici, P., Allan, B. J. M., Watson, S.-A., McCormick, M. I., and Munday, P. L. (2014). Shifting from right to left: the combined effect of elevated CO2 and temperature on behavioural lateralization in a coral reef fish. *PLoS One* 9:e87969. doi: 10.1371/journal.pone.0087969

Domenici, P., and Blake, R. W. (1997). The kinematics and performance of fish fast-start swimming. *J. Exp. Biol.* 200, 1165–1178.

Draper, B. W., Morcos, P. A., and Kimmel, C. B. (2001). Inhibition of zebrafish *fgf8* pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. *Genesis* 30, 154–156. doi: 10.1002/gene.1053

Dreosti, E., Vendrell Llopis, N., Carl, M., Yaksi, E., and Wilson, S. W. (2014). Left-right asymmetry is required for the habenulae to respond to both visual and olfactory stimuli. *Curr. Biol.* 24, 440–445. doi: 10.1016/j.cub.2014.01.016

Duboc, V., Dufourcq, P., Blader, P., and Roussigné, M. (2015). Asymmetry of the brain: development and Implications. *Annu. Rev. Genet.* 49, 647–672. doi: 10.1146/annurev-genet-112414-055322

Eaton, R. C., Lee, R. K., and Foreman, M. B. (2001). The Mauthner cell and other identified neurons of the brainstem escape network of fish. *Prog. Neurobiol.* 63, 467–485. doi: 10.1016/S0301-0082(00)00047-2

Erter, C. E., Solnica-Krezel, L., and Wright, C. V. (1998). Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the extraembryonic yolk syncytial layer. *Dev. Biol.* 204, 361–372. doi: 10.1006/dbio.1998.9097

Esbaugh, A. J. (2018). Physiological implications of ocean acidification for marine fish: emerging patterns and new insights. *J. Comp. Physiol. B* 188, 1–13. doi: 10.1007/s00360-017-1105-6

Escalante-Mead, P. R., Minshew, N. J., and Sweeney, J. A. (2003). Abnormal brain lateralization in high-functioning autism. *J. Autism Dev. Disord.* 33, 539–543. doi: 10.1023/A:1025887713788

Essner, J. J., Amack, J. D., Nyholm, M. K., Harris, E. B., and Yost, H. J. (2005). Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart and gut. *Development* 132, 1247–1260. doi: 10.1242/dev.01663

Facchin, L., Argenton, F., and Bisazza, A. (2009a). Lines of *Danio rerio* selected for opposite behavioural lateralization show differences in anatomical left-right asymmetries. *Behav. Brain Res.* 197, 157–165. doi: 10.1016/j.bbr.2008.08.033

Facchin, L., Burgess, H. A., Siddiqi, M., Granato, M., and Halpern, M. E. (2009b). Determining the function of zebrafish epithalamic asymmetry. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364, 1021–1032. doi: 10.1098/rstb.2008.0234

Facchin, L., Duboué, E. R., and Halpern, M. E. (2015). Disruption of epithalamic left-right asymmetry increases anxiety in zebrafish. *J. Neurosci.* 35, 15847–15859. doi: 10.1523/JNEUROSCI.2593-15.2015

Ferrari, M. C. O., McCormick, M. I., Allan, B. J. M., Choi, R., Ramasamy, R. A., Johansen, J. L., et al. (2015). Living in a risky world: the onset and ontogeny of an integrated antipredator phenotype in a coral reef fish. *Sci. Rep.* 5:15537. doi: 10.1038/srep15537

Fontana, B. D., Cleal, M., Clay, J. M., and Parker, M. O. (2019). Zebrafish (*Danio rerio*) behavioral laterality predicts increased short-term avoidance memory but not stress-reactivity responses. *Anim. Cogn.* 22, 1051–1061. doi: 10.1007/s10071-019-01296-9

Forsatkar, M. N., Dadda, M., and Nematollahi, M. A. (2015). Lateralization of aggression during reproduction in male siamese fighting fish. *Ethology* 121, 1039–1047. doi: 10.1111/eth.12418

Frasnelli, E., and Vallortigara, G. (2018). Individual-level and population-level lateralization: two sides of the same coin. *Symmetry* 10:739. doi: 10.3390/sym10120739

Frasnelli, E., Vallortigara, G., and Rogers, L. J. (2012). Left-right asymmetries of behaviour and nervous system in invertebrates. *Neurosci. Biobehav. Rev.* 36, 1273–1291. doi: 10.1016/j.neubiorev.2012.02.006

Gamse, J. T., Kuan, Y. S., Macurak, M., Brösamle, C., Thisse, B., Thisse, C., et al. (2005). Directional asymmetry of the zebrafish epithalamus guides dorsoventral innervation of the midbrain target. *Development* 132, 4869–4881. doi: 10.1242/dev.02046

Gamse, J. T., Thisse, C., Thisse, B., and Halpern, M. E. (2003). The parapineal mediates left-right asymmetry in the zebrafish diencephalon. *Development* 130, 1059–1068. doi: 10.1242/dev.00270

Garric, L., Ronsin, B., Roussigné, M., Booton, S., Gamse, J. T., Dufourcq, P., et al. (2014). Pitx2c ensures habenular asymmetry by restricting parapineal cell number. *Development* 141, 1572–1579. doi: 10.1242/dev.100305

Gatto, E., Agrillo, C., Brown, C., and Dadda, M. (2019). Individual differences in numerical skills are influenced by brain lateralization in guppies (*Poecilia reticulata*). *Intelligence* 74, 12–17. doi: 10.1016/j.intell.2019.04.004

Ghirlanda, S., Frasnelli, E., and Vallortigara, G. (2009). Intraspecific competition and coordination in the evolution of lateralization. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364, 861–866. doi: 10.1098/rstb.2008.0227

Ghirlanda, S., and Vallortigara, G. (2004). The evolution of brain lateralization: a game theoretical analysis of population structure. *Proc. Biol. Sci.* 271, 853–857. doi: 10.1098/rspb.2003.2669

Gourronc, F., Ahmad, N., Nedza, N., Eggleston, T., and Rebagliati, M. (2007). Nodal activity around Kupffer's vesicle depends on the T-box transcription factors notail and spadetail and on notch signaling. *Dev. Dyn.* 236, 2131–2146. doi: 10.1002/dvdy.21249

Green, L., and Jutfelt, F. (2014). Elevated carbon dioxide alters the plasma composition and behavior of shark. *Biol. Lett.* 10:20140538. doi: 10.1098/rsbl.2014.0538

Güntürkün, O., Diekamp, B., Manns, M., Nottelmann, F., Prior, H., Schwarz, A., et al. (2000). Asymmetry pays: visual lateralization improves discrimination success in pigeons. *Curr. Biol.* 10, 1079–1081. doi: 10.1016/S0960-9822(00)00671-0

Güntürkün, O., and Kesch, S. (1987). Visual lateralization during feeding in pigeons. *Behav. Neurosci.* 101, 433–435. doi: 10.1037/0735-7044.101.3.433

Güntürkün, O., and Ocklenburg, S. (2017). Ontogenesis of lateralization. *Neuron* 94, 249–263. doi: 10.1016/j.neuron.2017.02.045

Gutiérrez-Ibáñez, C., Reddon, A. R., Kreuzer, M. B., Wylie, D. R., and Hurd, P. L. (2011). Variation in asymmetry of the habenular nucleus correlates with behavioural asymmetry in a cichlid fish. *Behav. Brain. Res.* 221, 189–196. doi: 10.1016/j.bbr.2011.03.001

Hamilton, C. R., and Vermeire, B. A. (1988). Complementary hemispheric specialization in monkeys. *Science* 242, 1691–1694. doi: 10.1126/science.3201258

Hamilton, S. L., Logan, C. A., Fennie, H. W., Sogard, S. M., Barry, J. P., Makukhov, A. D., et al. (2017). Species-specific responses of juvenile rockfish to elevated pCO2: from behavior to genomics. *PLoS One* 12:e0169670. doi: 10.1371/journal.pone.0169670

Hashimoto, H., Rebagliati, M., Ahmad, N., Muraoka, O., Kurokawa, T., Hibi, M., et al. (2004). The Cerberus/Dan-family protein Charon is a negative regulator

of Nodal signaling during left-right patterning in zebrafish. *Development* 131, 1741–1753. doi: 10.1242/dev.01070

Hata, H., and Hori, M. (2011). Inheritance patterns of morphological laterality in mouth opening of zebrafish, *Danio rerio*. *Laterality* 17, 741–754. doi: 10.1080/1357650X.2011.626563

Hata, H., Takahashi, R., Ashiwa, H., Awata, S., Takeyama, T., Kohda, M., et al. (2012). Inheritance patterns of lateral dimorphism examined by breeding experiments with the Tanganyikan cichlid (*Julidochromis transcriptus*) and the Japanese Medaka (*Oryzias latipes*). *Zool. Sci.* 29, 49–53. doi: 10.2108/zsj.29.49

Hata, H., Yasugi, M., and Hori, M. (2011). Jaw laterality and related handedness in the hunting behavior of a scale-eating characin, *Exodon paradoxus*. *PLoS One* 6:e29349. doi: 10.1371/journal.pone.0029349

HedayatiRad, M., Nematollahi, M. A., Forsatkar, M. N., and Brown, V. (2017). Prozac impacts lateralization and aggression in male Siamese fighting fish. *Ecotoxicol. Environ. Saf.* 140, 84–88. doi: 10.1016/j.ecoenv.2017.02.027

Heim, S., and Keil, A. (2004). Large-scale neural correlates of developmental dyslexia. *Eur. Child Adolesc. Psychiatry* 13, 125–140. doi: 10.1007/s00787-004-0361-7

Herbert, M. R., Ziegler, D. A., Deutsch, C. K., O'Brien, L. M., Kennedy, D. N., Filipek, P. A., et al. (2005). Brain asymmetries in autism and developmental language disorder: a nested whole-brain analysis. *Brain J. Neurol.* 128, 213–226. doi: 10.1093/brain/awh330

Herbert-Read, J. E., Romanczuk, P., Krause, S., Strömbom, D., Couillaud, P., Domenici, P., et al. (2016). Proto-cooperation: group hunting sailfish improve hunting success by alternating attacks on grouping prey. *Proc. Biol. Sci.* 283:20162586. doi: 10.1098/rspb.2016.1671

Heuts, B. A. (1999). Lateralization of trunk muscle volume, and lateralization of swimming turns of fish responding to external stimuli. *Behav. Proc.* 47, 113–124. doi: 10.1016/S0376-6357(99)00056-X

Hews, D. K., and Worthington, R. A. (2001). Fighting from the right side of the brain: left visual field preference during aggression in free-ranging male tree lizards (*Urosaurus ornatus*). *Brain. Behav. Evol.* 58, 356–361. doi: 10.1159/000057576

Hojo, M., Takashima, S., Kobayashi, D., Sumeragi, A., Shimada, A., Tsukahara, T., et al. (2007). Right-elevated expression of *charon* is regulated by fluid flow in medaka Kupffer's vesicle. *Dev. Growth Differ.* 94, 395–405. doi: 10.1111/j.1440-169X.2007.00937.x

Hori, M. (1993). Frequency-dependent natural selection in the handedness of scale-eating cichlid fish. *Science* 260, 216–219. doi: 10.1126/science.260.5105.216

Hori, M., Nakajima, M., Hata, H., Yasugi, M., Takahashi, S., Nakae, M., et al. (2017). Laterality is universal among fishes but increasingly cryptic among derived groups. *Zool. Sci.* 34, 267–274. doi: 10.2108/zs160196

Hori, M., Ochi, H., and Kohda, M. (2007). Inheritance pattern of lateral dimorphism in two cichlids (a scale eater, *Perissodus microlepis*, and an herbivore, *Neolamprologus moorii*) in Lake Tanganyika. *Zool. Sci.* 24, 486–492. doi: 10.2108/zsj.24.486

Hüsken, U., and Carl, M. (2013). The Wnt/beta-catenin signaling pathway establishes neuroanatomical asymmetries and their laterality. *Mech. Dev.* 130, 330–335. doi: 10.1016/j.mod.2012.09.002

Hüsken, U., Stickney, H. L., Gestri, G., Bianco, I. H., Faro, A., Young, R. M., et al. (2014). Tcf7l2 is required for left-right asymmetric differentiation of habenular neurons. *Curr. Biol.* 24, 2217–2227. doi: 10.1016/j.cub.2014.08.006

Inbal, A., Kim, S. H., Shin, J., and Solnica-Krezel, L. (2007). Six3 represses nodal activity to establish early brain asymmetry in zebrafish. *Neuron* 55, 407–415. doi: 10.1016/j.neuron.2007.06.037

Izvekov, E. I., Kuternitskaya, E. A., Pankova, N. A., Malashichev, Y. B., and Nepomnyashchikh, V. A. (2014). Lateralisation of rotational swimming but not fast escape response in the juvenile sterlet sturgeon, *Acipenser ruthenus* (Chondrostei: Acipenseridae). *Laterality* 19, 302–324. doi: 10.1080/1357650X.2013.804080

Izvekov, E. I., and Nepomnyashchikh, V. A. (2010). Comparison of two kinds of functional asymmetry in the roach *Rutilus rutilus* (Teleostei: Cyprinidae). *J. Evol. Biochem. Physiol.* 46, 69–76. doi: 10.1134/S0022093010010081

Izvekov, E. I., Nepomnyashchikh, V. A., Medyantseva, E. N., Chebotareva, Y. V., and Izyumov, Y. G. (2012). Selection of direction of movement and bilateral morphological asymmetry in young roaches (*Rutilus rutilus*). *Biol. Bull. Rev.* 2, 364–370. doi: 10.1134/S2079086412040044

Jarrold, M. D., and Munday, P. L. (2018). Elevated temperature does not substantially modify the interactive effects between elevated CO2 and diel CO2 cycles on the survival, growth and behavior of a coral reef fish. *Front. Mar. Sci.* 5:458. doi: 10.3389/fmars.2018.00458

Jeffries, R. P. S., and Lewis, D. N. (1978). The English Silurian fossil *Placocystites forbesianus* and the ancestry of vertebrates. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 282, 205–323.

Jutfelt, F., de Souza, K. B., Vuylsteke, A., and Sturve, J. (2013). Behavioural disturbances in a temperate fish exposed to sustained high-CO2 levels. *PLoS One* 8:e65825. doi: 10.1371/journal.pone.0065825

Jutfelt, F., and Hedgärde, M. (2015). Juvenile Atlantic cod behavior appears robust to near-future CO2 levels. *Front. Zool.* 12:11. doi: 10.1186/s12983-015-0104-2

Kaarthigeyan, J., and Dharmaretnam, M. (2005). Relative levels of motivation and asymmetries of viewing and detour task in guppies (*Poecilia reticulata*). *Behav. Brain Res.* 159, 37–41. doi: 10.1016/j.bbr.2004.09.024

Kalueff, A. V., Stewart, A. M., and Gerlai, R. (2014). Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol. Sci.* 35, 63–75. doi: 10.1016/j.tips.2013.12.002

Keller, P. J., and Ahrens, M. B. (2015). Visualizing whole-brain activity and development at the single-cell level using light-sheet microscopy. *Neuron* 85, 462–483. doi: 10.1016/j.neuron.2014.12.039

Kendrick, K. M. (2006). Brain asymmetries for face recognition and emotion control in sheep. *Cortex* 42, 96–98. doi: 10.1016/S0010-9452(08)70328-9

Khuansuwan, S., Clanton, J. A., Dean, B. J., Patton, J. G., and Gamse, J. T. (2016). A transcription factor network controls cell migration and fate decisions in the developing zebrafish pineal complex. *Development* 143, 2641–2650. doi: 10.1242/dev.131680

Klar, A. J. (1999). Genetic models for handedness, brain lateralization, schizophrenia, and manic-depression. *Schizophr. Res.* 39, 207–218. doi: 10.1016/S0920-9964(99)00075-4

Klar, A. J. (2011). Breast cancer predisposition and brain hemispheric laterality specification likely share a common genetic cause. *Breast Dis.* 33, 49–52. doi: 10.3233/BD-2010-0318

Korn, H., and Faber, D. S. (2005). The Mauthner cell half a century later: a neurobiological model for decision-making? *Neuron* 47, 13–28. doi: 10.1016/j.neuron.2005.05.019

Krishnan, S., Mathuru, A. S., Kibat, C., Rahman, M., Lupton, C. E., Stewart, J., et al. (2014). The right dorsal habenula limits attraction to an odor in zebrafish. *Curr. Biol.* 24, 1167–1175. doi: 10.1016/j.cub.2014.03.073

Kurvers, R. H. J. M., Krause, S., Viblanc, P. E., Herbert-Read, J. E., Zaslansky, P., Domenici, P., et al. (2017). The evolution of lateralization in group hunting Sailfish. *Curr. Biol.* 27, 521–526. doi: 10.1016/j.cub.2016.12.044

Lagutin, O. V., Zhu, C. C., Kobayashi, D., Topczewski, J., Shimamura, K., Puelles, L., et al. (2003). Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. *Genes Dev.* 17, 368–379. doi: 10.1101/gad.1059403

Lai, F., Jutfelt, F., and Nilsson, G. E. (2015). Altered neurotransmitter function in CO2- exposed stickleback (*Gasterosteus aculeatus*): a temperate model species for ocean acidification research. *Conserv. Physiol.* 3:cov018. doi: 10.1093/conphys/cov018

Lee, H. J., Heim, V., and Meyer, A. (2015). Genetic and environmental effects on the morphological asymmetry in the scale-eating cichlid fish, *Perissodus microlepis*. *Ecol. Evol.* 5, 4277–4286. doi: 10.1002/ece3.1691

Lee, H. J., Kusche, H., and Meyer, A. (2012). Handed foraging behavior in scale-eating cichlid fish: its potential role in shaping morphological asymmetry. *PLoS One* 7:e44670. doi: 10.1371/journal.pone.0044670

Lekk, I., Duboc, V., Faro, A., Nicolaou, S., Blader, P., and Wilson, S. W. (2019). Sox1a mediates the ability of the parapineal to impart habenular left-right asymmetry. *eLife* 8:e47376. doi: 10.7554/eLife.47376

Levy, J. (1977). The mammalian brain and the adaptive advantage of cerebral asymmetry. *Ann. N. Y. Acad. Sci.* 299, 264–272. doi: 10.1111/j.1749-6632.1977.tb41913.x

Liang, J. O., Etheridge, A., Hantsoo, L., Rubinstein, A. L., Nowak, S. J., Izpisúa Belmonte, J. C., et al. (2000). Asymmetric nodal signaling in the zebrafish diencephalon positions the pineal organ. *Development* 127, 5101–5112.

Lippolis, G., Bisazza, A., Rogers, L. J., and Vallortigara, G. (2002). Lateralization of predator avoidance in three species of toads. *Laterality* 7, 163–183. doi: 10.1080/13576500143000221

Lippolis, G., Joss, J. M. P., and Rogers, L. J. (2009). Australian lungfish (*Neoceratodus forsteri*): a missing link in the evolution of complementary side biases for predator avoidance and prey capture. *Brain Behav. Evol.* 73, 295–303. doi: 10.1159/000230674

Lippolis, G., Westman, W., McAllan, B. M., and Rogers, L. J. (2005). Lateralisation of escape responses in the stripe-faced dunnart, *Sminthopsis macroura* (Dasyuridae: Marsupialia). *Laterality* 10, 457–470. doi: 10.1080/13576500442000210

Loffing, F. (2017). Left- handedness and time pressure in elite interactive ball games. *Biol. Lett.* 13:20170446. doi: 10.1098/rsbl.2017.0446

Long, S., Ahmad, N., and Rebagliati, M. (2003). The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Development* 130, 2303–2316. doi: 10.1242/dev.00436

Lopes, A. F., Morais, P., Pimentel, M., Rosa, R., Munday, P. L., Gonçalves, E. J., et al. (2016). Behavioural lateralization and shoaling cohesion of fish larvae altered under ocean acidification. *Mar. Biol.* 163:243. doi: 10.1007/s00227-016-3026-4

Lopes, S. S., Lourenço, R., Pacheco, L., Moreno, N., Kreiling, J., and Saúde, L. (2010). Notch signalling regulates left-right asymmetry through ciliary length control. *Development* 137, 3625–3632. doi: 10.1242/dev.054452

Lucon-Xiccato, T., Nati, J. J. H., Blasco, F. R., Johansen, J. L., Steffensen, J. F., and Domenici, P. (2014). Severe hypoxia impairs lateralization in a marine teleost fish. *J. Exp. Biol.* 217, 4115–4118. doi: 10.1242/jeb.111229

MacNeilage, P. F., Rogers, L. J., and Vallortigara, G. (2009). Origins of the left and right brain. *Sci. Am.* 301, 60–67. doi: 10.1038/scientificamerican0709-60

McManus, I. C. (1999). "Handedness, cerebral lateralisation and the evolution of language," in *The Descent of Mind: Psychological Perspective on Hominid Evolution*, eds M. C. Corballis, and S. E. G. Lea, (Oxford: Oxford University Press), 194–217.

Mehlis-Rick, M., Thünken, T., Krings, M., Käfer, S., and Rick, I. P. (2018). Cerebral lateralization and quantity discrimination abilities in the threespine stickleback (*Gasterosteus aculeatus*). *Evol. Ecol. Res.* 19, 487–501.

Melby, A. E., Warga, R. M., and Kimmel, C. B. (1996). Specification of cell fates at the dorsal margin of the zebrafish gastrula. *Development* 122, 2225–2237.

Mench, J., and Andrew, R. J. (1986). Lateralisation of a food search task in the domestic chick. *Behav. Neural Biol.* 46, 107–114. doi: 10.1016/S0163-1047(86)90570-4

Mikhailova, G. Z., Pavlik, V. D., Tiras, N. R., and Moshkow, D. A. (2005). Correlation of the dimensions of Mauthner neurons with the preference of goldfishes for rightward or leftward turns. *Morfologiya* 127, 16–19.

Miklosi, A., and Andrew, R. J. (1999). Right eye use associated with decision to bite in zebrafish. *Behav. Brain Res.* 105, 199–205. doi: 10.1016/S0166-4328(99)00071-6

Miklosi, A., Andrew, R. J., and Savage, H. (1997). Behavioural lateralisation of the tetrapod type in the zebrafish (*Brachydanio rerio*). *Physiol. Behav.* 63, 127–135. doi: 10.1016/S0031-9384(97)00418-6

Miyasaka, N., Morimoto, K., Tsubokawa, T., Higashijima, S., Okamoto, H., and Yoshihara, Y. (2009). From the olfactory bulb to higher brain centers: genetic visualization of secondary olfactory pathways in zebrafish. *J. Neurosci.* 29, 4756–4767. doi: 10.1523/JNEUROSCI.0118-09.2009

Miyashita, T., and Palmer, A. R. (2014). Handed behavior in hagfish-an ancient vertebrate lineage-and a survey of lateralized behaviors in other invertebrate chordates and elongate vertebrates. *Biol. Bull.* 226, 111–120.

Moscicki, M. K., Reddon, A. R., and Hurd, P. L. (2011). Lateralized behaviour of a non-social cichlid ?sh (*Amatitlania nigrofasciata*) in a social and non-social environment. *Behav. Proc.* 88, 27–32. doi: 10.1016/j.beproc.2011.07.004

Moulton, J. M., and Barron, S. E. (1967). Asymmetry in the Mauthner cells of the gold?sh brain. *Copeia* 4, 836–837.

Munday, P. L., Dixson, D. L., Donelson, J. M., Jones, G. P., Pratchett, M. S., Devitsina, G. V., et al. (2009). Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proc. Natl. Acad. Sci. U.S.A.* 106, 1848–1852. doi: 10.1073/pnas.0809996106

Neugebauer, J. M., and Yost, H. J. (2014). FGF signaling is required for brain left-right asymmetry and brain midline formation. *Dev. Biol.* 386, 123–134. doi: 10.1016/j.ydbio.2013.11.020

Neveu, P. J. (2002). Cerebral lateralization and the immune system. *Int. Rev. Neurobiol.* 52, 303–323. doi: 10.1016/S0074-7742(02)52014-6

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sørensen, C., Watson, S.-A., et al. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nat. Clim. Chang.* 2, 201–204. doi: 10.1038/nclimate1352

Nottebohm, F. (1971). Neural lateralization of vocal control in a passerine bird. I. Song. *J. Exp. Zool.* 177, 229–261. doi: 10.1002/jez.1401770210

Ocklenburg, S., Korte, M., Peterburs, J., Wolf, O. T., and Güntürkün, O. (2016). Stress and laterality - The comparative perspective. *Physiol. Behav.* 164, 321–329. doi: 10.1016/j.physbeh.2016.06.020

Ocklenburg, S., Ströckens, F., and Güntürkün, O. (2013). Lateralisation of conspecific vocalisation in non-human vertebrates. *Laterality* 18, 1–31. doi: 10.1080/1357650X.2011.626561

Paracchini, S., and Scerri, T. (2017). "Genetics of human handedness and laterality," in *Lateralized Brain Functions: Methods in Human and Non-Human Species*, eds L. J. Rogers, and G. Vallortigara, (Berlin: Springer), 523–552.

Raffini, F., Fruciano, C., Franchini, P., and Meyer, A. (2017). Towards understanding the genetic basis of mouth asymmetry in the scale-eating cichlid *Perissodus microlepis*. *Mol. Ecol.* 26, 77–91. doi: 10.1111/mec.13699

Raya, A., Kawakami, Y., Rodriguez-Esteban, C., Buscher, D., Koth, C. M., Itoh, T., et al. (2003). Notch activity induces Nodal expression and mediates the establishment of left-right asymmetry in vertebrate embryos. *Genes Dev.* 17, 1213–1218. doi: 10.1101/gad.1084403

Rebagliati, M. R., Toyama, R., Fricke, C., Haffter, P., and Dawid, I. B. (1998). Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev. Biol.* 199, 261–272. doi: 10.1006/dbio.1998.8935

Reddon, A. R., and Balshine, S. (2010). Lateralization in response to social stimuli in a cooperatively breeding cichlid fish. *Behav. Proc.* 85, 68–71. doi: 10.1016/j.beproc.2010.06.008

Reddon, A. R., and Hurd, P. L. (2008). Aggression, sex and individual differences in cerebral lateralization in a cichlid fish. *Biol. Lett.* 4, 338–340. doi: 10.1098/rsbl.2008.0206

Regan, J. C., Concha, M. L., Roussigne, M., Russell, C., and Wilson, S. W. (2009). An Fgf8-dependent bistable cell migratory event establishes CNS asymmetry. *Neuron* 61, 27–34. doi: 10.1016/j.neuron.2008.11.030

Reifers, F., Böhli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y., and Brand, M. (1998). Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* 125, 2381–2395.

Ribolsi, M., Koch, G., Magni, V., Di Lorenzo, G., Rubino, I. A., Siracusano, A., et al. (2009). Abnormal brain lateralization and connectivity in schizophrenia. *Rev. Neurosci.* 20, 61–70. doi: 10.1515/REVNEURO.2009.20.1.61

Robins, A., and Rogers, L. J. (2004). Lateralised prey catching responses in the toad (*Bufo marinus*): analysis of complex visual stimuli. *Anim. Behav.* 68, 567–575. doi: 10.1016/j.anbehav.2003.12.014

Rogers, L. J. (1990). Light input and the reversal of functional lateralization in the chicken brain. *Behav. Brain Res.* 38, 211–221. doi: 10.1016/0166-4328(90)90176-F

Rogers, L. J. (1997). Early experiential effects on laterality: research on chicks has relevance to other species. *Laterality* 2, 199–219. doi: 10.1080/713754277

Rogers, L. J. (2000). "Evolution of side biases: motor versus sensory lateralization," in *Side Bias: A Neuropsychological Perspective*, eds M. K. Mandal, M. B. Bulman-Fleming, and G. Tiwari, (Dordrecht: Kluwer Academic Publisher), 3–40.

Rogers, L. J. (2002). "Advantages and disadvantages of lateralization," in *Comparative Vertebrate Lateralization*, eds L. J. Rogers, and R. J. Andrew, (Cambridge: Cambridge University Press), 126–153.

Rogers, L. J. (2008). Development and function of lateralization in the avian brain. *Brain Res. Bull.* 76, 235–244. doi: 10.1016/j.brainresbull.2008.02.001

Rogers, L. J. (2010). "Interactive contributions of genes and early experience to behavioural development: sensitive periods and lateralized brain and behaviour," in *Handbook of Developmental Science, Behavior, and Genetics*, eds K. E. Hood, C. T. Halpern, G. Greenberg, and R. M. Lerner, (West Sussex: Wiley-Blackwell Publishing), 400–433.

Rogers, L. J. (2014). Asymmetry of brain and behavior in animals: its development, function, and human relevance. *Genesis* 52, 555–571. doi: 10.1002/dvg.22741

Rogers, L. J., and Anson, J. M. (1979). Lateralization of function in chicken forebrain. *Pharmacol. Biochem. Behav.* 10, 679–686. doi: 10.1016/0091-3057(79)90320-4

Rogers, L. J., and Deng, C. (2005). Corticosterone treatment of the chick embryo affects light stimulated development of the thalamofugal visual pathway. *Behav. Brain Res.* 159, 63–71. doi: 10.1016/j.bbr.2004.10.003

Rogers, L. J., and Vallortigara, G. (2015). When and why did brains break symmetry? *Symmetry* 7, 2181–2194. doi: 10.3390/sym7042181

Rogers, L. J., and Vallortigara, G. (2017). *Lateralized Brain Functions: Methods in Human and Non-Human Species*. Berlin: Springer. doi: 10.1007/978-1-4939-6725-4

Rogers, L. J., Vallortigara, G., and Andrew, R. J. (2013). *Divided Brains: The Biology and Behaviour of Brain Asymmetries*. New York, NY: Cambridge University Press.

Rogers, L. J., Zucca, P., and Vallortigara, G. (2004). Advantages of having a lateralized brain. *Proc. Biol. Sci.* 271, S420–S422. doi: 10.1098/rsbl.2004.0200

Rosa Salva, O., Regolin, L., Mascalzoni, E., and Vallortigara, G. (2012). Cerebral and behavioural asymmetry in animal social recognition. *Comp. Cogn. Behav. Rev.* 7, 110–138. doi: 10.3819/ccbr.2012.70006

Roussigné, M., Bianco, I. H., Wilson, S. W., and Blader, P. (2009). Nodal signaling imposes left-right asymmetry upon neurogenesis in the habenular nuclei. *Development* 136, 1549–1557. doi: 10.1242/dev.034793

Roussigné, M., Blader, P., and Wilson, S. W. (2012). Breaking symmetry: the zebrafish as a model for understanding left-right asymmetry in the developing brain. *Dev. Neurobiol.* 72, 269–281. doi: 10.1002/dneu.20885

Roux, N., Duran, E., Lanyon, R. G., Frédérich, B., Besson, M., Dixson, D. L., et al. (2016). Brain lateralization involved in visual recognition of conspecifics in coral reef fish at recruitment. *Anim. Behav.* 117, 3–8. doi: 10.1016/j.anbehav.2016.04.011

Sagasti, A. (2007). Three ways to make two sides: genetic models of asymmetric nervous system development. *Neuron* 55, 345–351. doi: 10.1016/j.neuron.2007.07.015

Sampath, K., Rubinstein, A. L., Cheng, A. M., Liang, J. O., Fekany, K., Solnica-Krezel, L., et al. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* 395, 185–189. doi: 10.1038/26020

Sandson, T. A., Wen, P. Y., and LeMay, M. (1992). Reversed cerebral asymmetry in women with breast cancer. *Lancet* 339, 523–524. doi: 10.1016/0140-6736(92)90341-Y

Schaafsma, S. M., and Groothuis, T. G. G. (2011). Sex specific effects of postnatal testosterone on lateralization in cichlid fish. *Anim. Behav.* 81, 283–288. doi: 10.1016/j.anbehav.2010.10.019

Schultze, H.-P. (1986). Dipnoans as sarcopterygians. *J. Morphol.* 1, 39–74. doi: 10.1002/jmor.1051900407

Schwarz, I. M., and Rogers, L. J. (1992). Testosterone: a role in the development of brain asymmetry in the chick. *Neurosci. Lett.* 146, 167–170. doi: 10.1016/0304-3940(92)90069-J

Seki, S., Kohda, M., and Hori, M. (2000). Asymmetry of mouth morph of a freshwater goby, *Rhinogobius flumineus*. *Zool. Sci.* 17, 1321–1325. doi: 10.2108/zsj.17.1321

Shu, D.-G., Luo, H.-L., Morris, S. C., Zhang, X.-L., Hu, S.-X., Chen, L., et al. (1999). Lower Cambrian vertebrates from south China. *Nature* 402, 42–46. doi: 10.1038/46965

Simpson, S. D., Munday, P. L., Wittenrich, M. L., Manassa, R., Dixson, D. L., Gagliano, M., et al. (2011). Ocean acidification erodes crucial auditory behaviour in a marine fish. *Biol. Lett.* 7, 917–920. doi: 10.1098/rsbl.2011.0293

Simpson, S. D., Purser, J., and Radford, A. N. (2015). Anthropogenic noise compromises antipredator behaviour in European eels. *Glob. Change Biol.* 21, 586–593. doi: 10.1111/gcb.12685

Snelson, C. D., and Gamse, J. T. (2009). Building an asymmetric brain: development of the zebrafish epithalamus. *Semin. Cell Dev. Biol.* 20, 491–497. doi: 10.1016/j.semcdb.2008.11.008

Sovrano, V. A. (2004). Visual lateralization in response to familiar and unfamiliar stimuli in fish. *Behav. Brain Res.* 152, 385–391. doi: 10.1016/j.bbr.2003.10.022

Sovrano, V. A., and Andrew, R. J. (2006). Eye use during viewing a reflection: behavioural lateralisation in zebrafish larvae. *Behav. Brain Res.* 167, 226–231. doi: 10.1016/j.bbr.2005.09.021

Sovrano, V. A., Bertolucci, C., Frigato, E., Foà, A., and Rogers, L. J. (2016). Influence of exposure in ovo to different light wavelengths on the lateralization of social response in zebrafish larvae. *Physiol. Behav.* 157, 258–264. doi: 10.1016/j.physbeh.2016.02.016

Sovrano, V. A., Bisazza, A., and Vallortigara, G. (2001). Lateralization of response to social stimuli in fishes: a comparison between different methods and species. *Physiol. Behav.* 74, 237–244. doi: 10.1016/S0031-9384(01)00552-2

Sovrano, V. A., Dadda, M., and Bisazza, A. (2005). Lateralized fish perform better than nonlateralized fish in spatial reorientation tasks. *Behav. Brain Res.* 163, 122–127. doi: 10.1016/j.bbr.2005.04.012

Sovrano, V. A., Rainoldi, C., Bisazza, A., and Vallortigara, G. (1999). Roots of brain specializations: preferential left-eye use during mirror-image inspection in six species of teleost fish. *Behav. Brain Res.* 106, 175–180. doi: 10.1016/S0166-4328(99)00105-9

Stancher, G., Sovrano, V. A., and Vallortigara, G. (2018). Motor asymmetries in fishes, amphibians and reptiles, in cerebral lateralization and cognition: evolutionary and developmental investigations of behavioral biases. *Prog. Brain Res.* 238, 35–56. doi: 10.1016/bs.pbr.2018.06.002

Stennett, C. R., and Strauss, R. E. (2010). Behavioural lateralization in zebrafish and four related species of minnows (Osteichthyes: Cyprinidae). *Anim. Behav.* 79, 1339–1342. doi: 10.1016/j.anbehav.2010.03.007

Stewart, A. M., Braubach, O., Spitsbergen, J., Gerlai, R., and Kalueff, A. V. (2014). Zebrafish models for translational neuroscience research: from tank to bedside. *Trends Neurosci.* 37, 264–278. doi: 10.1016/j.tins.2014.02.011

Stewart, T., and Albertson, R. C. (2010). Evolution of a unique predatory feeding apparatus: functional anatomy, development and a genetic locus for jaw laterality in Lake Tanganyika scale-eating cichlids. *BMC Biol.* 8:8. doi: 10.1186/1741-7007-8-8

Ströckens, F., Güntürkün, O., and Ocklenburg, S. (2013). Limb preferences in non-human vertebrates. *Laterality* 18, 536–575. doi: 10.1080/1357650X.2012.723008

Sundin, J., and Jutfelt, F. (2015). 9–28 days of exposure to elevated pCO2 reduces avoidance of predator odour but had no effect on behavioural lateralization or swimming activity in a temperate wrasse (*Ctenolabrus rupestris*). *ICES J. Mar. Sci.* 73, 620–632. doi: 10.1093/icesjms/fsv101

Sundin, J., and Jutfelt, F. (2018). Effects of elevated carbon dioxide on male and female behavioural lateralization in a temperate goby. *R. Soc. Open Sci.* 5:171550. doi: 10.1098/rsos.171550

Takeuchi, J. K., Lickert, H., Bisgrove, B. W., Sun, X., Yamamoto, M., Chawengsaksophak, K., et al. (2007). Baf60c is a nuclear Notch signaling component required for the establishment of left-right asymmetry. *Proc. Natl. Acad. Sci. U.S.A.* 104, 846–851. doi: 10.1073/pnas.0608118104

Takeuchi, Y., and Hori, M. (2008). Behavioural laterality in the shrimp-eating cichlid fish *Neolamprologus fasciatus* in Lake Tanganyika. *Anim. Behav.* 75, 1359–1366. doi: 10.1016/j.anbehav.2007.09.008

Takeuchi, Y., Hori, M., Myint, O., and Kohda, M. (2010). Lateral bias of agonistic responses to mirror images and morphological asymmetry in the Siamese fighting fish (*Betta splendens*). *Behav. Brain Res.* 208, 106–111. doi: 10.1016/j.bbr.2009.11.021

Takeuchi, Y., Hori, M., and Oda, Y. (2012). Lateralized kinematics of predation behavior in a Lake Tanganyika scale-eating cichlid fish. *PLoS One* 7:e29272. doi: 10.1371/journal.pone.0029272

Turner, K. J., Hawkins, T. A., Yáñez, J., Anadón, R., Wilson, S. W., and Folgueira, M. (2016). Afferent connectivity of the zebrafish habenulae. *Front. Neural Circuits* 10:30. doi: 10.3389/fncir.2016.00030

Vallortigara, G. (1992). Right hemisphere advantage for social recognition in the chick. *Neuropsychologia* 30, 761–768. doi: 10.1016/0028-3932(92)90080-6

Vallortigara, G. (2000). Comparative neuropsychology of the dual brain: a stroll through left and right animals' perceptual worlds. *Brain Lang.* 73, 189–219. doi: 10.1006/brln.2000.2303

Vallortigara, G. (2006a). "The evolution of behavioural and brain asymmetries: bridging together neuropsychology and evolutionary biology," in *Behavioral and Morphological Asymmetries in Vertebrates*, eds Y. Malashichev, and W. Deckel, (Austin TX: Landes Bioscience), 1–20.

Vallortigara, G. (2006b). The evolutionary psychology of left and right: costs and benefits of lateralization. *Dev. Psychobiol.* 48, 418–427. doi: 10.1002/dev.20166

Vallortigara, G. (2019). Phenotypes in hemispheric functional segregation as by-products of the evolution of lateralization population structure: comment on "Phenotypes in hemispheric functional segregation? Perspectives and challenges" by Guy Vingerhoets. *Phys. Life Rev.* 30, 38–40. doi: 10.1016/j.plrev.2019.07.008

Vallortigara, G., and Andrew, R. J. (1991). Lateralization of response by chicks to change in a model partner. *Anim. Behav.* 41, 187–194. doi: 10.1016/S0003-3472(05)80470-1

Vallortigara, G., and Andrew, R. J. (1994). Differential involvement of right and left hemisphere in individual recognition in the domestic chick. *Behav. Proc.* 33, 41–58. doi: 10.1016/0376-6357(94)90059-0

Vallortigara, G., and Bisazza, A. (2002). "How ancient is brain lateralization?," in *Comparative Vertebrate Lateralization*, eds R. J. Andrew, and L. J. Rogers, (Cambridge: Cambridge University Press), 9–69.

Vallortigara, G., Chiandetti, C., and Sovrano, V. A. (2011). Brain asymmetry (animal). *Wiley Interdiscip. Rev. Cogn. Sci.* 2, 146–157. doi: 10.1002/wcs.100

Vallortigara, G., Cozzutti, C., Tommasi, L., and Rogers, L. J. (2001). How birds use their eyes: opposite left-right specialisation for the lateral and frontal visual hemifield in the domestic chick. *Curr. Biol.* 11, 29–33. doi: 10.1016/S0960-9822(00)00027-0

Vallortigara, G., and Rogers, L. J. (2005). Survival with an asymmetrical brain: advantages and disadvantages of cerebral lateralization. *Behav. Brain Sci.* 28, 575–633. doi: 10.1017/S0140525X05350102

Vallortigara, G., and Rogers, L. J. (2020). A function for the bicameral mind. *Cortex* 124, 274–285.

Vallortigara, G., Rogers, L. J., Bisazza, A., Lippolis, G., and Robins, A. (1998). Complementary right and left hemifield use for predatory and agonistic behaviour in toads. *Neuroreport* 9, 3341–3344.

Vallortigara, G., and Versace, E. (2017). "Laterality at the neural, cognitive, and behavioral levels," in *APA Handbook of Comparative Psychology: Basic Concepts, Methods, Neural Substrate, and Behavior*, Vol. 1, eds J. Call, G. M. Burghardt, I. M. Pepperberg, C. T. Snowdon, and T. Zentall, (Washington DC: American Psychological Association), 557–577.

Van Dooren, T., van Goor, H., and van Putten, M. (2010). Handedness and asymmetry in scale-eating cichlids: antisymmetries of different strength. *Evolution* 64, 2159–2165. doi: 10.1111/j.1558-5646.2010.00977.x

Versace, E., Morgante, M., Pulina, G., and Vallortigara, G. (2007). Behavioural lateralization in sheep (*Ovis aries*). *Behav. Brain Res.* 184, 72–80. doi: 10.1016/j.bbr.2007.06.016

Vossen, L. E., Jutfelt, F., Cocco, A., Thörnqvist, P.-O., and Winberg, S. (2016). Zebrafish (*Danio rerio*) behaviour is largely unaffected by elevated pCO2. *Conserv. Physiol.* 4:cow065. doi: 10.1093/conphys/cow065

Watkins, J., Miklosi, A., and Andrew, R. J. (2004). Early asymmetries in the behaviour of zebrafish larvae. *Behav. Brain Res.* 151, 177–183. doi: 10.1016/j.bbr.2003.08.012

Wijers, A. A., Been, P. H., and Romkes, K. S. (2005). Dyslexics show a deviant lateralization of attentional control: a brain potential study. *Neurosci. Lett.* 374, 87–91. doi: 10.1016/j.neulet.2004.10.072

Wiper, M. L., Lehnert, S. J., Heath, D. D., and Higgs, D. M. (2017). Neutral genetic variation in adult Chinook salmon (*Oncorhynchus tshawytscha*) affects brain-to-body trade-off and brain laterality. *R. Soc. Open. Sci.* 4:170989. doi: 10.1098/rsos.170989

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroanatomy

REVIEW
published: 15 April 2020
doi: 10.3389/fnana.2020.00012

Understanding the Molecular and Cell Biological Mechanisms of Electrical Synapse Formation

E. Anne Martin†, Abagael M. Lasseigne*† and Adam C. Miller**

Department of Biology, Institute of Neuroscience, University of Oregon, Eugene, OR, United States

In this review article, we will describe the recent advances made towards understanding the molecular and cell biological mechanisms of electrical synapse formation. New evidence indicates that electrical synapses, which are gap junctions between neurons, can have complex molecular compositions including protein asymmetries across joined cells, diverse morphological arrangements, and overlooked similarities with other junctions, all of which indicate new potential roles in neurodevelopmental disease. Aquatic organisms, and in particular the vertebrate zebrafish, have proven to be excellent models for elucidating the molecular mechanisms of electrical synapse formation. Zebrafish will serve as our main exemplar throughout this review and will be compared with other model organisms. We highlight the known cell biological processes that build neuronal gap junctions and compare these with the assemblies of adherens junctions, tight junctions, non-neuronal gap junctions, and chemical synapses to explore the unknown frontiers remaining in our understanding of the critical and ubiquitous electrical synapse.

Keywords: electrical synapse, synaptogenesis, cell biology, connexin, Cx36, cytoskeleton, junction, zebrafish

OPEN ACCESS

Edited by:
Paolo De Girolamo,
University of Naples Federico II, Italy

Reviewed by:
Joachim H. R. Lübke,
Julich Research Centre, Germany
Christian Stigloher,
Julius Maximilian University of Würzburg, Germany

***Correspondence:**
E. Anne Martin
emartin4@uoregon.edu
Adam C. Miller
acmiller@uoregon.edu

†*These authors have contributed equally to this work*

Received: *31 December 2019*
Accepted: *09 March 2020*
Published: *15 April 2020*

Citation:
Martin EA, Lasseigne AM and Miller AC (2020) Understanding the Molecular and Cell Biological Mechanisms of Electrical Synapse Formation. Front. Neuroanat. 14:12. doi: 10.3389/fnana.2020.00012

INTRODUCTION

Electrical synapses are specialized connections between neurons that facilitate direct ionic and small metabolite communication (**Figure 1**). They are composed of tens to thousands of gap junction channels clustered together into plaques that are present throughout developing and adult brains. Electrical synapses contribute towards initial neural circuit function including driving the earliest animal behaviors (Rekling et al., 2000; Saint-Amant and Drapeau, 2000; Marin-Burgin et al., 2006; Su et al., 2017) and continue to function broadly throughout life in neural circuits controlling sensory processing (Li et al., 2009; Huang et al., 2010; Yaksi and Wilson, 2010; Pouille et al., 2017), rhythmic behavior in central pattern generators and motor systems (Eisen and Marder, 1982; Song et al., 2016; Traub et al., 2020), and cortical processing in mammals (Galarreta and Hestrin, 2001, 2002; Connors and Long, 2004; Gibson et al., 2005; Hestrin and Galarreta, 2005; Mancilla et al., 2007). Despite these well-documented and diverse circuit functions (reviewed in Nagy et al., 2018), the electrical synapse is commonly thought of as a necessary, but simple and temporary, precursor in development to the later-forming chemical synapse. However, emerging evidence supports an alternative view, namely that electrical and chemical synapses are essential life-long collaborators in both invertebrate and vertebrate neural circuits where they work synergistically to dynamically shape brain function (reviewed extensively in Pereda, 2014). Indeed, the best-studied electron-microscope reconstructed connectomes, of *C. elegans* and the rabbit retina, reveal that electrical synapses make up about 20% of connections in these mature circuits (White et al., 1986; Anderson et al., 2011; Jarrell et al., 2012; Cook et al., 2019).

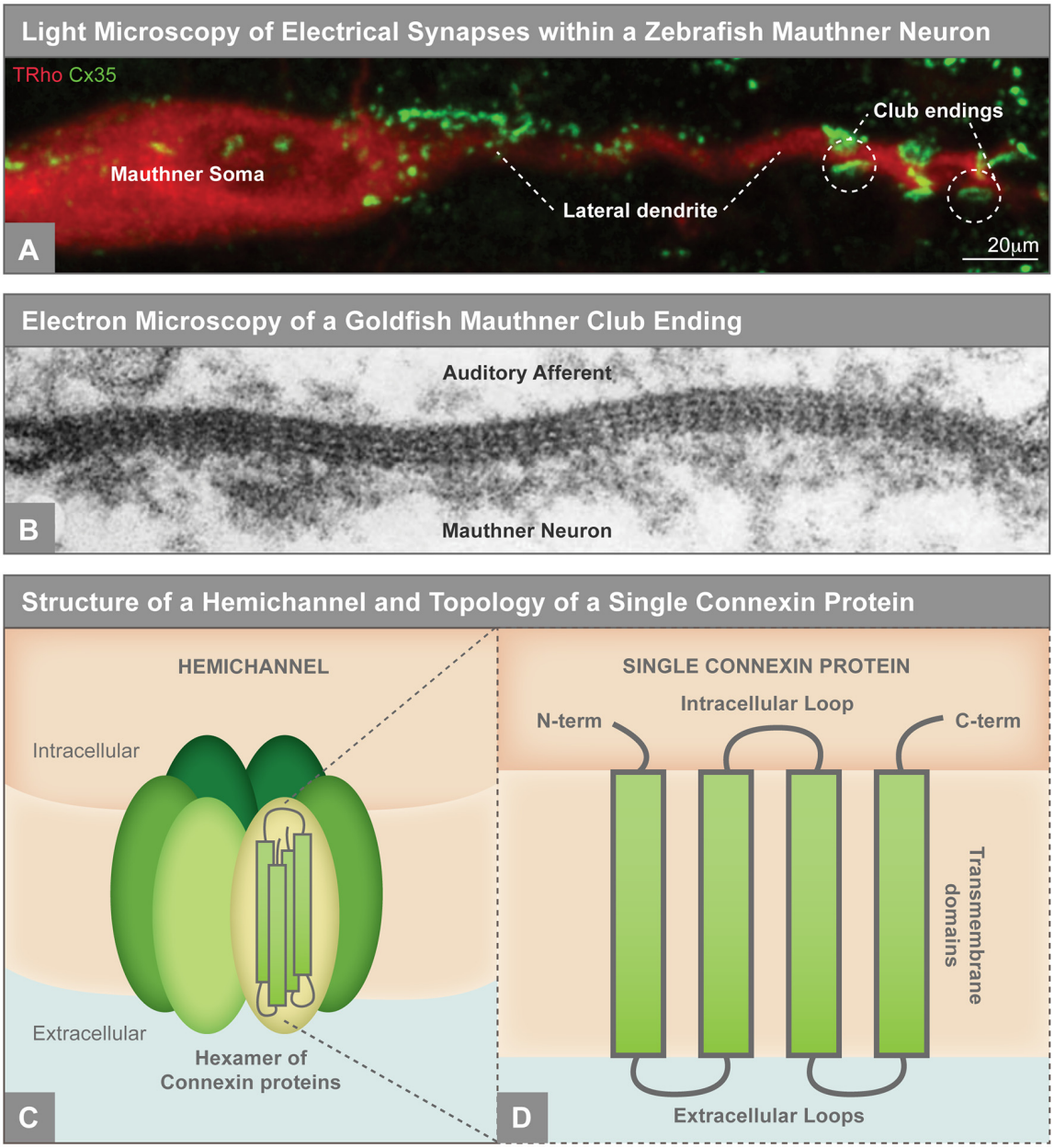


FIGURE 1 | (A) Electrical synapses visualized by light microscopy on the larval zebrafish Mauthner neuron. Mauthner, labeled with tetramethylrhodamine-dextran (TRho, red), makes electrical synapses, labeled by Connexin35 (Cx35, green), on its soma and lateral dendrite. The so-called club ending synapses represent uniquely identifiable electrical connections with auditory afferents. The Mauthner neuron has served as a key model for electrical synapse formation and function and the principles learned have applied to both invertebrate and vertebrate systems (Nagy et al., 2018). Image modified from Yao et al. (2014), reproduced with permission. **(B)** Electron microscopy showing gap junctions at the club endings between the postsynaptic Mauthner neuron and the presynaptic auditory afferents in adult goldfish. The electron density between the neurons and the characteristic intermembrane spacing are hallmarks of gap junctions. X 285,000. Republished with permission of Rockefeller University Press, from Brightman and Reese (1969); permission conveyed through Copyright Clearance Center, Inc. **(C)** Illustration of an unpaired gap junction hemichannel inserted into the plasma membrane, composed of a hexamer of Connexin proteins. **(D)** A single Connexin protein is illustrated to show protein topology.

Also, electrical synapses have emerged as complex biochemical structures, with their proteomic diversity supporting sophisticated neuronal functions including activity-dependent plasticity (reviewed in Miller and Pereda, 2017). These findings lead to exciting new ideas about the role of electrical synapses in brain development, function, and disease. However, while abundant literature has explored the mechanisms that build both non-neuronal gap junction and chemical synapse formation, the field still has only furtive glances into the cell biological mechanisms that control electrical synapse formation and

function. Given that electrical synapses are formed within the elaborate architecture of neurons and that they are optimized for fast transmission and plasticity, we expect that complex cell biological rules regulate the formation and homeostasis of these gap junction channels. Here we focus on emerging evidence that provides the first glimpse of electrical synapse cell biology *in vivo*. We apologize for the many excellent articles we were unable to cite in this review due to space constraints, but the explosion of renewed interest in these structures has generated many recent reviews that provide excellent resources to examine the many aspects of electrical synapse structure and function (Dong et al., 2018; Harris, 2018; Jabeen and Thirumalai, 2018; O'Brien and Bloomfield, 2018; Traub et al., 2018; Alcamí and Pereda, 2019; Totland et al., 2020).

The Formation of Intercellular Junctions

While the mechanisms that build an electrical synapse are not well understood, critical clues to how the process might work are likely to be found in the known mechanisms that build other junction types such as adherens junctions, tight junctions, non-neuronal gap junctions, and chemical synapses. This process of junction formation requires: (1) selecting the junction site; (2) adhering to the cellular membranes in close apposition; (3) anchoring to the cytoskeleton; and (4) coordinating protein recruitment between the two cells to form a functional junction. Every junction type must create molecular solutions to these problems, and while each junction has its unique features, they share a common foundation (**Figure 2**).

In both neuronal and non-neuronal tissues, adherens, tight, and gap junctions exist to link cells to one another. Adherens junctions essentially take on the role of molecular glue between cells (**Figure 2A**). These structures mediate cell-cell adhesion *via* the extracellular binding of cell adhesion molecules (CAMs), which include transmembrane cadherins and nectins (Troyanovsky, 2014). Intracellularly, CAMs anchor the cell membrane to actin *via* cytoskeleton-interacting linkers and scaffolding proteins such as catenins and afadin (Indra et al., 2013). By contrast, tight junctions bind cells to one another to create a seal that generates a mesh-like barrier with small pores between tissues. These junctions largely use the claudin CAM family as their transcellular connector and link to intracellular scaffolding proteins such as ZO proteins (**Figure 2B**; Zihni et al., 2016). Unlike adherens and tight junctions, gap junctions create a physical intercellular channel connecting the two cell cytoplasms and making a direct passage for ions and other small molecules to pass from cell to cell. Gap junctions are created by coupled hemichannels contributed by each cell, with each hemichannel, in vertebrates, being comprised of a hexamer of Connexin proteins (**Figures 1C**, **2C**). Invertebrates accomplish the same task by using an evolutionarily distinct class of proteins called Innexins to form gap junctions (reviewed in Phelan, 2005; Güiza et al., 2018). Much like the CAMs at adherens and tight junctions, Connexins are intracellularly connected to scaffolding and cytoskeletal linkage proteins including ZO proteins and EB1 (Li et al., 2004; Epifantseva and Shaw, 2018). Thus, while there is some molecular overlap, each junction's unique morphology and function requires specialized membrane proteins, and fundamentally each must have a form of CAM, a scaffold, and an anchor to the cytoskeleton. How does this change within a neuronal environment?

Neurons use their special intercellular junctions to support the fast communication needs of neural network function. Moreover, the cell biological demands of their complex and diverse morphology (far-reaching axons and dendrites) require a carefully orchestrated protein delivery and control system (Tahirovic and Bradke, 2009). In particular, neuronal cells have two specialized junctions to manage fast information flow: chemical and electrical synapses. Chemical synapses (**Figure 2D**) are fundamentally asymmetric structures, with the presynaptic side, the so-called active zone, specialized for fast synaptic vesicle release in response to neuronal action potentials (Südhof, 2012). Synaptic vesicle exocytosis at the active zone releases neurotransmitters into the synaptic cleft between the neurons to activate receptors on the postsynaptic cell. The postsynapse also termed the postsynaptic density, is specialized to manage the localization, organization, and function of neurotransmitter receptors to control communication (Frank and Grant, 2017). As with their non-neuronal junction counterparts, common mechanistic themes control the formation of all chemical synapses. Synaptic CAMs are thought to initiate synaptogenesis and offer trans-synaptic structural support; intracellular synaptic scaffolding molecules organize and stabilize both the pre- and postsynaptic compartments; and adaptor proteins link to the cytoskeleton to manage trafficking, anchoring, and later plasticity. Proteomic work on pre- and postsynaptic chemical synapses have revealed hundreds and thousands of proteins, respectively, in each compartment (Collins et al., 2006; Bayés and Grant, 2009; Ryan and Grant, 2009; Dieterich and Kreutz, 2016). While there is great protein diversity in these connections, each of the molecular aspects of building a chemical synapse relates to the fundamental themes of adhesion, scaffolding, and cytoskeletal anchoring, and these are critical to the structure, function, and plasticity of these connections.

While we know relatively little about the molecular mechanisms that regulate electrical synapses (**Figure 2E**), their observed functional diversity and plasticity suggests complex cell biological rules must control their formation and function, presumably using similar mechanisms as the other junction types. The notion of electrical synapse complexity is supported by several observations. First, we know that these neuronal gap junctions appear throughout the nervous system, from sensory neurons to central processing circuits to motor outputs (Galarreta and Hestrin, 2001; Connors and Long, 2004; Nagy et al., 2018). Besides, circuits build these connections in development and then refine them to form the final set of electrical synapses used in adulthood (Rash et al., 2000; Galarreta and Hestrin, 2002; Pereda, 2014). Thus, there must exist critical gene regulatory networks controlling when and where electrical synapse genes are expressed. Second, electron microscopy shows that the cell biological construction of electrical synapses is varied, and these structures can form between all neuronal compartments: there are axo-dendritic, somato-somatic,

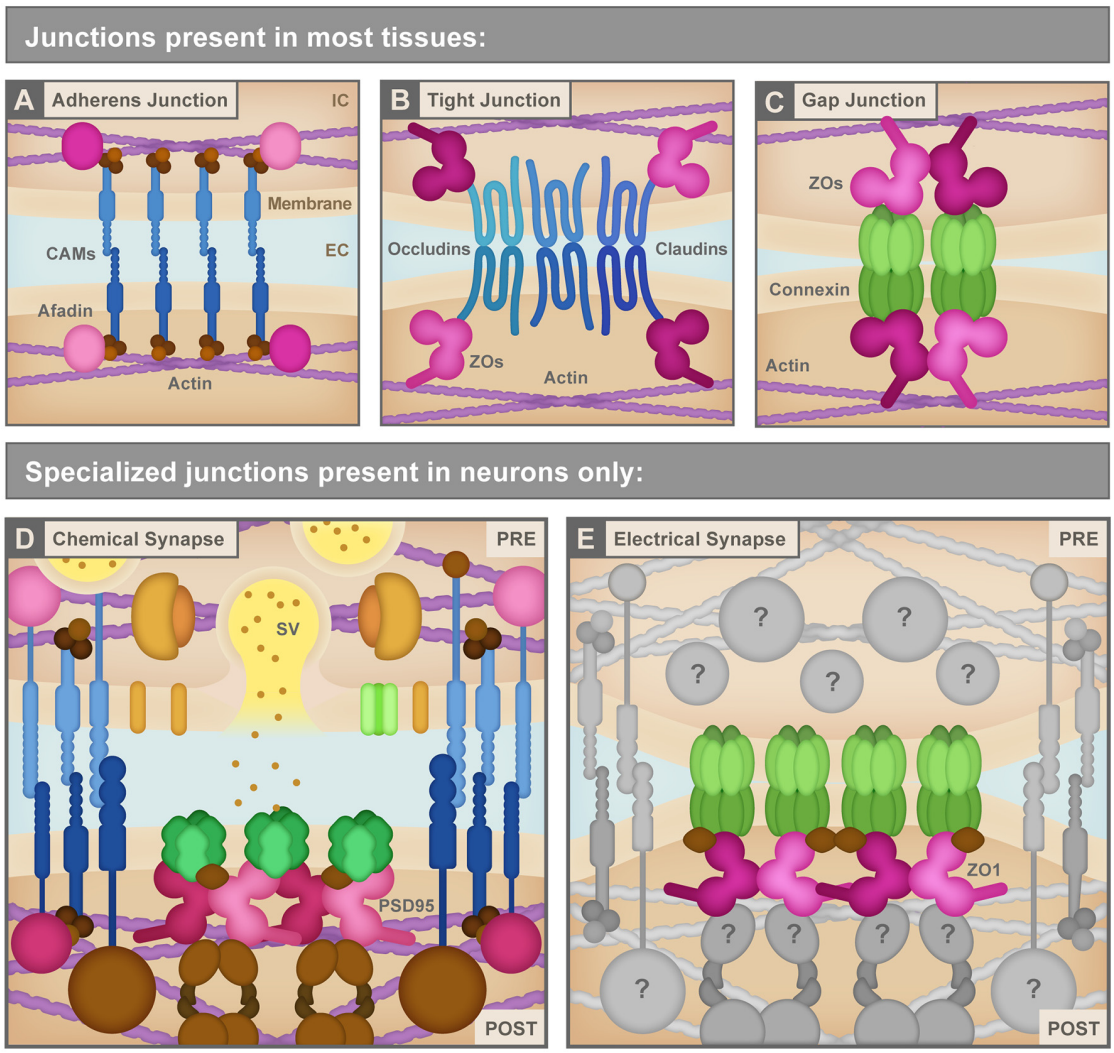


FIGURE 2 | (A) Adherens junctions are the simplest junctions consisting of cell adhesion molecules (CAMs, blue) such as cadherins and nectins, and scaffolding proteins like Afadin (pink) combined with linker proteins (brown) such as catenins that connect cellular membranes to the actin cytoskeleton (purple). IC, Intracellular; EC, Extracellular. **(B)** Tight junctions use different CAMs (blue) including claudins and occludins to bring the neighboring cell membranes tightly together. These CAMs connect to the actin cytoskeleton (purple) *via* several scaffolding molecules (pink) including ZO proteins. **(C)** Non-neuronal gap junctions use Connexin proteins arranged in hexameric hemichannels (green) to intercellularly connect cells. Connexins also use scaffolding proteins (pink) including ZO proteins to link to other signaling molecules and the actin cytoskeleton (purple). **(D)** Chemical synapses, such as the glutamatergic excitatory chemical synapse represented here, have a vast assortment of proteins composing their structure including a variety of CAMs (blue), scaffolding molecules such as PSD95 (pink), neurotransmitters and synaptic vesicles (SV) and associated proteins (yellow and orange), neurotransmitter receptors and calcium channels (green), cytoskeletal adaptor proteins and other signaling molecules (brown), etc. PRE, Presynapse; POST, Postsynapse. **(E)** Electrical synapses are neuronal gap junction channels and use Connexins (green) to directly interconnect two neurons. Electrical synapses are often thought of as molecularly symmetric, but they can have asymmetric protein localization, as depicted here. At asymmetric electrical synapses, two postsynaptic proteins, ZO1 (pink) and Ca2+/calmodulin-dependent protein kinase II (CAMKII, brown) are observed to directly interact with Connexin C-terminal tails in the postsynapse to provide scaffolding and kinase activity. Due to the cell-biological specificity of electrical synapse formation within the complexity of neuronal morphology, and given their sophisticated functions in fast interneuronal communication, we expect that a large assortment of unknown proteins (gray) exists to manage electrical synapse formation and function. See the text for details. Republished with permission of Rockefeller University Press, from Brightman and Reese (1969); permission conveyed through Copyright Clearance Center, Inc.

axo-axonic, and dendro-dendritic electrical synapses (Kosaka and Hama, 1985; Hamzei-Sichani et al., 2007; Nagy et al., 2018). These varied configurations suggest molecular specificity mechanisms to ensure electrical synapses are made in the right places and at the right times. Finally, electrical synapses are found in multiple morphological arrangements, such as in dense plaques, lacey plaques, wide ribbons, and thin strings (Nagy et al., 2018), suggesting that individual synapses are differentially regulated to achieve their unique functional needs. Here, we will explore the cell biological and molecular mechanisms which likely exist to manage each of these processes, beginning with gene expression control, then how gap junction proteins arrive

at the synapse, followed by an analysis of electrical synapse organization, then by addressing how an electrical synapse site may be specified, and finally by exploring how electrical synapses may contribute to disease. Our goal is to highlight critical areas of unexplored biology with the hope that this spurs efforts to identify the molecules and mechanisms that build, maintain, and allow for the modification of the electrical synapse.

EXPRESSION AND LOCALIZATION OF GAP JUNCTION FORMING GENES

To make electrical synapses, neurons must express genes that support gap junction formation. In chordates, gap junctions are created by Connexins while in non-chordate animals Innexins make the channels (Slivko-Koltchik et al., 2019). While chordates retain Innexin genes, called Pannexins in these genomes, these proteins only make hemichannels and do not form intercellular junctions (Abascal and Zardoya, 2013). Despite evolution devising two molecular solutions to forming gap junctions, Connexin and Innexin structure and function are strikingly conserved (Goodenough and Paul, 2009; Pereda and Macagno, 2017). All animal genomes contain large numbers of gap junction forming genes, each expressed in cell-type-specific patterns and encoding proteins that facilitate unique functions. Therefore, to understand the electrical synapses of the nervous system, it is critical to examine the molecular complexities of the gap junctions. In *C. elegans*, 17 of the 25 Innexin genes are neuronally expressed, and they display highly complex and overlapping patterns that suggest incredible electrical synapse molecular complexity (Bhattacharya et al., 2019). Analogously, vertebrate genomes encode many Connexins; for example, zebrafish contain ∼40 unique genes (Watanabe, 2017). Most Connexin genes are not expressed within neurons, such as the gene *gap junction a1 (gja1)* encoding the Connexin43 (Cx43) protein, which is expressed in non-neuronal tissue including epithelia and glia (Janssen-Bienhold et al., 1998; Güldenagel et al., 2000; Misu et al., 2016). A subset of Connexins are expressed in neurons, though each gene has a unique expression profile within the nervous system (Li et al., 2009; Rash et al., 2013; Klaassen et al., 2016; Song et al., 2016; Miller et al., 2017). For example, the *gjd2*/Cx36 family of genes are the most broadly expressed neuronal Connexins, found in neurons from the forebrain to the spinal cord within zebrafish and mouse brains (Condorelli et al., 1998; Connors and Long, 2004; Li et al., 2009; Söhl et al., 2010; Miller et al., 2017). By contrast, the mammalian *gja10*/Cx57 gene and its homologs in zebrafish are expressed exclusively in retinal horizontal cells (Söhl et al., 2010; Klaassen et al., 2016; Greb et al., 2018). Thus, while a complete accounting of vertebrate Connexin expression in the nervous system has not yet been achieved, it is clear that regulated expression contributes to the specificity of the electrical connectome.

In addition to gene regulatory mechanisms contributing to electrical synapse specificity, there are complexities as to whether two different Connexins can form a gap junction. For example, Cx43 expressed within glia cannot form gap junctions with neuronally expressed Cx36 (Rash et al., 2001; Koval et al., 2014). By contrast, many Connexin types can interact with one another, either within a hemichannel or between apposed cells. Given that many neurons express multiple Connexin proteins, there is the potential for a variety of Connexin arrangements within neuronal gap junctions (O'Brien et al., 2004; Rash et al., 2013; Palacios-Prado et al., 2014; Miller et al., 2017). These rules of engagement are certainly important for creating specific connectivity, yet we still lack the complete set of compatibility guidelines between the large family of Connexins. The spatial and temporal control of Connexin expression, coupled with the rules of Connexin engagement, provide both specificity and opportunities for complexity in the formation of electrical synapses. Future work is required to elucidate the complete molecular map of electrical synapse gene expression and protein usage in a complex vertebrate brain such as zebrafish.

While Connexin incompatibilities and expression are important for specificity, it is also clear that neurons are selective in where they form electrical synapses. An intriguing example of this is found within the mouse retina where the rod and cone photoreceptors express Cx36 and make electrical synapses with one another (Deans et al., 2002; Li et al., 2014; Asteriti et al., 2017). The photoreceptors also make chemical synapses with bipolar neurons, which themselves are coupled to other retinal neurons by Cx36-mediated electrical synapses (Deans et al., 2002; Trenholm and Awatramani, 2019). However, the photoreceptors do not make electrical synapses with bipolar neurons, despite their ability to form chemical synapses with one another and their mutual expression of Cx36. How can this be? The answer must arise from cell biological mechanisms that specify where the Connexins travel within the cell to form gap junctions. Yet we know little about the trafficking mechanisms of Connexins within neurons.

TRAFFICKING OF CONNEXINS WITHIN NEURONAL COMPARTMENTS

Most of our understanding of Connexin trafficking comes from studies of Cx43-based gap junctions (reviewed in Epifantseva and Shaw, 2018). In essence, Cx43 hemichannels are packaged into vesicles, travel along microtubules to an adherens junction situated near an established gap junction plaque, and are deposited into the membrane where they then migrate to and are incorporated into the plaque. However, in considering how electrical synapses are built, neurons offer additional trafficking challenges given their distinct cellular compartments. In most vertebrate neurons, axons are far-reaching processes that control information transmission at the presynapse, while dendrites are highly branched processes that typically stay relatively near the cell soma and manage information reception at the postsynapse. Axons and dendrites use analogous yet distinct processes to manage specific protein trafficking to their pre- and postsynaptic contact points. While chemical synapse contacts are necessarily asymmetric, electrical synapses can be either symmetric or asymmetric, and the flow of information at the electrical synapse can be bi-directional or biased (rectified; Phelan et al., 2008; Rash et al., 2013; Miller et al., 2017; Bhattacharya et al., 2019). In this review article, we will often refer to presynaptic (axonal) and postsynaptic (dendritic) electrical

synapse components, and we do so only concerning the polarized neuronal compartments in which each side of the synapse resides. Given that electrical synapses occur on dendrites, cell bodies, and axons, and that axons and dendrites use different methods to traffic proteins, the trafficking of Connexins and other electrical synapse components within neurons must be controlled to build the appropriate electrical connections.

A striking example of the molecular organization of Connexins within distinct neuronal compartments was recently revealed using the power of zebrafish genetics. In zebrafish Mauthner neurons, two Connexins, Cx34.1 and Cx35.5, both homologous to mammalian Cx36, are necessary for electrical synapse formation (Miller et al., 2017). Surprisingly, Cx34.1 is specifically required in the postsynapse while Cx35.5 is exclusively required in the presynapse, but the mechanisms guiding compartment-specific Connexin localization are unknown. This asymmetric compartmentalization of Connexins suggests that molecular rules must exist to guide specific Connexin types to particular sub-neuronal regions. Connexin proteins are four-pass transmembrane domain proteins with N- and C-termini located intracellularly (**Figure 1D**). Postsynaptic Cx34.1 and presynaptic Cx35.5 are ∼90% amino acid identical, yet they have tantalizing differences in their intracellular loops and C-terminal tails which must, in some as yet undiscovered way, support their separate requirement in dendrites and axons. If we look to the chemical synapse for clues, we find that the trafficking and stabilization of postsynaptic AMPA neurotransmitter receptor subtypes are regulated through interactions between its C-terminal domain and intracellular scaffolding proteins, which connects them to the cytoskeleton and other signaling molecules (reviewed in Anggono and Huganir, 2012). But how do neurons target Connexins to these different neuronal compartments?

To traffic along axons and dendrites, Connexins first need to be packaged into vesicles which sort them into neuronal compartments according to the proteins on the vesicle surface. Identifying the types of vesicles in which Connexins transit would help us to understand their trafficking pathway, but these vesicles are yet to be identified. The vesicles must next engage with the intrinsic neuronal polarity mechanisms that define dendrites and axons, particularly the motor proteins that direct traffic along microtubules to these specific regions. These compartmental motors are distinctly organized: guidance to the presynapse along the axon requires kinesin motor proteins, and guidance to the postsynapse along the dendrite requires tethering to both kinesins and dyneins, with short-range, synaptic delivery in each compartment guided by actin-trafficked myosin motor proteins (for a detailed analysis of axon and dendrite polarity differences see Rolls and Jegla, 2015). Both tubulin (Brown et al., 2019) and actin (Wang, 2015) are required for proper trafficking of Cx36 to the membrane. Yet we still do not know the types of motor proteins Connexins or other electrical synapse components use to direct electrical synapse protein trafficking. However, recently some clues have started to point the field in the right direction.

Connexins likely rely on adaptor proteins to regulate their transport to the synapse. In a forward genetic screen using zebrafish, the epilepsy- and autism-associated gene Neurobeachin was identified as necessary for both electrical and chemical synapse formation (Iossifov et al., 2014; Miller et al., 2015; Mulhern et al., 2018). Neurobeachin is localized on vesicles which are found at the trans side of the Golgi, along dendrites, and also at chemical postsynapses (Wang et al., 2000; Miller et al., 2015). Its localization at electrical synapses is currently unknown. Past studies show Neurobeachin regulates membrane protein trafficking of chemical synapse scaffolds including PSD95 and SAP102 which in turn control the trafficking of neurotransmitter receptors (Medrihan et al., 2009; Niesmann et al., 2011; Nair et al., 2013; Farzana et al., 2016; Gromova et al., 2018). In zebrafish Mauthner neurons, Neurobeachin loss results in the failure of Connexin and electrical synapse scaffold ZO1 localization. Intriguingly, Neurobeachin is both necessary and sufficient postsynaptically for electrical synapse formation in this circuit (Miller et al., 2015). This supports a model wherein Neurobeachin controls the polarized trafficking of electrical components within the postsynaptic dendrite, although the molecular mechanism remains unknown. It is attractive to speculate that perhaps Neurobeachin acts to define dendritically targeted vesicles carrying electrical synapse cargo and that it may bridge them to the motor proteins required for postsynaptic delivery. Future experiments are required to identify how Neurobeachin functions in the dendrite to control synapse formation. The coordination of electrical and chemical synapses through a master synapse regulator such as Neurobeachin has critical implications for understanding the etiology of neurodevelopmental disorders (further discussed at the end of this review).

Once arriving at the synapse, Connexin vesicles must undergo exocytosis to become inserted into the membrane, allowing them to find their partner hemichannels in the neighboring neuron. Chemical synapses use v-SNAREs, present on pre- and postsynaptic vesicles, to bind t-SNAREs on the neuronal membrane and fuse the vesicles at the synapse. Work in goldfish Mauthner neurons examined the effect of SNAP-25 peptides, which block the formation of SNARE-complexes, on the mixed electrical-chemical synapses of the Mauthner club endings (Flores et al., 2012). Mixed electrical-chemical synapses at single synaptic termini represent another fascinating synaptic organization, and each component appears to be separately organized (Pereda, 2014; Nagy et al., 2019). Intra-dendritic application of these SNAP-25 peptides reduced both the electrical and the glutamatergic component of synaptic transmission suggesting the SNARE complex may function in Connexin insertion at the membrane (Flores et al., 2012). If the SNARE complex functions to fuse Connexin vesicles, there must be v-SNARE proteins within Connexin vesicles. But again, the composition of Connexin-containing vesicles and its protein constituents remain unknown. Insight into the molecular control of Connexin vesicle trafficking and membrane insertion in neurons will be critical to understanding electrical synapse formation and plasticity.

Further insights into the cell biological framework of electrical synapses will require an identification of the type of vesicles that contain Connexins; the motor, adaptor, and vesicle fusion proteins required for their transport and membrane fusion; and

to determine if these features change between electrical synapse formation and plasticity. The elucidation of the cell biological pathways regulating electrical synapse protein trafficking will reveal whether they are the same or distinct from those of chemical synapses. The fact that electrical and chemical synapses have known distinct protein constituents suggests that at least some components will be unique, but the involvement of both Neurobeachin and SNAP-25 suggests some molecular overlap is also present. Besides, several trafficking conundrums remain. If Neurobeachin manages the postsynaptic trafficking of Connexins, what guides Connexin to the axon and the presynapse? And, in mammals, given that Cx36 is used within both the axon and the dendrite, how does a neuron resolve specific trafficking to these compartments? One possibility is that Connexin trafficking depends upon posttranslational modifications to the protein, such as phosphorylation (Li et al., 2009, 2013), to direct its localization. Or instead, Neurobeachin and other adaptor proteins may bind a scaffold protein which traffics with Connexin, as is observed with chemical synapse components (Tao-Cheng, 2007; Vukoja et al., 2018). Thus, cell-type-specific expression of these scaffolds and adaptors could result in different trafficking patterns and thus different cell biological construction of electrical synapses. This leads us to our next question: how do electrical synapse scaffolds control electrical synapse development?

ORGANIZING THE GROWING ELECTRICAL SYNAPSE

To fully appreciate electrical synapse cell biology, we must understand that each electrical synapse is composed of plaques of tens to thousands of gap junction channels (Flores et al., 2012; Rash et al., 2012, 2013, 2015; Yao et al., 2014). These plaques of gap junction channels can take on many different conformations such as wide or thin ribbons and large circular regions of channels, either densely collected or with lace-like holes (Nagy et al., 2018). Connexins arrive at the synapse as hemichannels that are inserted at the boundaries of existing gap junction plaques where they then find a partner hemichannel in the adjoining neuron. Over time, the channels migrate towards the center of the plaque where they are endocytosed and sent to the lysosome for degradation (Lauf et al., 2002; Flores et al., 2012; Wang et al., 2015). The half-life of Cx36 is estimated to be between 1 and 3 h *in vivo*, so to maintain the electrical synapse, Cx36 must continuously be made and trafficked to the correct location (Flores et al., 2012; Wang et al., 2015). The known organizational principles of the plaque, and the turnover demand of Connexins, requires complex and ongoing molecular machinery to ensure appropriate development and homeostasis. But what ensures that the components of the electrical synapse, including Connexins, unite at the same place over time?

The gene *tjp1* encodes the ZO1 protein, a membrane-associated guanylate kinase (MAGUK) historically known for its necessity at tight junctions (Umeda et al., 2006) and epithelial gap junctions (Singh et al., 2005; Bao et al., 2019), and first identified at electrical synapses in the mouse brain (Li et al., 2004; Penes et al., 2005). Recent work in zebrafish shows that ZO1 is required for electrical synapse formation (Marsh et al., 2017) as larval fish mutant for the ZO1 homolog *tjp1b* lack Connexin localization resulting in functional deficits at electrical synapses. This suggests Tjp1b/ZO1 is required to either recruit, traffic, or stabilize Connexins at electrical synapses. Strikingly, the broad class of MAGUK scaffold proteins are well-known for their ability to aggregate protein components at other well-studied cell-cell junctions (see **Figures 2B–E**, MAGUKs shown in pink). For example, PSD95, SAP102, and PSD93 are all postsynaptic MAGUK proteins that localize at glutamatergic chemical synapses, make up a majority of proteins in the postsynaptic density, and interact either directly or indirectly with glutamatergic neurotransmitter receptors. Simultaneous knock-down of these three scaffolds results in smaller postsynaptic densities and a substantial reduction in chemical synapse transmission (Chen et al., 2015). These findings support MAGUKs, including ZO1, as master organizers of intercellular junctions. The unique features that facilitate their shared function at different cell-cell adhesions are exhaustively reviewed elsewhere (e.g., Zhu et al., 2016; Ye et al., 2018), but we will highlight several key characteristics that inform our understanding of ZO1 at the electrical synapse.

First, MAGUK proteins contain one or more PDZ (PSD95, Dlg1, and ZO1) domains. These domains interact with short ligand sequences, called PDZ binding motifs (PBMs), usually found at the C-terminus of the interacting protein. At cell-cell junctions, MAGUK PDZ domains bring together the C-termini of transmembrane (or auxiliary) proteins to create a carefully organized hub of molecular interactions (reviewed in Lee and Zheng, 2010). Although all PDZs share a canonical structure, amino acid differences in the binding surface of the PDZ and PBM confer interaction specificity (Giallourakis et al., 2006; Liu et al., 2019). Additionally, these specific interactions can be regulated by posttranslational modifications to either the PDZ or the ligand motif. At the electrical synapse, Cx36 and its teleost homologs all contain a C-terminal SAYV motif that interacts directly with the first PDZ domain of ZO1 (Li et al., 2004; Flores et al., 2008). It has, therefore, been proposed that electrical synapse formation and function requires a ZO1-PDZ1/Cx36-PBM interaction, but this has yet to be explicitly shown *in vivo*.

Second, in addition to transmembrane proteins, MAGUKs also interact with other scaffolds, regulatory proteins, signaling proteins, the cytoskeleton, and even in some cases the plasma membrane. This array of interactions allows MAGUKs to aggregate the pieces necessary to create, maintain, and regulate a functional junction. ZO1 is found in complex with numerous proteins found at the electrical synapse including neuronal Connexins (Li et al., 2004; Flores et al., 2008), CAMKII, which is responsible for some forms of electrical synapse plasticity (Alev et al., 2008; Flores et al., 2010; Li et al., 2012), and actin (Fanning et al., 2012). Thus, ZO1 appears poised to act as the central hub for electrical synapse protein organization and to act as a direct link to the cytoskeleton, yet the details of how it achieves this molecular coordination remain unknown.

Finally, recent studies have shown that many MAGUK proteins are capable of phase separating, creating dynamic and selective non-membrane bound organelles. All MAGUKs include

a PDZ-SH3-GUK (PSG) tandem set of domains that function in regulated oligomerization (Pan et al., 2011; Rademacher et al., 2019), thus creating highly concentrated nanodomains that can aggregate various proteins to a specific site within a cell. At chemical synapses, phase separation within the presynaptic active zone clusters synaptic vesicle fusion proteins while at the postsynaptic density phase separation concentrates neurotransmitter receptors (reviewed in Chen et al., 2020). Recent work has found that ZO1 is capable of phase separation facilitated by its PSG tandem, and loss of ZO1's phase separating capabilities in mammalian cell culture and the larval fish results in a loss of aggregation near the epithelial membrane and impairments in tight junction integrity (Beutel et al., 2019; Schwayer et al., 2019). Thus, it is attractive to propose a model of electrical synapse formation led by ZO1 phase separation which provides a local, specialized domain to capture Connexins and other molecular machinery through both direct and indirect interactions. This presents an exciting new avenue for future exploration.

Our knowledge of ZO1 and other MAGUKs at cell-cell junctions suggests a model in which ZO1 is oligomerized into nanodomains at the cell membrane destined to become Connexin plaques. As Connexins are rapidly turned over throughout the life of the electrical synapse, ZO1 stabilizes them, aggregates necessary regulatory proteins such as kinases, and links the structure to the cytoskeleton. Intriguingly, ZO1 has been shown to interact with numerous neuronally expressed Connexins, in addition to Cx36, suggesting that this mechanism may be common across all electrical synapses (reviewed in Hervé et al., 2012). The emerging evidence suggests ZO1 acts as a master organizer of electrical synapses once it is recruited to the site of the future electrical synapse. This, however, leads us to the question: what tells ZO1 where the electrical synapse should be?

SPECIFYING WHEN AND WHERE ELECTRICAL SYNAPSES ARE CREATED

Although it is possible that site specification initially occurs *via* extracellularly secreted signals, we know that synaptic initiation and maintenance requires cell adhesion molecules (CAMs). These membrane-spanning proteins have extracellular domains allowing for intercellular interactions with CAMs on an opposing cell. Additionally, they have intracellular domains that interact with the cytoskeleton, scaffolds, and other proteins that can trigger signaling cascades and the recruitment of other molecules. Thus, it is highly likely that neurons use CAMs to choose the right place and the right time to create an electrical synapse.

Could the Connexin proteins act as the CAM for electrical synaptogenesis? Connexins are indeed CAMs, and, in certain circumstances such as radial migration of neurons in the mouse cortex, the adhesive properties appear to be more important than the channel itself (Elias et al., 2007). So it is tempting to question if Connexins coordinate the recruitment of ZO1 and other required proteins to the electrical synapse. The gap junction channel as director of synapse formation appears to be the case in the leech, where the diversity of gap junction forming Innexin proteins drives the site-specific formation of electrical synapses (Baker and Macagno, 2014). However, in vertebrates, which use Connexins for their gap junctions, this may not be the case. In *Cx36* mutant mice that lack many neuronal gap junctions, electron microscopic analysis of the stereotyped dendro-dendritic electrical connections between olivary neurons found recognizable intercellular junctions still formed, but they lacked the classic electron-dense, gap junction morphology (De Zeeuw et al., 2003). A similar conclusion was found using immunohistochemistry at the MesV nucleus in *Cx36* null mice, where the stereotyped electrical synapse lacked neuronal Connexin staining, yet ZO1 was still localized to the putative electrical synaptic sites (Nagy and Lynn, 2018). Taken together, these results suggest that electrical synapses are specified by mechanisms other than Connexins, yet the nature of the signal remains unknown.

So what are the CAMs that specify electrical synapse sites? Vertebrate genomes contain thousands of genes that encode CAMs (Zhong et al., 2015), making it no small feat to identify the correct molecules that initiate electrical synapse site specification. Yet particular CAMs, such as the Nectins, may be the key as they play a critical role in establishing initial cell-cell adhesions and are known for their instructive role in adherens junction and tight junction formation in epithelia. At these locations, they precede the cadherin-based or claudin-based adhesions that are recruited later to these sites. Nectins build up a macromolecular complex by interacting with Afadin, an intracellular scaffold that directly interfaces with the actin cytoskeleton and other important scaffolds, such as alpha-catenin and ZO1, required for adherens junction and tight junction formation respectively (Yamada et al., 2006; Ooshio et al., 2010). In neurons, the loss of Nectins results in altered axon targeting whereas loss of Afadin results in greatly decreased neuronal N-cadherin and β- and αN-catenin puncta along with extensive reductions in excitatory synapse density (Honda et al., 2006; Beaudoin et al., 2012). The effects on electrical synapses have not been assessed. The relationship between Nectins and Afadins is likely cell type-specific, but these results support that, much like at tight junctions, these complexes form initial adhesions that lay a foundation for cadherin recruitment to the synaptic site.

But are Nectins responsible for specifying the locations of electrical synapses? Cx36, ZO1, and Afadin, but not Nectin, colocalize at electrical synapses in the rat/mouse brain. Moreover, Cx36 co-immunoprecipitates with Afadin in both whole-brain and retinal homogenates (Li et al., 2012), most likely through direct interaction with ZO1. Adjacent to electrical synapses, Afadin is also present at adherens junctions where it colocalizes with Nectin and N-cadherin (Li et al., 2012; Nagy and Lynn, 2018). This suggests a potential model where initial Nectin/Afadin adherens junctions form between neurons before electrical, or chemical, synapse formation and they recruit in cadherins to maintain the synapse, however, this has not been explicitly tested. How specification proceeds to differentiate between these future structures to guide specific molecular complex formation or whether these are causally required for formation remains unclear.

Alternatively, electrical synapses may use different complements of CAMs in their formation and maintenance,

and to potentiate their functional plasticity. Chemical synapses use a multitude of synaptic CAMs not only to specify separate synaptic types (e.g., excitatory and inhibitory) but also to solidify and modulate synapse connections between neurons over time (Jang et al., 2017; Rawson et al., 2017). Other CAMs, such as claudins, occludins, and N-cadherin, all are found to interact with Connexins in epithelia alluding to their potential roles at the electrical synapse (reviewed in Hervé et al., 2012). However, attempting to elucidate the requirement of these CAMs *in vivo* is difficult due to the pleiotropic nature of these proteins and their use at many cellular junctions. So how can the electrical synapse CAMs be identified and studied? Zebrafish offer some advantages, particularly given the new methods in CRISPR-based reverse genetic screening (Shah et al., 2015), which provides a fast method for knocking out a large battery of potential CAMs to identify those that regulate electrical synapses. For the field, identifying the CAMs that specify the temporal and spatial electrical synapse dynamics is an essential hurdle that needs to be overcome to move forward in understanding the cell biology of the electrical synapse.

DISCUSSION AND CONCLUSION

Here we have explored several critical open questions surrounding the cell biology of the electrical synapse. Filling these gaps in knowledge will greatly impact our understanding of the development and homeostasis of electrical synapses and will provide new frontiers in regards to the etiology of neurological disorders.

Numerous human disorders are characterized by the loss of gap junction channels, and they span tissues including the skin, heart, joints, teeth, and immune system, to name just a few (Jongsma and Wilders, 2000; van Steensel, 2004; Kleopa and Scherer, 2006; Laird, 2006, 2010; Wong et al., 2017; Donahue et al., 2018). Indeed, the leading cause of deafness is due to the loss of Connexins expressed in the ear, which is currently, and extremely controversially, earmarked for a possible human CRISPR trial (Batissoco et al., 2018; Cyranoski, 2019). These pathologies seemingly emerge from the disruption of wide-ranging gap junction roles within cell proliferation and differentiation, morphogenesis, cell migration, growth control, and many other cell biological processes (McGonnell et al., 2001; Vinken et al., 2006; Kardami et al., 2007; Marins et al., 2009). If we turn our gaze to the nervous system, we find that in *Cx36* knockout mice there are brain-wide electrical synapse defects such as within the cerebellum where motor function is impaired, in the hippocampus where perturbed long-term potentiation and network oscillations impact learning and memory, in the cortex where cortical interneurons become desynchronized, and in both visual and olfactory systems which are dysfunctional (Güldenagel et al., 2001; Frisch et al., 2005; Bissiere et al., 2011; Wang and Belousov, 2011; Zolnik and Connors, 2016; Pouille et al., 2017). Similar disruptions are mirrored in zebrafish, where elimination of *Cx36* homologs results in delayed responses to threatening stimuli and motor coordination defects (Miller et al., 2017). These behavioral defects in animal models lacking a broad class of electrical synapses are exactly what the field of neurodevelopment would expect for genes linked to disease phenotypes (Mas et al., 2004; Hempelmann et al., 2006; Solouki et al., 2010; Li et al., 2015; Kunceviciene et al., 2018). Namely, that many disorders of neurodevelopment result not in large effects with gross dysfunction, but instead are comprised of subtle molecular differences that slightly shift the functional outcomes. Indeed, many so-called synaptopathies are thought to affect synapse formation and perturb excitatory/inhibitory balances (Grant, 2012). We suggest that the perspective should be broadened to the electrical/excitatory/inhibitory balance, as disruptions to any of these components lead to subsequent abnormal circuit function which develops to have larger behavioral ramifications over time. Indeed, electrical synapse disruptions are proposed to contribute to the etiology of disorders such as autism (Welsh et al., 2005) and epilepsy (Cunningham et al., 2012). However, Connexin loss is not yet a well-appreciated contributor to such disorders. We think it is likely that the growing awareness and attention electrical synapses are receiving in neural circuit formation, function, and behavior will bring to light their links to a large set of neurodevelopmental disorders.

In this review, we have made the case that Connexins are not the full story in considering the form and function of the electrical synapse. Indeed, our work on Neurobeachin, which itself is linked with both autism and epilepsy in human patients, suggests that as we begin to understand the totality of electrical synapse formation, how these structures are related to disorders of neural function will become ever more apparent. Therefore, we fundamentally need to expand our understanding of the cell biological mechanisms that develop, maintain, and regulate electrical synapses. And we need to improve our knowledge of the mechanistic relationship between electrical and chemical synapse formation to clarify the contributions of each synapse type to development and adult neural circuit function. In conclusion, we predict that the continuing studies of electrical synapse structure and function will provide a new framework for understanding fundamental mechanisms of brain structure and function as well as the etiology of the disease.

AUTHOR CONTRIBUTIONS

EM, AL, and AM discussed and wrote the review. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

Funding was provided by the NIH Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), Developmental Biology Training Grant, T32HD007348 to AL, NIH National Institute of Neurological Disorders and Stroke (NINDS) R01NS105758, NIH Office of the Director (OD) R24OD026591, and the University of Oregon to AM.

REFERENCES

Abascal, F., and Zardoya, R. (2013). Evolutionary analyses of gap junction protein families. *Biochim. Biophys. Acta* 1828, 4–14. doi: 10.1016/j.bbamem.2012.02.007

Alcamí, A. B., and Pereda, A. E. (2019). Beyond plasticity: the dynamic impact of electrical synapses on neural circuits. *Nat. Rev. Neurosci.* 20, 253–271. doi: 10.1038/s41583-019-0133-5

Alev, C., Urscheld, S., Sonntag, S., Zoidl, G., Fort, A. G., Höher, T., et al. (2008). The neuronal connexin36 interacts with and is phosphorylated by CaMKII in a way similar to CaMKII interaction with glutamate receptors. *Proc. Natl. Acad. Sci. U S A* 105, 20964–20969. doi: 10.1073/pnas.0805408105

Anderson, J. R., Jones, B. W., Watt, C. B., Shaw, M. V., Yang, J. H., DeMill, D., et al. (2011). Exploring the retinal connectome. *Mol. Vis.* 17, 355–379. doi: 10.3390/molecules17010355

Anggono, V., and Huganir, R. L. (2012). Regulation of AMPA receptor trafficking and synaptic plasticity. *Curr. Opin. Neurobiol.* 22, 461–469. doi: 10.1016/j.conb.2011.12.006

Asteriti, S., Gargini, C., and Cangiano, L. (2017). Connexin 36 expression is required for electrical coupling between mouse rods and cones. *Vis. Neurosci.* 34:E006. doi: 10.1017/s0952523817000037

Baker, M. W., and Macagno, E. R. (2014). Control of neuronal morphology and connectivity: Emerging developmental roles for gap junctional proteins. *FEBS Letters* 588, 1470–1479. doi: 10.1016/j.febslet.2014.02.010

Bao, H., Yang, S., Li, H., Yao, H., Zhang, Y., Zhang, J., et al. (2019). The interplay between E-Cadherin, connexin 43 and zona occludens 1 in retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 60, 5104–5111. doi: 10.1167/iovs.19-27768

Batissoco, A. C., Salazar-Silva, R., Oiticica, J., Bento, R. F., Mingroni-Netto, R. C., and Haddad, L. A. (2018). A cell junctional protein network associated with connexin-26. *Int. J. Mol. Sci.* 19:2535. doi: 10.3390/ijms19092535

Bayés, A., and Grant, S. G. N. (2009). Neuroproteomics: understanding the molecular organization and complexity of the brain. *Nat. Rev. Neurosci.* 10, 635–646. doi: 10.1038/nrn2701

Beaudoin, G. M. J., Schofield, C. M., Nuwal, T., Zang, K., Ullian, E. M., Huang, B., et al. (2012). Afadin, a Ras/Rap effector that controls cadherin function, promotes spine and excitatory synapse density in the hippocampus. *J. Neurosci.* 32, 99–110. doi: 10.1523/jneurosci.4565-11.2012

Beutel, O., Maraspini, R., Pombo-García, K., Martin-Lemaitre, C., and Honigmann, A. (2019). Phase separation of zonula occludens proteins drives formation of tight junctions. *Cell* 179, 923–936. doi: 10.1016/j.cell.2019.10.011

Bhattacharya, A., Aghayeva, U., Berghoff, E. G., and Hobert, O. (2019). Plasticity of the electrical connectome of *C. elegans*. *Cell* 176, 1174–1189. doi: 10.1016/j.cell.2018.12.024

Bissiere, S., Zelikowsky, M., Ponnusamy, R., Jacobs, N. S., Blair, H. T., and Fanselow, M. S. (2011). Electrical synapses control hippocampal contributions to fear learning and memory. *Science* 331, 87–91. doi: 10.3410/f.8704956.9221054

Brightman, M. W., and Reese, T. S. (1969). Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* 40, 648–677. doi: 10.1083/jcb.40.3.648

Brown, C. A., del Corsso, C., Zoidl, C., Donaldson, L. W., Spray, D. C., and Zoidl, G. (2019). Tubulin-dependent transport of Connexin-36 potentiates the size and strength of electrical synapses. *Cells* 8:1146. doi: 10.3390/cells8101146

Chen, X., Levy, J. M., Hou, A., Winters, C., Azzam, R., Sousa, A. A., et al. (2015). PSD-95 family MAGUKs are essential for anchoring AMPA and NMDA receptor complexes at the postsynaptic density. *Proc. Natl. Acad. Sci. U S A* 112, E6983–E6992. doi: 10.1073/pnas.1517045112

Chen, X., Wu, X., Wu, H., and Zhang, M. (2020). Phase separation at the synapse. *Nat. Neurosci.* 23, 301–310. doi: 10.1038/s41593-019-0579-9

Collins, M. O., Husi, H., Yu, L., Brandon, J. M., Anderson, C. N. G., Blackstock, W. P., et al. (2006). Molecular characterization and comparison of the components and multiprotein complexes in the postsynaptic proteome. *J. Neurochem.* 97, 16–23. doi: 10.1111/j.1471-4159.2005.03507.x

Condorelli, D. F., Parenti, R., Spinella, F., Salinaro, A. T., Belluardo, N., Cardile, V., et al. (1998). Cloning of a new gap junction gene (Cx36) highly expressed in mammalian brain neurons. *Eur. J. Neurosci.* 10, 1202–1208. doi: 10.1046/j.1460-9568.1998.00163.x

Connors, B. W., and Long, M. A. (2004). Electrical synapses in the mammalian brain. *Annu. Rev. Neurosci.* 27, 393–418. doi: 10.1146/annurev.neuro.26.041002.131128

Cook, S. J., Jarrell, T. A., Brittin, C. A., Wang, Y., Bloniarz, A. E., Yakovlev, M. A., et al. (2019). Whole-animal connectomes of both *Caenorhabditis elegans* sexes. *Nature* 571, 63–71. doi: 10.1038/s41586-019-1352-7

Cunningham, M. O., Roopun, A., Schofield, I. S., Whittaker, R. G., Duncan, R., Russell, A., et al. (2012). Glissandi: transient fast electrocorticographic oscillations of steadily increasing frequency, explained by temporally increasing gap junction conductance. *Epilepsia* 53, 1205–1214. doi: 10.1111/j.1528-1167.2012.03530.x

Cyranoski, D. (2019). Russian "CRISPR-baby" scientist has started editing genes in human eggs with goal of altering deaf gene. *Nature* 574, 465–466. doi: 10.1038/d41586-019-03018-0

De Zeeuw, C. I., Chorev, E., Devor, A., Manor, Y., Van Der Giessen, R. S., De Jeu, M. T., et al. (2003). Deformation of network connectivity in the inferior olive of connexin 36-deficient mice is compensated by morphological and electrophysiological changes at the single neuron level. *J. Neurosci.* 23, 4700–4711. doi: 10.1523/jneurosci.23-11-04700.2003

Deans, M. R., Volgyi, B., Goodenough, D. A., Bloomfield, S. A., and Paul, D. L. (2002). Connexin36 is essential for transmission of rod-mediated visual signals in the mammalian retina. *Neuron* 36, 703–712. doi: 10.1016/s0896-6273(02)01046-2

Dieterich, D. C., and Kreutz, M. R. (2016). Proteomics of the synapse—a quantitative approach to neuronal plasticity. *Mol. Cell. Proteomics* 15, 368–381. doi: 10.1074/mcp.r115.051482

Donahue, H. J., Qu, R. W., and Genetos, D. C. (2018). Joint diseases: from connexins to gap junctions. *Nat. Rev. Rheumatol.* 14, 42–51. doi: 10.1038/nrrheum.2017.204

Dong, A., Liu, S., and Li, Y. (2018). Gap junctions in the nervous system: probing functional connections using new imaging approaches. *Front. Cell. Neurosci.* 12:320. doi: 10.3389/fncel.2018.00320

Eisen, J. S., and Marder, E. (1982). Mechanisms underlying pattern generation in lobster stomatogastric ganglion as determined by selective inactivation of identified neurons. III. Synaptic connections of electrically coupled pyloric neurons. *J. Neurophysiol.* 48, 1392–1415. doi: 10.1152/jn.1982.48.6.1392

Elias, L. A. B., Wang, D. D., and Kriegstein, A. R. (2007). Gap junction adhesion is necessary for radial migration in the neocortex. *Nature* 448, 901–907. doi: 10.3410/f.1089485.551927

Epifantseva, I., and Shaw, R. M. (2018). Intracellular trafficking pathways of Cx43 gap junction channels. *Biochim. Biophys. Acta Biomembr.* 1860, 40–47. doi: 10.1016/j.bbamem.2017.05.018

Fanning, A. S., Van Itallie, C. M., and Anderson, J. M. (2012). Zonula occludens-1 and -2 regulate apical cell structure and the zonula adherens cytoskeleton in polarized epithelia. *Mol. Biol. Cell* 23, 577–590. doi: 10.3410/f.14039963.15500068

Farzana, F., Zalm, R., Chen, N., Li, K. W., Grant, S. G. N., Smit, A. B., et al. (2016). Neurobeachin regulates glutamate- and GABA-receptor targeting to synapses *via* distinct pathways. *Mol. Neurobiol.* 53, 2112–2123. doi: 10.1007/s12035-015-9164-8

Flores, C. E., Cachope, R., Nannapaneni, S., Ene, S., Nairn, A. C., and Pereda, A. E. (2010). Variability of distribution of Ca^{2+}/calmodulin-dependent kinase II at mixed synapses on the mauthner cell: colocalization and association with connexin 35. *J. Neurosci.* 30, 9488–9499. doi: 10.1523/jneurosci.4466-09.2010

Flores, C. E., Li, X., Bennett, M. V. L., Nagy, J. I., and Pereda, A. E. (2008). Interaction between connexin35 and zonula occludens-1 and its potential role in the regulation of electrical synapses. *Proc. Natl. Acad. Sci. U S A* 105, 12545–12550. doi: 10.1073/pnas.0804793105

Flores, C. E., Nannapaneni, S., Davidson, K. G. V., Yasumura, T., Bennett, M. V. L., Rash, J. E., et al. (2012). Trafficking of gap junction channels at a vertebrate electrical synapse *in vivo*. *Proc. Natl. Acad. Sci. U S A* 109, E573–E582. doi: 10.3410/f.13997959.15454062

Frank, R. A., and Grant, S. G. (2017). Supramolecular organization of NMDA receptors and the postsynaptic density. *Curr. Opin. Neurobiol.* 45, 139–147. doi: 10.1016/j.conb.2017.05.019

Frisch, C., Souza-Silva, M. A. D., Söhl, G., Güldenagel, M., Willecke, K., Huston, J. P., et al. (2005). Stimulus complexity dependent memory impairment and changes in motor performance after deletion of the neuronal gap junction protein connexin36 in mice. *Behav. Brain Res.* 157, 177–185. doi: 10.1016/j.bbr.2004.06.023

Galarreta, M., and Hestrin, S. (2001). Electrical synapses between GABA-releasing interneurons. *Nat. Rev. Neurosci.* 2, 425–433. doi: 10.1038/35077566

Galarreta, M., and Hestrin, S. (2002). Electrical and chemical synapses among parvalbumin fast-spiking GABAergic interneurons in adult mouse neocortex. *Proc. Natl. Acad. Sci. U S A* 99, 12438–12443. doi: 10.1073/pnas.192159599

Giallourakis, C., Cao, Z., Green, T., Wachtel, H., Xie, X., Lopez-Illasaca, M., et al. (2006). A molecular-properties-based approach to understanding PDZ domain proteins and PDZ ligands. *Genome Res.* 16, 1056–1072. doi: 10.1101/gr.5285206

Gibson, J. R., Beierlein, M., and Connors, B. W. (2005). Functional properties of electrical synapses between inhibitory interneurons of neocortical layer 4. *J. Neurophysiol.* 93, 467–480. doi: 10.1152/jn.00520.2004

Goodenough, D. A., and Paul, D. L. (2009). Gap junctions. *Cold Spring Harb. Perspect. Biol.* 1. doi: 10.1101/cshperspect.a002576

Grant, S. G. N. (2012). Synaptopathies: diseases of the synaptome. *Curr. Opin. Neurobiol.* 22, 522–529. doi: 10.1101/cshperspect.a002576

Greb, H., Klaassen, L. J., Schultz, K., Kamermans, M., Zoidl, G., Weiler, R., et al. (2018). An alternative splice variant of zebrafish Cx52.6 is expressed in retinal horizontal cells. *Neuroscience* 388, 191–202. doi: 10.1016/j.neuroscience.2018.07.024

Gromova, K. V., Muhia, M., Rothammer, N., Gee, C. E., Thies, E., Schaefer, I., et al. (2018). Neurobeachin and the kinesin KIF21B are critical for endocytic recycling of NMDA receptors and regulate social behavior. *Cell Rep.* 23, 2705–2717. doi: 10.1016/j.celrep.2018.04.112

Güiza, J., Barría, I., Sáez, J. C., and Vega, J. L. (2018). Innexins: expression, regulation, and functions. *Front. Physiol.* 9:1414. doi: 10.3389/fphys.2018.01414

Güldenagel, M., Ammermüller, J., Feigenspan, A., Teubner, B., Degen, J., Söhl, G., et al. (2001). Visual transmission deficits in mice with targeted disruption of the gap junction gene connexin36. *J. Neurosci.* 21, 6036–6044. doi: 10.1523/jneurosci.21-16-06036.2001

Güldenagel, M., Söhl, G., Plum, A., Traub, O., Teubner, B., Weiler, R., et al. (2000). Expression patterns of connexin genes in mouse retina. *J. Comp. Neurol.* 425, 193–201. doi: 10.1002/1096-9861(20000918)425:2<193::AID-CNE3>3.0.CO;2-N

Hamzei-Sichani, F., Kamasawa, N., Janssen, W. G. M., Yasumura, T., Davidson, K. G. V., Hof, P. R., et al. (2007). Gap junctions on hippocampal mossy fiber axons demonstrated by thin-section electron microscopy and freeze-fracture replica immunogold labeling. *Proc. Natl. Acad. Sci. U S A* 104, 12548–12553. doi: 10.1073/pnas.0705281104

Harris, A. L. (2018). Electrical coupling and its channels. *J. Gen. Physiol.* 150, 1606–1639. doi: 10.1085/jgp.201812203

Hempelmann, A., Heils, A., and Sander, T. (2006). Confirmatory evidence for an association of the connexin-36 gene with juvenile myoclonic epilepsy. *EEpilepsy Res.* 71, 223–228. doi: 10.1016/j.eplepsyres.2006.06.021

Hervé, J. C., Derangeon, M., Sarrouilhe, D., Giepmans, B. N. G., and Bourmeyster, N. (2012). Gap junctional channels are parts of multiprotein complexes. *Biochim. Biophys. Acta* 1818, 1844–1865. doi: 10.1016/j.bbamem.2011.12.009

Hestrin, S., and Galarreta, M. (2005). Electrical synapses define networks of neocortical GABAergic neurons. *Trends Neurosci.* 28, 304–309. doi: 10.1016/j.tins.2005.04.001

Honda, T., Sakisaka, T., Yamada, T., Kumazawa, N., Hoshino, T., Kajita, M., et al. (2006). Involvement of nectins in the formation of puncta adherentia junctions and the mossy fiber trajectory in the mouse hippocampus. *Mol. Cell. Neurosci.* 31, 315–325. doi: 10.1016/j.mcn.2005.10.002

Huang, J., Zhang, W., Qiao, W., Hu, A., and Wang, Z. (2010). Functional connectivity and selective odor responses of excitatory local interneurons in *Drosophila* antennal lobe. *Neuron* 67, 1021–1033. doi: 10.1016/j.neuron.2010.08.025

Indra, I., Hong, S., Troyanovsky, R., Kormos, B., and Troyanovsky, S. (2013). The adherens junction: a mosaic of cadherin and nectin clusters bundled by actin filaments. *J. Invest. Dermatol.* 133, 2546–2554. doi: 10.1038/jid.2013.200

Iossifov, I., O'Roak, B. J., Sanders, S. J., Ronemus, M., Krumm, N., Levy, D., et al. (2014). The contribution of *de novo* coding mutations to autism spectrum disorder. *Nature* 515, 216–221. doi: 10.1038/nature13908

Jabeen, S., and Thirumalai, V. (2018). The interplay between electrical and chemical synaptogenesis. *J. Neurophysiol.* 120, 1914–1922. doi: 10.1152/jn.00398.2018

Jang, S., Lee, H., and Kim, E. (2017). Synaptic adhesion molecules and excitatory synaptic transmission. *Curr. Opin. Neurobiol.* 45, 45–50. doi: 10.1016/j.conb.2017.03.005

Janssen-Bienhold, U., Dermietzel, R., and Weiler, R. (1998). Distribution of Connexin43 immunoreactivity in the retinas of different vertebrates. *J. Comp. Neurol.* 396, 310–321. doi: 10.1002/(sici)1096-9861(19980706)396:3<310::aid-cne3>3.0.co;2-5

Jarrell, T. A., Wang, Y., Bloniarz, A. E., Brittin, C. A., Xu, M., Thomson, J. N., et al. (2012). The connectome of a decision-making neural network. *Science* 337, 437–444. doi: 10.1126/science.1221762

Jongsma, H. J., and Wilders, R. (2000). Gap junctions in cardiovascular disease. *Circ. Res.* 86, 1193–1197. doi: 10.1161/01.res.86.12.1193

Kardami, E., Dang, X., Iacobas, D. A., Nickel, B. E., Jeyaraman, M., Srisakuldee, W., et al. (2007). The role of connexins in controlling cell growth and gene expression. *Prog. Biophys. Mol. Biol.* 94, 245–264. doi: 10.1016/j.pbiomolbio.2007.03.009

Klaassen, L. J., De Graaff, W., Van Asselt, J. B., Klooster, J., and Kamermans, M. (2016). Specific connectivity between photoreceptors and horizontal cells in the zebrafish retina. *J. Neurophysiol.* 116, 2799–2814. doi: 10.1152/jn.00449.2016

Kleopa, K. A., and Scherer, S. S. (2006). Molecular genetics of X-linked charcot-marie-tooth disease. *Neuromolecular Med.* 8, 107–122. doi: 10.1385/nmm:8:1-2:107

Kosaka, T., and Hama, K. (1985). Gap junctions between non-pyramidal cell dendrites in the rat hippocampus (CA1 and CA3 regions): a combined Golgi-electron microscopy study. *J. Comp. Neurol.* 231, 150–161. doi: 10.1002/cne.902310203

Koval, M., Molina, S. A., and Burt, J. M. (2014). Mix and match: investigating heteromeric and heterotypic gap junction channels in model systems and native tissues. *FEBS Letters* 588, 1193–1204. doi: 10.3410/f.718285864.793491698

Kunceviciene, E., Sriubiene, M., Liutkeviciene, R., Miceikiene, I. T., and Smalinskiene, A. (2018). Heritability of myopia and its relation with GDJ2 and RASGRF1 genes in Lithuania. *BMC Ophthalmol.* 18:124. doi: 10.1186/s12886-018-0787-1

Laird, D. W. (2006). Life cycle of connexins in health and disease. *Biochem. J.* 394, 527–543. doi: 10.1042/bj20051922

Laird, D. W. (2010). The gap junction proteome and its relationship to disease. *Trends Cell Biol.* 20, 92–101. doi: 10.1016/j.tcb.2009.11.001

Lauf, U., Giepmans, B. N. G., Lopez, P., Braconnot, S., Chen, S. C., and Falk, M. M. (2002). Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. *Proc. Natl. Acad. Sci. U S A* 99, 10446–10451. doi: 10.1073/pnas.162055899

Lee, H. J., and Zheng, J. J. (2010). PDZ domains and their binding partners: structure, specificity and modification. *Cell Communication and Signaling* 8:8. doi: 10.1186/1478-811x-8-8

Li, H., Chuang, A. Z., and O'Brien, J. (2009). Photoreceptor coupling is controlled by connexin 35 phosphorylation in zebrafish retina. *J. Neurosci.* 29, 15178–15186. doi: 10.1523/jneurosci.3517-09.2009

Li, H., Chuang, A. Z., and O'brien, J. (2014). Regulation of photoreceptor gap junction phosphorylation by adenosine in zebrafish retina. *Vis. Neurosci.* 31, 237–243. doi: 10.1017/s095252381300062x

Li, H., Zhang, Z., Blackburn, M. R., Wang, S. W., Ribelayga, C. P., and O'Brien, J. (2013). Adenosine and dopamine receptors coregulate photoreceptor coupling *via* gap junction phosphorylation in mouse retina. *J. Neurosci.* 33, 3135–3150. doi: 10.1523/jneurosci.2807-12.2013

Li, J., Jiang, D., Xiao, X., Li, S., Jia, X., Sun, W., et al. (2015). Evaluation of 12 myopia-associated genes in Chinese patients with high myopia. *Invest. Ophthalmol. Vis. Sci.* 56, 722–729. doi: 10.1167/iovs.14-14880

Li, X., Lynn, B. D., and Nagy, J. I. (2012). The effector and scaffolding proteins AF6 and MUPP1 interact with connexin36 and localize at gap junctions that form electrical synapses in rodent brain. *Eur. J. Neurosci.* 35, 166–181. doi: 10.1111/j.1460-9568.2011.07947.x

Li, X., Olson, C., Lu, S., Kamasawa, N., Yasumura, T., Rash, J. E., et al. (2004). Neuronal connexin36 association with zonula occludens-1 protein (ZO-1) in mouse brain and interaction with the first PDZ domain of ZO-1. *Eur. J. Neurosci.* 19, 2132–2146. doi: 10.1111/j.0953-816x.2004.03283.x

Liu, X., Golden, L. C., Lopez, J. A., Shepherd, T. R., Yu, L., and Fuentes, E. J. (2019). Conformational dynamics and cooperativity drive the specificity of a protein-ligand interaction. *Biophys. J.* 116, 2314–2330. doi: 10.1016/j.bpj.2019.05.008

Mancilla, J. G., Lewis, T. J., Pinto, D. J., Rinzel, J., and Connors, B. W. (2007). Synchronization of electrically coupled pairs of inhibitory interneurons in neocortex. *J. Neurosci.* 27, 2058–2073. doi: 10.1523/jneurosci.2715-06.2007

Marin-Burgin, A., Eisenhart, F. J., Kristan, W. B., and French, K. A. (2006). Embryonic electrical connections appear to prefigure a behavioral circuit in the leech CNS. *J. Comp. Physiol. A* 192, 123–133. doi: 10.1007/s00359-005-0055-8

Marins, M., Xavier, A. L. R., Viana, N. B., Fortes, F. S. A., Fróes, M. M., and Menezes, J. R. L. (2009). Gap junctions are involved in cell migration in the early postnatal subventricular zone. *Dev. Neurobiol.* 69, 715–730. doi: 10.1002/dneu.20737

Marsh, A. J., Michel, J. C., Adke, A. P., Heckman, E. L., and Miller, A. C. (2017). Asymmetry of an intracellular scaffold at vertebrate electrical synapses. *Curr. Biol.* 27, 3561–3567. doi: 10.1016/j.cub.2017.10.011

Mas, C., Taske, N., Deutsch, S., Guipponi, M., Thomas, P., Covanis, A., et al. (2004). Association of the connexin36 gene with juvenile myoclonic epilepsy. *J. Med. Genet.* 41:e93. doi: 10.1136/jmg.2003.017954

McGonnell, I. M., Green, C. R., Tickle, C., and Becker, D. L. (2001). Connexin43 gap junction protein plays an essential role in morphogenesis of the embryonic chick face. *Dev. Dyn.* 222, 420–438. doi: 10.1002/dvdy.1208

Medrihan, L., Rohlmann, A., Fairless, R., Andrae, J., Döring, M., Missler, M., et al. (2009). Neurobeachin, a protein implicated in membrane protein traffic and autism, is required for the formation and functioning of central synapses. *J. Physiol. Lond.* 587, 5095–5106. doi: 10.1113/jphysiol.2009.178236

Miller, A. C., and Pereda, A. E. (2017). The electrical synapse: molecular complexities at the gap and beyond. *Dev. Neurobiol.* 77, 562–574. doi: 10.1002/dneu.22484

Miller, A. C., Voelker, L. H., Shah, A. N., and Moens, C. B. (2015). Neurobeachin is required postsynaptically for electrical and chemical synapse formation. *Curr. Biol.* 25, 16–28. doi: 10.1016/j.cub.2014.10.071

Miller, A. C., Whitebirch, A. C., Shah, A. N., Marsden, K. C., Granato, M., O'Brien, J., et al. (2017). A genetic basis for molecular asymmetry at vertebrate electrical synapses. *ELife* 6:e25364. doi: 10.7554/elife.25364.024

Misu, A., Yamanaka, H., Aramaki, T., Kondo, S., Skerrett, I. M., Iovine, M. K., et al. (2016). Two different functions of Connexin43 confer two different bone phenotypes in zebrafish. *J. Biol. Chem.* 291, 12601–12611. doi: 10.1074/jbc.m116.720110

Mulhern, M. S., Stumpel, C., Stong, N., Brunner, H. G., Bier, L., Lippa, N., et al. (2018). NBEA: developmental disease gene with early generalized epilepsy phenotypes. *Ann. Neurol.* 84, 788–795. doi: 10.1002/ana.25350

Nagy, J. I., and Lynn, B. D. (2018). Structural and intermolecular associations between Connexin36 and protein components of the adherens junction-neuronal gap junction complex. *Neuroscience* 384, 241–261. doi: 10.1016/j.neuroscience.2018.05.026

Nagy, J. I., Pereda, A. E., and Rash, J. E. (2018). Electrical synapses in mammalian CNS: past eras, present focus and future directions. *Biochim. Biophys. Acta Biomembr.* 1860, 102–123. doi: 10.1016/j.bbamem.2017.05.019

Nagy, J. I., Pereda, A. E., and Rash, J. E. (2019). On the occurrence and enigmatic functions of mixed (chemical plus electrical) synapses in the mammalian CNS. *Neurosci. Lett.* 695, 53–64. doi: 10.1016/j.neulet.2017.09.021

Nair, R., Lauks, J., Jung, S. Y., Cooke, N. E., de Wit, H., Brose, N., et al. (2013). Neurobeachin regulates neurotransmitter receptor trafficking to synapses. *J. Cell. Biol.* 200, 61–80. doi: 10.1085/jgp1411oia2

Niesmann, K., Breuer, D., Brockhaus, J., Born, G., Wolff, I., Reissner, C., et al. (2011). Dendritic spine formation and synaptic function require neurobeachin. *Nat. Commun.* 2:557. doi: 10.1038/ncomms1565

O'Brien, J., and Bloomfield, S. A. (2018). Plasticity of retinal gap junctions: roles in synaptic physiology and disease. *Annu. Rev. Vis. Sci.* 4, 79–100. doi: 10.1146/annurev-vision-091517-034133

O'Brien, J., Nguyen, H. B., and Mills, S. L. (2004). Cone photoreceptors in bass retina use two connexins to mediate electrical coupling. *J. Neurosci.* 24, 5632–5642. doi: 10.1523/jneurosci.1248-04.2004

Ooshio, T., Kobayashi, R., Ikeda, W., Miyata, M., Fukumoto, Y., Matsuzawa, N., et al. (2010). Involvement of the interaction of afadin with ZO-1 in the formation of tight junctions in Madin-Darby canine kidney cells. *J. Biol. Chem.* 285, 5003–5012. doi: 10.1074/jbc.m109.043760

Palacios-Prado, N., Huetteroth, W., and Pereda, A. E. (2014). Hemichannel composition and electrical synaptic transmission: molecular diversity and its implications for electrical rectification. *Front. Cell. Neurosci.* 8:324. doi: 10.3389/fncel.2014.00324

Pan, L., Chen, J., Yu, J., Yu, H., and Zhang, M. (2011). The structure of the PDZ3-SH3-GuK tandem of ZO-1 protein suggests a supramodular organization of the membrane-associated guanylate kinase (MAGUK) family scaffold protein core. *J. Biol. Chem.* 286, 40069–40074. doi: 10.1074/jbc.c111.293084

Penes, M. C., Li, X., and Nagy, J. I. (2005). Expression of zonula occludens-1 (ZO-1) and the transcription factor ZO-1-associated nucleic acid-binding protein (ZONAB)-MsY3 in glial cells and colocalization at oligodendrocyte and astrocyte gap junctions in mouse brain. *Eur. J. Neurosci.* 22, 404–418. doi: 10.1111/j.1460-9568.2005.04225.x

Pereda, A. E. (2014). Electrical synapses and their functional interactions with chemical synapses. *Nat. Rev. Neurosci.* 15, 250–263. doi: 10.1038/nrn3708

Pereda, A. E., and Macagno, E. (2017). Electrical transmission: two structures, same functions? *Dev. Neurobiol.* 77, 517–521. doi: 10.1002/dneu.22488

Phelan, P. (2005). Innexins: members of an evolutionarily conserved family of gap-junction proteins. *Biochim. Biophys. Acta* 1711, 225–245. doi: 10.1016/j.bbamem.2004.10.004

Phelan, P., Goulding, L. A., Tam, J. L. Y., Allen, M. J., Dawber, R. J., Davies, J. A., et al. (2008). Molecular mechanism of rectification at identified electrical synapses in the *Drosophila* giant fiber system. *Curr. Biol.* 18, 1955–1960. doi: 10.1016/j.cub.2008.10.067

Pouille, F., McTavish, T. S., Hunter, L. E., Restrepo, D., and Schoppa, N. E. (2017). Intraglomerular gap junctions enhance interglomerular synchrony in a sparsely connected olfactory bulb network. *J. Physiol.* 595, 5965–5986. doi: 10.1113/jp274408

Rademacher, N., Kuropka, B., Kunde, S. A., Wahl, M. C., Freund, C., and Shoichet, S. A. (2019). Intramolecular domain dynamics regulate synaptic MAGUK protein interactions. *Elife* 8. doi: 10.7554/elife.41299.018

Rash, J. E., Kamasawa, N., Vanderpool, K. G., Yasumura, T., O'Brien, J., Nannapaneni, S., et al. (2015). Heterotypic gap junctions at glutamatergic mixed synapses are abundant in goldfish brain. *Neuroscience* 285, 166–193. doi: 10.1016/j.neuroscience.2014.10.057

Rash, J. E., Staines, W. A., Yasumura, T., Patel, D., Furman, C. S., Stelmack, G. L., et al. (2000). Immunogold evidence that neuronal gap junctions in adult rat brain and spinal cord contain connexin-36 but not connexin-32 or connexin-43. *Proc. Natl. Acad. Sci. U S A* 97, 7573–7578. doi: 10.1073/pnas.97.13.7573

Rash, J. E., Curti, S., Vanderpool, K. G., Kamasawa, N., Nannapaneni, S., Palacios-Prado, N., et al. (2013). Molecular and functional asymmetry at a vertebrate electrical synapse. *Neuron* 79, 957–969. doi: 10.1016/j.neuron.2013.06.037

Rash, J. E., Kamasawa, N., Davidson, K. G. V., Yasumura, T., Pereda, A. E., and Nagy, J. I. (2012). Connexin composition in apposed gap junction hemiplaques revealed by matched double-replica freeze-fracture replica immunogold labeling. *J. Membr. Biol.* 245, 333–344. doi: 10.1007/s00232-012-9454-2

Rash, J. E., Yasumura, T., Dudek, F. E., and Nagy, J. I. (2001). Cell-specific expression of connexins and evidence of restricted gap junctional coupling between glial cells and between neurons. *J. Neurosci.* 21, 1983–2000. doi: 10.1523/jneurosci.21-06-01983.2001

Rawson, R. L., Martin, E. A., and Williams, M. E. (2017). Mechanisms of input and output synaptic specificity: finding partners, building synapses, and fine-tuning communication. *Curr. Opin. Neurobiol.* 45, 39–44. doi: 10.1016/j.conb.2017.03.006

Rekling, J. C., Shao, X. M., and Feldman, J. L. (2000). Electrical coupling and excitatory synaptic transmission between rhythmogenic respiratory neurons in the prebötzinger complex. *J. Neurosci.* 20, 20–23. doi: 10.1523/jneurosci.20-23-j0003.2000

Rolls, M. M., and Jegla, T. J. (2015). Neuronal polarity: an evolutionary perspective. *J. Exp. Biol.* 218, 572–580. doi: 10.1242/jeb.112359

Ryan, T. J., and Grant, S. G. N. (2009). The origin and evolution of synapses. *Nat. Rev. Neurosci.* 10, 701–712. doi: 10.1038/nrn2717

Saint-Amant, L., and Drapeau, P. (2000). Motoneuron activity patterns related to the earliest behavior of the zebrafish embryo. *J. Neurosci.* 20, 3964–3972. doi: 10.1523/jneurosci.20-11-03964.2000

Schwayer, C., Shamipour, S., Pranjic-Ferscha, K., Schauer, A., Balda, M., Tada, M., et al. (2019). Mechanosensation of tight junctions depends on ZO-1 phase separation and flow. *Cell* 179, 937–952.e18. doi: 10.1016/j.cell.2019.10.006

Shah, A. N., Davey, C. F., Whitebirch, A. C., Miller, A. C., and Moens, C. B. (2015). Rapid reverse genetic screening using CRISPR in zebrafish. *Nat. Methods* 12, 535–540. doi: 10.1038/nmeth.3360

Singh, D., Solan, J. L., Taffet, S. M., Javier, R., and Lampe, P. D. (2005). Connexin 43 interacts with zona occludens-1 and -2 proteins in a cell cycle stage-specific manner. *J. Biol. Chem.* 280, 30416–30421. doi: 10.1074/jbc.m506799200

Slivko-Koltchik, G. A., Kuznetsov, V. P., and Panchin, Y. V. (2019). Are there gap junctions without connexins or pannexins? *BMC Evol. Biol.* 19:46. doi: 10.1186/s12862-019-1369-4

Söhl, G., Joussen, A., Kociok, N., and Willecke, K. (2010). Expression of connexin genes in the human retina. *BMC Ophthalmol.* 10:27. doi: 10.1186/1471-2415-10-27

Solouki, A. M., Verhoeven, V. J. M., Van Duijn, C. M., Verkerk, A. J. M. H., Ikram, M. K., Hysi, P. G., et al. (2010). A genome-wide association study identifies a susceptibility locus for refractive errors and myopia at 15q14. *Nat. Genet.* 42, 897–901. doi: 10.1038/ng.663

Song, J., Ampatzis, K., Björnfors, E. R., and El Manira, A. (2016). Motor neurons control locomotor circuit function retrogradely *via* gap junctions. *Nature* 529, 399–402. doi: 10.1038/nature16497

Su, X., Chen, J.-J., Liu, L.-Y., Huang, Q., Zhang, L.-Z., Li, X.-Y., et al. (2017). Neonatal *CX26* removal impairs neocortical development and leads to elevated anxiety. *Proceedings of the National Academy of Sciences* 114, 3228–3233. doi: 10.1073/pnas.1613237114

Südhof, T. C. (2012). The presynaptic active zone. *Neuron* 75, 11–25. doi: 10.1016/j.neuron.2012.06.012

Tahirovic, S., and Bradke, F. (2009). Neuronal polarity. *Cold Spring Harb. Perspect. Biol.* 1:a001644. doi: 10.1101/cshperspect.a001644

Tao-Cheng, J. H. (2007). Ultrastructural localization of active zone and synaptic vesicle proteins in a preassembled multi-vesicle transport aggregate. *Neuroscience* 150, 575–584. doi: 10.1016/j.neuroscience.2007.09.031

Totland, M. Z., Rasmussen, N. L., Knudsen, L. M., and Leithe, E. (2020). Regulation of gap junction intercellular communication by connexin ubiquitination: physiological and pathophysiological implications. *Cell. Mol. Life Sci.* 77, 573–591. doi: 10.1007/s00018-019-03285-0

Traub, R. D., Whittington, M. A., Gutiérrez, R., and Draguhn, A. (2018). Electrical coupling between hippocampal neurons: contrasting roles of principal cell gap junctions and interneuron gap junctions. *Cell Tissue Res.* 373, 671–691. doi: 10.1007/s00441-018-2881-3

Traub, R. D., Whittington, M. A., Maier, N., Schmitz, D., and Nagy, J. I. (2020). Could electrical coupling contribute to the formation of cell assemblies? *Rev. Neurosci.* 31, 121–141. doi: 10.1515/revneuro-2019-0059

Trenholm, S., and Awatramani, G. B. (2019). "Myriad roles for gap junctions in retinal circuits," in *Webvision: The Organization of the Retina and Visual System*, eds H. Kolb, E. Fernandez and R. Nelson (Salt Lake City, UT: University of Utah Health Sciences Center). doi: 10.1007/3-540-28621-7_10

Troyanovsky, S. (2014). Adherens junction assembly. *Subcell. Biochem.* 60, 89–108. doi: 10.1007/978-94-007-4186-7_5

Umeda, K., Ikenouchi, J., Katahira-Tayama, S., Furuse, K., Sasaki, H., Nakayama, M., et al. (2006). ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* 126, 741–754. doi: 10.1016/j.cell.2006.06.043

van Steensel, M. A. M. (2004). Gap junction diseases of the skin. *Am. J. Med. Genet. C Semin. Med. Genet.*, 131, 12–19. doi: 10.1002/ajmg.c.30030

Vinken, M., Vanhaecke, T., Papeleu, P., Snykers, S., Henkens, T., and Rogiers, V. (2006). Connexins and their channels in cell growth and cell death. *Cell. Signal.* 18, 592–600. doi: 10.1016/j.cellsig.2005.08.012

Vukoja, A., Rey, U., Petzoldt, A. G., Ott, C., Vollweiter, D., Quentin, C., et al. (2018). Presynaptic biogenesis requires axonal transport of lysosome-related vesicles. *Neuron* 99, 1216–1232.e7. doi: 10.1016/j.neuron.2018.08.004

Wang, H. Y., Lin, Y. P., Mitchell, C. K., Ram, S., and O'Brien, J. (2015). Two-color fluorescent analysis of connexin 36 turnover: relationship to functional plasticity. *J. Cell Sci.* 128, 3888–3897. doi: 10.1242/jcs.162586

Wang, X., Herberg, F. W., Laue, M. M., Wullner, C., Hu, B., Petrasch-Parwez, E., et al. (2000). Neurobeachin: a protein kinase A-anchoring, beige/Chediak-Higashi protein homolog implicated in neuronal membrane traffic. *J. Neurosci.* 20, 8551–8565. doi: 10.1523/jneurosci.20-23-08551.2000

Wang, Y. (2015). Two-Color Fluorescent Analysis of Connexin 36 Turnover and Trafficking—Relationship to Functional Plasticity. Ph.D. thesis, The University of Texas Graduate School of Biomedical Sciences at Houston. Available online at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations/589/.

Wang, Y., and Belousov, A. B. (2011). Deletion of neuronal gap junction protein connexin 36 impairs hippocampal LTP. *Neurosci. Lett.* 502, 30–32. doi: 10.1016/j.neulet.2011.07.018

Watanabe, M. (2017). Gap junction in the teleost fish lineage: duplicated connexins may contribute to skin pattern formation and body shape determination. *Front. Cell Dev. Biol.* 5. doi: 10.3389/fcell.2017.00013

Welsh, J. P., Ahn, E. S., and Placantonakis, D. G. (2005). Is autism due to brain desynchronization? *Int.J. Dev. Neurosci.* 23, 253–263. doi: 10.1016/j.ijdevneu.2004.09.002

White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*314, 1–340. doi: 10.1098/rstb.1986.0056

Wong, P., Laxton, V., Srivastava, S., Chan, W. W. F., and Tse, G. (2017). The role of gap junctions in inflammatory and neoplastic disorders (Review). *Int. J. Mol. Med.* 39, 498–506. doi: 10.3892/ijmm.2017.2859

Yaksi, E., and Wilson, R. I. (2010). Electrical coupling between olfactory glomeruli. *Neuron* 67, 1034–1047. doi: 10.1016/j.neuron.2010.11.012

Yamada, A., Fujita, N., Sato, T., Okamoto, R., Ooshio, T., Hirota, T., et al. (2006). Requirement of nectin, but not cadherin, for formation of claudin-based tight junctions in annexin II-knockdown MDCK cells. *Oncogene* 25, 5085–5102. doi: 10.1038/sj.onc.1209525

Yao, C., Vanderpool, K. G., Delfiner, M., Eddy, V., Lucaci, A. G., Soto-Riveros, C., et al. (2014). Electrical synaptic transmission in developing zebrafish: properties and molecular composition of gap junctions at a central auditory synapse. *J. Neurophysiol.* 112, 2102–2113. doi: 10.1152/jn.00397.2014

Ye, F., Zeng, M., and Zhang, M. (2018). Mechanisms of MAGUK-mediated cellular junctional complex organization. *Curr. Opin. Struct. Biol.* 48, 6–15. doi: 10.1016/j.sbi.2017.08.006

Zhong, X., Drgonova, J., Li, C. Y., and Uhl, G. R. (2015). Human cell adhesion molecules: annotated functional subtypes and overrepresentation of addiction-associated genes. *Ann. N. Y. Acad. Sci.* 1349, 83–95. doi: 10.1111/nyas.12776

Zhu, J., Shang, Y., and Zhang, M. (2016). Mechanistic basis of MAGUK-organized complexes in synaptic development and signalling. *Nat. Rev. Neurosci.* 17, 209–223. doi: 10.1038/nrn.2016.18

Zihni, C., Mills, C., Matter, K., and Balda, M. S. (2016). Tight junctions: from simple barriers to multifunctional molecular gates. *Nat. Rev. Mol. Cell Biol.* 17, 564–580. doi: 10.1038/nrm.2016.80

Zolnik, T. A., and Connors, B. W. (2016). Electrical synapses and the development of inhibitory circuits in the thalamus. *J. Physiol.* 594, 2579–2592. doi: 10.1113/jp271880

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroanatomy

ORIGINAL RESEARCH
published: 21 April 2020
doi: 10.3389/fnana.2020.00015

Calsequestrins New Calcium Store Markers of Adult Zebrafish Cerebellum and Optic Tectum

*Sandra Furlan[1], Marina Campione[1], Marta Murgia[2,3], Simone Mosole[4], Francesco Argenton[5], Pompeo Volpe[2] and Alessandra Nori[2]**

[1]*Consiglio Nazionale delle Ricerche, Institute of Neuroscience, Padova, Italy,* [2]*Department of Biomedical Sciences, University of Padova, Istituto Interuniversitario di Miologia, Padova, Italy,* [3]*Department of Proteomics and Signal Transduction, Max-Planck-Institute of Biochemistry, Martinsried, Germany,* [4]*Institute of Oncology Research (IOR), Bellinzona, Switzerland,* [5]*Department of Biology, University of Padova, Padova, Italy*

Calcium stores in neurons are heterogeneous in compartmentalization and molecular composition. *Danio rerio* (zebrafish) is an animal model with a simply folded cerebellum similar in cellular organization to that of mammals. The aim of the study was to identify new endoplasmic reticulum (ER) calcium store markers in zebrafish adult brain with emphasis on cerebellum and optic tectum. By quantitative polymerase chain reaction, we found three RNA transcripts coding for the intra-ER calcium binding protein calsequestrin: *casq1a*, *casq1b*, and *casq2*. In brain homogenates, two isoforms were detected by mass spectrometry and western blotting. Fractionation experiments of whole brain revealed that Casq1a and Casq2 were enriched in a heavy fraction containing ER microsomes and synaptic membranes. By *in situ* hybridization, we found the heterogeneous expression of *casq1a* and *casq2* mRNA to be compatible with the cellular localization of calsequestrins investigated by immunofluorescence. Casq1 was expressed in neurogenic differentiation 1 expressing the granule cells of the cerebellum and the periventricular zone of the optic tectum. Casq2 was concentrated in parvalbumin expressing Purkinje cells. At a subcellular level, Casq1 was restricted to granular cell bodies, and Casq2 was localized in cell bodies, dendrites, and axons. Data are discussed in relation to the differential cellular and subcellular distribution of other cerebellum calcium store markers and are evaluated with respect to the putative relevance of calsequestrins in the neuron-specific functional activity.

Keywords: granule cell, Purkinje cell, calcium stores, Zebrafish, Ca-binding protein

OPEN ACCESS

Edited by:
Paolo De Girolamo, University of Naples Federico II, Italy

Reviewed by:
Naresh Chandra Bal, KIIT University, India
Ernesto Mollo, Italian National Research Council, Italy

****Correspondence:***
Alessandra Nori
alessandra.nori@unipd.it

Received: *31 October 2019*
Accepted: *18 March 2020*
Published: *21 April 2020*

Citation:
Furlan S, Campione M, Murgia M, Mosole S, Argenton F, Volpe P and Nori A (2020) Calsequestrins New Calcium Store Markers of Adult Zebrafish Cerebellum and Optic Tectum. Front. Neuroanat. 14:15. doi: 10.3389/fnana.2020.00015

Abbreviations: Casq, calsequestrin protein; *casq*, calsequestrin gene and RNA; CC antibody, antibody to canine cardiac Casq Pa1–913; CCe, corpus cerebelli; COPI, coatomer protein complex; DCN, deep cerebella nuclei; ER, endoplasmic reticulum; GCL, granule cell layer; Kd, dissociation constant; IP3, inositol triphosphate; ITPR1, inositol triphosphate-sensitive Ca^{2+} channel type 1; LCa, lobus caudalis cerebelli; LTD, long-term depression; MC antibody, antibody to mouse cardiac Casq C3868; ML, molecular layer; MON, medial octavolateralis nucleus; NeuroD1, neurogenic differentiation 1; OTML, optic tectum marginal layer; PCL, Purkinje cell layer; PDI, protein disulfide isomerase; SPV, stratum periventriculare; Pvalb, parvalbumin; *Ryr*, ryanodine-sensitive Ca^{2+} channels gene; S1, S2, S3, S4, surnatant fractions 1–4; P2, P3, P4, pellet fractions 2–4; SERCA, sarco-endoplasmic reticulum calcium pump; SOCE, store operating calcium entry; STIM1, stromal interaction molecule 1; TeO, optic tectum; TL, torus longitudinalis; Val-Vam, valvula cerebelli.

INTRODUCTION

Zebrafish (*Danio rerio*) is an emerging animal model whose developed brain, cells, and neuronal circuits are similar to those of mammals and thus is suitable to study human brain pathology (Saleem and Kannan, 2018). Calcium (Ca^{2+}) storage in neurons is crucial for cell activity and death (Zündorf and Reiser, 2011). Three major compartments are involved in intracellular Ca^{2+} homeostasis: endoplasmic reticulum (ER), mitochondria, and cytosol. The ER Ca^{2+} store is not homogeneous: families of channels, pumps, and storage proteins are key players in the regulation of Ca^{2+} concentration and cycling in specific compartments to ensure integration and/or strictly localized responses to multiple stimuli. The ER Ca^{2+} stores are involved in neuronal physiology by multiple mechanisms. The IP3-sensitive Ca^{2+} stores are involved in neural plasticity and memory formation in mammals (Inoue et al., 1998; Rose and Konnerth, 2001) and are suggested to elevate the resting Ca^{2+} concentration of the pre-synaptic compartment upon Ca^{2+} depletion of the synaptic space. Moreover, the release of Ca^{2+} from IP3-sensitive Ca^{2+} stores has been involved in long-term depression (LTD) mechanisms. Calcium-induced calcium release from ER Ca^{2+} stores has been proposed as the mechanism for the transmission of a Ca^{2+} signal from the periphery to the cell body in order to regulate gene transcription in long-term potentiation [extensively reviewed by Verkhratsky (2005) and Brini et al. (2014)]. The capacity of Ca^{2+} stores depends both on the intra-ER Ca^{2+} binding proteins that provide releasable Ca^{2+} in specific cell regions and the prolonged stimulations. The identification and the localization of Ca^{2+} handling proteins expressed selectively and/or in combination within a specific neuronal class are essential to identify different cells and their Ca^{2+} handling processes during development and in adult tissues. Cytoplasmic neuronal Ca^{2+} binding proteins have been identified in *D. rerio*: for example, parvalbumin 7 (Pvalb7) is selectively expressed in Purkinje cells of the cerebellum and cerebellum-like structures during development and in adult individuals (Bae et al., 2009; Takeuchi et al., 2015), calretinin (29-kDa calbindin or calbindin 2) is expressed in the eurydendroid cells of the cerebellum (Castro et al., 2006), calbindin 2 is a specific marker for granule cells of the LCa area of the cerebellum (Table 1 in Bae et al., 2009), calbindin 1 (D28k) is expressed in ciliate and microvillous cells of the olfactory sensor neurons (Kress et al., 2015), and S100a1 is found in Purkinje cells (Germanà et al., 2008). In addition, different Ca^{2+} binding proteins are expressed in the olfactory bulb and in the spinal cord (Kress et al., 2015; Berg et al., 2018). All these cytoplasmic binding proteins are involved in cytosolic Ca^{2+} buffering during transmission of the action potential.

Neuronal ER Ca^{2+} Binding Proteins

At rest, the majority of ER Ca^{2+} content is protein bound and rapidly releasable. Intra-ER Ca^{2+} binding proteins are characterized for their low affinity (Kd ∼1 mM) and high capacity for Ca^{2+} (40–80 mol/mol). Calreticulin, expressed in neurons and many other cells, is a multifunctional chaperone involved in protein quality control of secreted proteins. Calsequestrin (Casq) is a key component of sarcoplasmic reticulum Ca^{2+} store in skeletal and in cardiac muscles (Reddish et al., 2017). Casq facilitates Ca^{2+} uptake by sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) in the ER by decreasing free intra-ER Ca^{2+}concentration. Casq is a peculiar Ca^{2+}-binding protein since it has high capacity (from 40 to 80 mol Ca^{2+}/mol protein) and low Ca^{2+} affinity (Kd around 1 mM). Binding of Ca^{2+} causes protein oligomerization (Sanchez et al., 2012), allowing strong and dynamic buffering power at low protein concentration. Casq is able to condense at critical sites where ryanodine-sensitive Ca^{2+} release channels are concentrated by specific anchor proteins (Shin et al., 2000). Such a condensation property is missing in other lumenal proteins, widely distributed throughout the ER lumen. Very recently, a role for cardiac Casq in stress response has been proposed (Wang et al., 2019). By and large, these biophysical and physiological properties define and control Ca^{2+} store capacity in the ER of neuronal cells: nonetheless, Casq expression in the brain of fish and of mammals has been poorly investigated. Distinct genes codify different Casq isoforms: in mammals, two genes, *Casq1* and *Casq2* (mouse), encode for two isoforms preferentially expressed in the skeletal muscle and in the heart, respectively. In zebrafish, three genes, *casq1a*, *casq1b*, and *casq2*, are expressed in the skeletal muscle (Furlan et al., 2016) and one (*casq2*) in the cerebellum (Takeuchi et al., 2017). Up to now, Casq2 has been identified as a marker of chicken Purkinje cells only in association with the IP3-sensitive Ca^{2+} store and excluded from the ryanodine-sensitive Ca^{2+} store (Volpe et al., 1990, 1991). In zebrafish brain, multiple RNA isoforms of the ryanodine-sensitive Ca^{2+} channel (*Ryr*) have been identified (Darbandi and Franck, 2009; Holland et al., 2017; Tse et al., 2018), but the relative protein products have been partially analyzed (Wu et al., 2011). Zebrafish Purkinje cells express the IP3-sensitive Ca^{2+} channel (ITPR1) in the cell body, the axon, the dendritic shaft, and the spines (Koulen et al., 2000), and adult zebrafish brain proteomic studies have identified Casq2 protein in total homogenates (Nolte et al., 2014; Smidak et al., 2016), but cell-type expression and localization remain to be elucidated. Based on current proteomic and transcriptomic studies, it is evident that multiple isoforms of Ca^{2+} store components are expressed in adult zebrafish brain so that the latter is a suitable model to identify neuronal Ca^{2+} stores in different cells and compartments. A crucial question regards the identification and combination of different molecular markers for a specific Ca^{2+} store and its neuronal-type association. The present article addresses the question pertaining to the expression and the cellular localization of the intra-ER Ca^{2+} binding protein Casq in zebrafish brain. The specific aim of the study is to investigate the differential distribution of Casq isoforms in neurons belonging to the cerebellum and the optic tectum in order to expand knowledge on neuronal Ca^{2+} store markers in zebrafish as a prerequisite to assess their specific functions.

MATERIALS AND METHODS

Ethical Approval

Experiments were carried out on adult (3–6 months) zebrafish under the approval of the University of Padua Ethical Committee

on Animal Experimentation and Ministero della Salute (Project Number D2784.N.BGL). The adult fish were maintained and raised in 5-L tanks with freshwater at 28°C, with a 12-h light/12-h dark cycle.

Quantitative PCR

Total RNA was obtained from pools of adult brains, hearts, and skeletal muscles using TRIzol® extraction method. Reverse transcription, primer sequences, and qPCR are described in Furlan et al. (2016). Normalization was performed by ΔCT method using B2M and EF1alpha as reference genes. Values are expressed as mean ($n = 3$) ± SEM.

Protein Methods (Preparation of Tissue Extracts, Subcellular Fractionation, Western Blotting, and *in vitro* Deglycosylation)

Whole homogenates were prepared as previously described (Salvatori et al., 1997). Briefly, the tissues were homogenized with a Teflon pestle-equipped Potter-Elvehjem tissue grinder in the presence of a medium containing 3% (wt/vol) SDS, 0.1 mM EGTA, pH 7.0, and a cocktail of protease inhibitors. The homogenates were then boiled for 5 min and clarified at 15,000 *g* for 10 min. The supernatants were used as whole-protein extracts. Subcellular brain fractionation was carried out essentially as described (Furlan et al., 2016). The brains were homogenized in a homogenization buffer (10 mM Hepes-NaOH, pH 7.4, and 0.32 M sucrose) supplemented with a cocktail of protease inhibitors. The total homogenate was centrifuged for 10 min at 950 *g* and the post-nuclear supernatant (S1) was collected and spun again. S2 was saved and combined with S1, centrifuged at 17,000 *g* for 15 min to yield a pellet corresponding to the mitochondrial fraction (P3) and a supernatant (S3). The S3 containing the remaining organelles from the total homogenate was centrifuged at 30,000 *g* for 1 h to yield a high-speed supernatant (S4) and a pellet (P4) enriched in membranes. Protein concentration was estimated by the method of Lowry using bovine serum albumin as a standard. *In vitro* deglycosylation of glycoproteins was performed on 10 μg of P4 sub-fractions obtained from muscle and brain tissues, using N-glycosydase F deglycosylation kit (Roche) according to the manifacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were carried out as previously described.

PA1-913 (CC) is a polyclonal antibody produced using native canine cardiac Casq as immunogen. In zebrafish, it recognizes mostly Casq2 and, at lower intensity, Casq1a and Casq1b. On the contrary, polyclonal MC reacts mostly with the zebrafish skeletal isoforms; in fact, immunizing peptide for C3868 shows homology with zebrafish Casq1a (67% identity) and Casq1b (44% identity) by BLAST-P analysis, but no homology with zebrafish Casq2 and calreticulin, another Ca^{2+}-binding protein enriched in brain that shares some properties (molecular weight and isoelectric point) with Casqs.

Antibodies		
Calsequestrin (CC)	PA1-913	Thermo Fisher Scientific
Calsequestrin (MC)	C3868	Sigma–Aldrich
Calreticulin	PA3-900	Thermo Fisher Scientific
Synaptotagmin1/2	105002	Synaptic System
NDUFS3	3459130	Thermo Fisher Scientific
NeuroD1	ab60704	Abcam
Parvalbumin1	MAB1572	Merck Millipore
Serca2	MA3-910	Thermo Fisher Scientific
ITPR 1	Polyclonal D130	(Volpe et al., 1991)
Stim1	D88E10	Cell signaling
Ryanodine receptor1	MA3-925	Thermo Fisher Scientific

Mass Spectrometry

Zebrafish brain P4 and S4 protein fractions to be analyzed by MS were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue G-250. Following de-staining, gel slices were washed with 50 mm ammonium bicarbonate and shrunk with ethanol. The reduction/alkylation of proteins was performed with 10 mM dithiothreitol and 55 mM iodoacetamide. After two steps of washing with ammonium bicarbonate/ethanol, the gel was dried with ethanol and incubated with 12.5 ng/μl Lys-C in 50 mM ammonium bicarbonate at 4°C for 15 min. The supernatant was then replaced with fresh 50 mM ammonium bicarbonate, and the reaction allowed to proceed overnight at 37°C. The reaction was stopped with 1% (v/v) trifluoroacetic acid, 0.5% (v/v) acetic acid, and 3% (v/v) acetonitrile, and the supernatant recovered. Additional peptide extraction steps were performed with 30% (v/v) acetonitrile and 100% acetonitrile. The supernatants were concentrated and then diluted with 0.5% (v/v) acetic acid, 30% (v/v) acetonitrile, and 1% (v/v) trifluoroacetic acid. The peptides were desalted and concentrated on reverse-phase C18 StageTips (Rappsilber et al., 2003). Reverse-phase chromatography was performed on a Thermo Easy nLC 1000 system connected to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) through a nanoelectrospray ion source. The peptides were separated on a 50-cm column with an inner diameter of 75 μm packed in house with 1.9 μm C18 resin (Dr. Maisch GmbH). The peptides were eluted with a linear gradient of acetonitrile 0.1% formic acid at a constant flow rate of 250 nl/min. The column temperature was kept at 50°C by an oven (Sonation GmbH). The eluted peptides from the column were directly electrosprayed into the mass spectrometer.

Mass spectra were acquired in a data-dependent mode to automatically switch between full scan MS and up to 15 data-dependent MS/MS scans. The maximum injection time for full scans was 100 ms, with a target value of 3e6 at a resolution of 120,000 at m/z = 200. The target values for MS/MS were set to

1e5, with a maximum injection time of 100 ms at a resolution of 15,000 at m/z = 200. To avoid repetitive sequencing, the dynamic exclusion of the sequenced peptides was set to 20 s.

The spectra were analyzed using MaxQuant (version 1.6.6.2). Peak lists were searched against the UNIPROT databases for *D. rerio* (release 2019_08) with common contaminants added. The search included carbamidomethylation of cysteines as fixed modification as well as methionine oxidation and N-terminal acetylation as variable modifications. The maximum allowed mass deviation for MS peaks was set to 6 and 20 ppm for MS/MS peaks. The maximum missed cleavages were two. The false discovery rate was determined by searching a reverse database. The maximum false discovery rates were 0.01 both on the peptide and the protein levels. The minimum required peptide length was six residues. Peptide identification was performed with an allowed initial precursor mass deviation of up to 7 ppm and an allowed fragment mass deviation of 20 ppm. Match between runs was used. The mass spectrometry (MS) proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD015577. Bioinformatic analyses were performed with the Perseus software (version 1.5.4.2), part of the MaxQuant environment[1].

Immunofluorescence

After sacrifice, the adult zebrafish were quickly peeled to expose the brain in skull, briefly washed in phosphate buffered saline, pH 7.4 (phosphate-buffered saline, PBS), and fixed overnight with 4% paraformaldehyde in PBS. The fixed zebrafish brains were carefully removed from the skull, dehydrated through graded ethanol, and embedded in paraffin as previously described (Moorman et al., 2001). Immunofluorescence analysis was performed on serial 10-μm paraffin wax-embedded sections. After deparaffinization and rehydration, the sections were boiled for 20 min in sodium citrate buffer to induce epitope retrieval (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and briefly washed in PBS before immunofluorescence assay. The sections were blocked with PBS-Tw-N (PBS, 0.1% Tween20, 5% goat serum) for 30 min to avoid non-specific staining. The sections were then incubated in primary antibodies diluted in PBS-Tw-N for 2–16 h and, after washing, were incubated for 1 h in fluorescently conjugated secondary antibody diluted in PBS-Tw-N. After washing as detailed above, the sections were mounted with ProLong Gold antifade reagent with DAPI (Life Technologies) and cover-slipped. Epi-fluorescence analysis was performed under a Leica DMR microscope using the software Leica Application Suite Advanced Fluorescence 4.0.0.11706 (LASAF). Confocal analysis was performed under Leica SP5 confocal inverted microscope and Zeiss LSM 700 confocal microscope.

Probe Design

Probes specific for *casq1a* and *casq2* mRNAs were designed by homology search using NCBI-BLAST. Probes were mapped at nucleotides 1,991–2,780 within exon 12 and the 3′ untranslated region for *casq1a* (GenBank accession no. NM_001003620) and at nucleotides 1,038–1,887 within exon 8 and the 3′ untranslated region for *casq2* (GenBank accession no. NM_001002682), respectively. The primers used were *casq1a* (forward primer: 5′-TCCCATTGACCCAATGTTCT-3′, reverse primer: 5′-CC CTTGTGACCAAAGGAAAA-3′, probe size 789 bp) and *casq2* (forward primer: 5′-CGTTTGCTGAAGAGGAGGAC-3′, reverse primer 5′-TGGGTTTTTGCCTTTATTCG-3′, probe size 849 bp). The PCR products were amplified from zebrafish brain cDNA and then cloned into pCR 2.1 and pCR II vectors. Antisense labeled-mRNA was *in vitro* transcribed using digoxygenin-RNA labeling Mix SP6/T7 (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions.

In situ Hybridization

In situ hybridization was performed on serial 10-μm paraffin wax-embedded sections as previously described (Moorman et al., 2001). Briefly, the sections were treated with 20 μg/ml of proteinase K for 15 min at 37°C and postfixed with 4% paraformaldehyde for 20 min. The sections were then washed again before a prehybridization step of 1 h at 70°C with a hybridization solution [50% formamide, 5× SSC, 1% blocking powder (Roche), 0.1% Tween, 0.1% CHAPS, 1 mg/ml yeast tRNA, and 5 mM EDTA]. Next, the fresh solution was added with each of the digoxigenin-labeled RNA probes at the proper dilution. The probes were left to hybridize overnight at 70°C. On the next day, three highly stringent washes were carried out for 30 min each with 50% formamide in SSC. After blocking with B-block for 1 h, the sections were incubated with 1:2,000 sheep anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche) in blocking solution overnight, and the reaction was revealed by BCIP/NBT as substrates.

RESULTS

Expression and Immunological Identification of Casqs in Adult Brain

The relative levels of three transcripts (*casq1a*, *casq1b*, and *casq2*) were measured by qPCR, comparing three tissues (brain, skeletal muscle, and heart). As shown in **Figure 1A**, the higher expression of *casq1a* was found in the muscle, whereas *casq2* was the most expressed isoform in the heart. In the brain, both *casq1a* and *casq2* were expressed, the lowest expression being observed for *casq1b*.

In order to identify Casqs at the protein level, equal amounts of crude extracts from the skeletal muscle, the heart, and the brain were separated by gel electrophoresis and analyzed by specific antibodies. Two commercially available antibodies, Pa1-913 (CC) and C3868 (MC) raised against mammalian Casqs, recognize zebrafish homologs with different specificity (see Furlan et al., 2016 and below). As shown in **Figure 1B**, CC antibody mostly reacted with a 58-kDa protein, previously identified as Casq2 in zebrafish skeletal and cardiac muscle. In the brain, a 58-kDa band co-migrating with Casq2 was clearly detected. In the skeletal muscle, the same antibody recognized Casq1 isoforms (doublet at 83 kDa and single band at 74 kDa) in addition to Casq2 (**Figure 1B** and Furlan et al., 2016). In the

[1]http://www.perseus-framework.org

A

Quantitative determination of Casq mRNA transcripts

		relative to B2M (±SE)	relative to EF1α (±SE)
total brain	*casq1a*	0,23±0,092	0,012±0,005
	casq1b	0,06±0,023	0,005±0,002
	casq2	0,22±0,073	0,013±0,005
muscle	*casq1a*	9,30±2,462	0,775±0,183
	casq1b	0,84±0,128	0,087±0,016
	casq2	0,11±0,044	0,010±0,002
heart	*casq1a*	0,07±0,017	0,005±0,002
	casq1b	0,14±0,029	0,012±0,002
	casq2	1,02±0,237	0,058±0,008

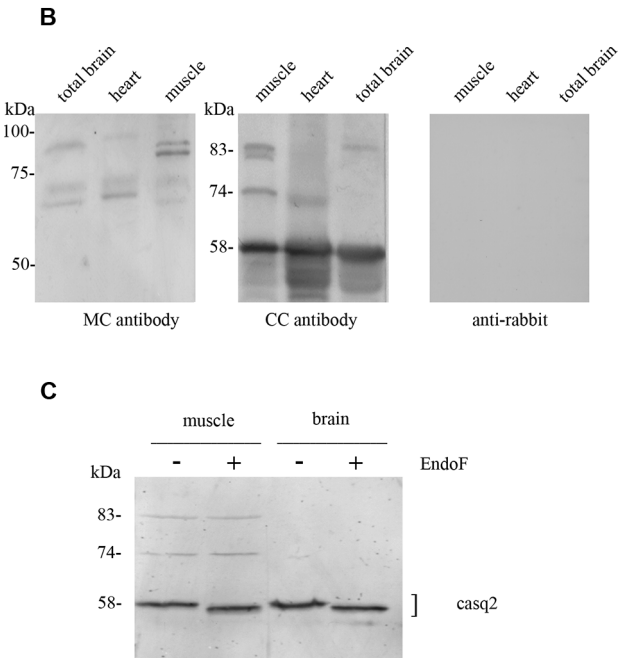


FIGURE 1 | Casq mRNA in selected tissues from adult zebrafish **(A)**. The levels of RNA expression are relative to two different housekeeping genes, beta-2-microglobulin (B2M) and elongation factor1-α (EF1α). Mean values and standard error were determined from triplicate runs of the qPCR assay in three different tissue preparations. **(B)**. Anti-Casq antibodies reactivity on total homogenates (40 μg per lane) obtained from pooled (N = 3) skeletal muscle, heart, and brain of adult zebrafish. In the control lanes, (anti-rabbit) blot incubation was performed in the presence of anti-rabbit conjugate with alkaline phosphatase only. Molecular mass markers (in kDa) are indicated. **(C)** *In vitro* deglycosylation of Casqs. Immunodetection of Casq by CC antibody in membrane-enriched fraction P4 (see "Materials and Methods" section) after endoglycosidase F digestion, with 10 μg loaded per lane.

brain, a single band at around 83 kDa was also detected. The second antibody, C3868 (MC), showed reactivity at 83 kDa and at around 74 kDa in the brain, similarly to that found in the heart and in muscle homogenates. The MC antibody did not react with the 58-kDa isoform. Signal specificity was tested by omitting anti-Casq antibodies in a control immunoblot. As reported in **Figure 1**, (anti-rabbit) signals were absent.

A distinctive feature of Casq1 and Casq2 is the *N*-glycosylation consensus sequence (Asn-*X*-Ser/Thr) at the C terminus. An analysis of zebrafish Casq sequences by NetNGlyc[2] and GlycoEP[3] webservers identified a consensus sequence in position Asn336 (NVT) that is highly conserved in Casq2 among different species. In order to check the N-glycosylation state of native zebrafish Casqs, we performed digestion with N-glycosidase F on skeletal muscle and brain Casq2-enriched fractions. As shown in **Figure 1C**, after N-glycosidase F treatment, Casq2 apparent molecular weight was shifted (about 3 kDa) both in the skeletal muscle and in the brain, suggesting a native glycosylated form of Casq2 in both tissues, whereas Casq1a and Casq1b (detectable in muscle fractions) did not change mobility as they lack a specific consensus sequence. Taken together, mRNA analysis, immunological, and glycosylation data indicate that more than one Casq isoform is expressed in the brain.

Identification of Casqs in Subcellular Fractions of Zebrafish Brain

In order to obtain protein fractions enriched in Casq, differential centrifugation was performed on total brain homogenates derived from pooled adult zebrafish brains. Three membrane fractions (P1-2, P3, and P4—the latter being the lightest of the three) and a supernatant fraction S4 were obtained as described in "Materials and Methods" section. The different sub-fractions were characterized by specific immunological markers. As shown in **Figure 2**, synaptotagmin1 (SYT1), an abundant integral membrane protein of synaptic vesicles, was clearly identified in all membrane-containing fractions. Densitometric analysis (see **Supplementary Table S1**) showed an enrichment of the synaptotagmin signal in P2, P3, and P4 of 10.7, 17.6, and 26.1-fold, respectively, in comparison with S4, indicating that P4 was significantly enriched in membranes of synaptic origin. On the contrary, a mitochondrial marker protein associated to the inner mitochondrial membrane and matrix, NADH dehydrogenase [ubiquinone] iron-sulfur protein 3 (NDUFS3; Dieteren et al., 2008), was fully recovered in the P3 fraction, intermediate-speed pellet fraction, confirming the enrichment in mitochondria. Calreticulin was found in P4 pellet but was also abundant in the soluble fraction S4 according to previous reports (Holaska et al., 2001; Labriola et al., 2010). The distribution of Casq isoforms was not homogeneous among the fractions: the 58-kDa isoform, identified by CC antibody, was found in all membrane fractions and especially enriched (5.1-fold) in P4 in comparison to S4. On the contrary, the 83-kDa isoform was enriched in fraction S4 (6.9-fold) in comparison to P4. Finally, the 74-kDa isoform Casq1b, previously identified in the skeletal muscle, was not detectable in the brain fractions by immunoblotting. In conclusion, we obtained two fractions enriched in both the 58- and the 83-kDa proteins.

Quantitative MS-based proteomics was applied to P4 and S4 fractions to confirm the identity of Casqs. Both isoforms were identified by several peptides spanning 9% of Casq1 and 22% of Casq2 (**Figure 3B**). In the P4 fraction, Casq2 was more abundant than Casq1a since the respective ranking by cumulative abundance was 1,074 for Casq2 and 1, 360 for Casq1a

[2]http:http://www.cbs.dtu.dk/services/NetNGlyc
[3]https://webs.iiitd.edu.in/raghava/glycoep/submit.html

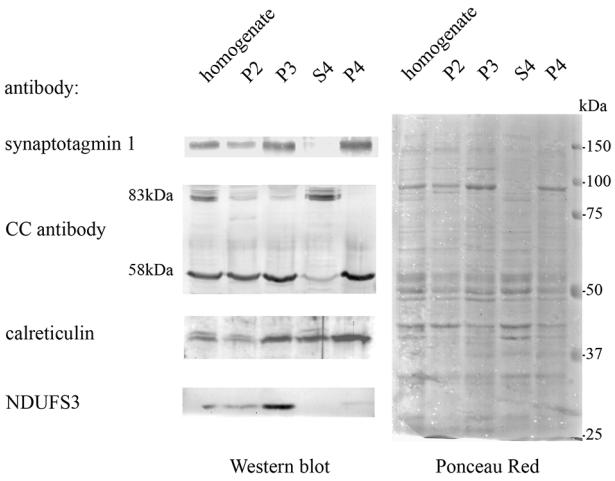


FIGURE 2 | Adult brain subcellular fractionation: immunochemical profile. Immunoblot analysis of brain subcellular fractions. Equal protein amounts (40 μg) from each fraction were analyzed with CC antibodies to recognize Casqs and with antibodies specific for synaptotagmin1 (SYT1), calreticulin, NDUFS3 (as described in "Materials and Methods" section). Raw data derived from densitometric analysis are presented in **Supplementary Table S1**.

A

Accession number	Gene name	Protein name	% in S4	% in P4	n. peptides
F1QNI9	casq1a	calsequestrin 1a	13	87	7
Q6DI16	casq2	calsequestrin 2	3	97	8

B

F1QXT6|F1QXT6_DANRE Calsequestrin OS=Danio rerio GN=casq1a
MKWNLVFLGLLLTFGQLSWGQKGMDIPEYDGKDRVHELNAKNYKSVMKKYDVMVVYYHEH
VGSSKVAQKQFQIEELALELAAQVLADFDDEDIGIGLLDEKTDKAVAKKLGLDEADSIFI
FIEDEVIEYDGELAADTLVEFIYDVIEDPVEIIGNVREMKGFNNIEEDIKLVGFFKSAKS
DHYHEYEDAAEEFHPHIKFFATFNPKVALKLGLKLNEVDFYEPFMDKPVVIPGKPYSEKE
LVRFIEDNDRPTLRKMQPHNMYEIWEDALDGEHIIAFAEEGDPDGFEFLEIVKEVAEDNT
ENPDLSIIWIDPDDFPLLVHYWEKTFDIDLSSPQIGVVEVDDAESIWFDMDDDDMPTVDE
LEDWLEDVLENKIDPDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDD
DDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDD
DDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDNDDDDDDDDDDDDDDDDDDDD
DDDDDDDDDDDD

Q6DI16|Q6DI16_DANRE Calsequestrin OS=Danio rerio GN=casq2
MHTIWILLLASMAFFTLASAKKGLEFPRYDGEDRVLDIDDKNYRKALKKYDMLCLFYHAP
PPAAKELQKQLHLTELVLELAAQVLEEKDIGFGMVDSQKDAKVAKKLGLHEEGSVYIFKD
DRVIEFDGLPSADTLVEFLLDLLEDPVEIIDNALELRAFDRMEEDIKLIGFFKSQSEHY
LAFQEAAEQFQPFIKFFATFEKSVAKELTLKMNEVDFYEPFMEEPVTIPDKPHSEEELVA
FISEHRRPTLRKLKAEDMFETWEDDLNGIHIVAFAEEEDPDGFEFLEILKEVARDNTHNP
DLSIVWIDPDNFPLLIPYWEMTFKVDLFRPQIGVVNVTDADSVWLEIPNDDELPSAEELE
NWIEDVLSGTVNTEDDDDDDDDDDDDDDDDDDDNDDDDDDDDDDDDDDE

FIGURE 3 | Identification of Casqs in adult brain fractions. **(A)** Relative abundance of the two detected isoforms. **(B)** Mapping of peptides identified by mass spectrometry on Casq sequences. In red is the consensus site for N-glycosylation.

in 3,916 proteins (see **Supplementary Table S2**). Conversely, Casq1a was more abundant than Casq2 in S4, where the two isoforms ranked 1,910 and 2,141, respectively. No peptide from Casq1b was detected, either in S4 or in P4 fractions. Consistent with SDS-PAGE analysis, the MS results confirm the identification of Casq1a and Casq2 and suggest a different cellular compartmentation of the two isoforms.

Casq mRNA Is Expressed in Multiple Brain Regions

Expression of Casq RNA was studied by *in situ* hybridization. Isoform-specific riboprobes for *casq1* and *casq2* genes were used to examine whole sagittal sections, although focus was placed on the cerebellum and the optic tectum regions. In **Supplementary Figure S1**, sections of the whole brain processed with anti-sense RNA for *casq1* and *casq2* are shown paired with a control experiment performed without the riboprobes. **Figure 4A** shows the expression pattern of *casq1a* mRNA in the cerebellum and the optic tectum (TeO). Hybridization signals with casq1a riboprobe were found in the granule cell layer (GCL) of the cerebellum and the torus longitudinalis and in the stratum periventriculare (SPV) of the optic tectum. Higher magnification images of these regions are illustrated in **Figures 4B–D** that show a blue signal compatible with densely packed granular cells and absent in the control experiments (**Supplementary Figures S1D–F**). **Figure 4E** shows the expression pattern of *casq2* mRNA in a parasagittal section serial to that of **Figure 4A**. Casq2 mRNA

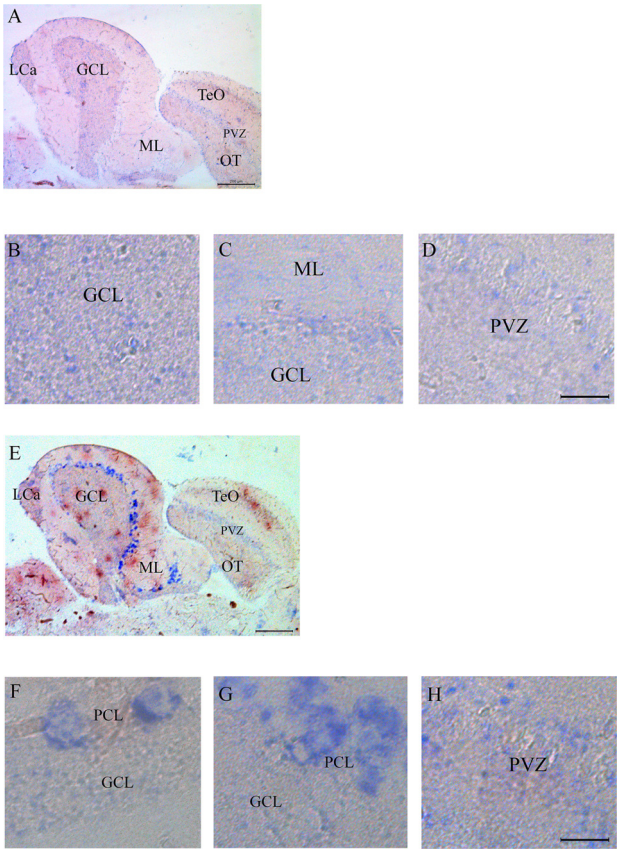


FIGURE 4 | *In situ* analysis of *casq1a* [**(A–D)** and casq2 panels **(E–H)**] transcripts in sagittal brain sections (**A,E**, rostral to the right). Whole-brain sections and control sections are presented in **Supplementary Figure S1**. Anatomic terminology was inferred from Ullmann et al. (2010). CCe, corpus cerebelli; GCL, granule cell layer; Lca, caudal lobe of cerebellum; ML, molecular layer; PCL, Purkinje cell layer; PVZ, periventricular zone of optic tectum; TeO, optic tectum. **(A,E)** Bar: 200 μm. **(B–D,F–H)** Bar: 20 μm.

was detected in the cerebellum, with a strong blue signal at the Purkinje cell layer and a weak signal in the optic tectum (SPV). Higher-magnification images show a strong signal at the level of the Purkinje cell bodies (panel F and G) and a weaker labeling in TeO SPV (panel H). In summary, these results indicate that the granule cells of the cerebellum and the optic tectum express Casq1, whereas the Purkinje cells of the cerebellum express Casq2.

Differential Localization of Casqs in Zebrafish Cerebellum and Optic Tectum

The different localization of *casq1* and *casq2* RNA in zebrafish brain suggested by *in situ* hybridization raises the intriguing possibility that Casq1a and Casq2 might be expressed in distinct cells. Cellular localization was investigated in parasagittal brain sections by immunofluorescence with MC and CC antibodies that recognize Casq1 and Casq2, respectively. With the CC antibody, a lively reaction was detectable in the cerebellum area (both corpus, CCe and valvula cerebelli; Val–Vam), being more intense at the level of the Purkinje cell layer and the molecular layer (ML; **Figure 5A**). Signal specificity was confirmed by processing a brain section with the same immunofluoresence protocol but excluding the CC antibody (**Figure 5B**). Confocal analysis (**Figures 5C–E**) of the fluorescence pattern showed, at higher magnification, drop-shaped large neurons located between the ML and the GCL, heavily stained in the cell bodies, except the nuclei, and organized in simple and in multiple layers according to the Purkinje cell distribution in the zebrafish cerebellum (Miyamura and Nakayasu, 2001). Dendrites extending into the ML of the cerebellum were also clearly stained with a punctuate pattern (E). In Purkinje cell bodies (D), a patchy reticulate pattern characteristic of Casq was clearly detectable. The granule cells were not stained (**Supplementary Figure S2**). Rare, thin, and dotted fluorescence, organized in a linear arrangement, was also detectable in the GCL (**Figure 5E**; see also **Figure 6**) in continuity with positive cell bodies. These linear structures are similar to the axons of chicken and mammal Purkinje cells (Villa et al., 1991; Sacchetto et al., 1995; Koulen et al., 2000). In cerebellum circuits of mammals and birds, the Purkinje cells send inhibitory projections (axons) to the deep cerebellar nuclei (DCN) whereas in zebrafish Purkinje the cell axons target eurydendroid cells, which are equivalent to mammalian DCN. The zebrafish Purkinje axons are shorter than the mammalian ones since the eurydendroid cells are big parvalbumin 7-negative neurons located in the granular layer in proximity to the Purkinje cells (Bae et al., 2009). A fluorescence pattern, similar to the CC pattern described above, was previously observed for ITPR1-positive Purkinje cells in zebrafish brain (Koulen et al., 2000), strongly suggesting that the CC-positive cells are Purkinje neurons. Additional parasagittal brain sections were processed by immunofluorescence with the MC antibody that recognizes exclusively Casq skeletal isoform in zebrafish muscle. The MC antibody (**Figure 5F**) strongly reacted at the cerebellum areas, mainly localized to the GCL and at the SPV of the optic tectum. The specificity of the signal was confirmed by processing a brain section with the same immunofluoresence protocol but excluding the MC antibody

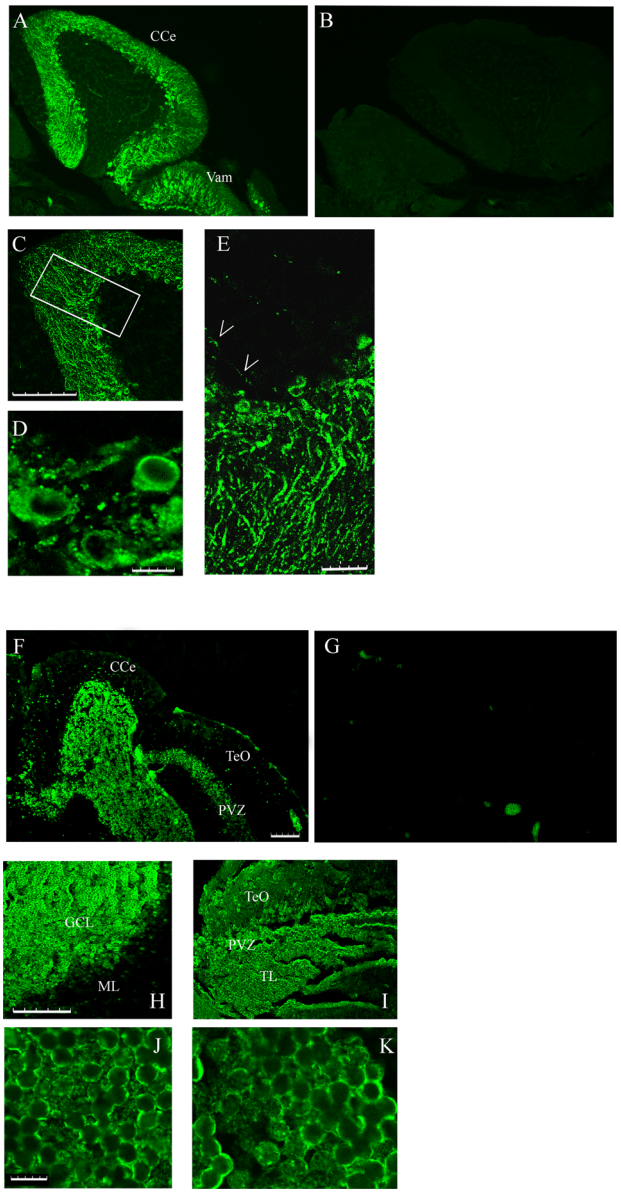


FIGURE 5 | Immunofluorescence staining of sagittal brain sections area restricted to the cerebellum and the optic tectum (rostral to the right) decorated with CC **(A,C–E)** and MC **(F,H–K)** antibodies. **(B,G)** Images collected from control sections (see "Results" section) and acquired with the same conditions as in **(A)** and **(F)**, respectively. **(A,B,F,G)** Epi-fluorescence signals. **(C–E,H–K)** Images obtained by single optic section of confocal analysis. The box in **(C)** indicates the area shown at higher magnification in **(E)**. **(D)** PCL in a different region and focal plane. Arrows: axons of Purkinje cells. The abbreviations are the same as in **Figure 4**. **(A,C,F,H,I)** Bar: 100 μm. **(E)** Bar: 25 μm. **(D,J,K)** Bar: 7.5 μm.

(**Figure 5G**). At higher magnification (**Figures 5H,I**), a reaction was clearly detectable in the peripheral area of granule cells. The confocal images of the cerebellum and the optic tectum SPV showed granule cells stained in the perinuclear area (**Figures 5J,K**). Some positive cells were also detected at the ML.

Identification of Casq-positive neurons was carried out by immunofluorescence in sagittal sections double-

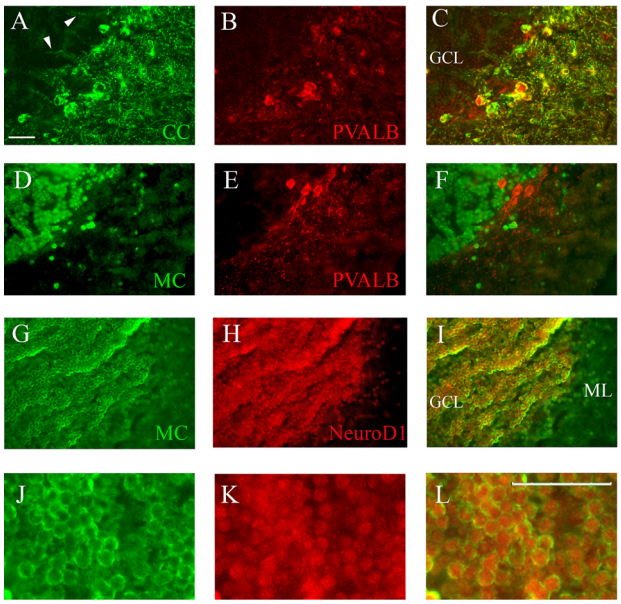


FIGURE 6 | Co-staining of cerebellum with Casq and neuron-specific markers. **(A–C)** Epi-fluoresence signal obtained by double immunofluorescence with CC antibody (green) and anti-parvalbunin antibodies (red) in the cerebellum area. **(C)** A merge of the two images. Arrowheads indicate the axons of Purkinje cells. **(D–F)** Epi-fluoresence images obtained by double-staining with MC antibody (green) and anti-parvalbunin antibodies (red). **(D)** A merge of the two images. **(G–I)** Confocal images obtained by double-staining with MC antibody (green) and anti-NeuroD1 antibody (red). **(J–L)** Higher magnification of granule cells in the central area of the cerebellum double-labeled with MC (green) and anti-NeuroD1 antibody (red). Bar: 25 μm.

labeled with antibodies for cell-specific markers. Double immunofluorescence with specific parvalbumin (Pvalb) and MC antibodies (**Figures 6A–C**) showed that all neurons stained by CC were also Pvalb-positive, particularly at cell bodies and at proximal dendrites. Since Pvalb is a well-known marker for zebrafish and teleost cerebellum (Alonso et al., 1992; Takeuchi et al., 2015), these results show for the first time that the Purkinje cells of zebrafish cerebellum express Casq. Since in the western blot of brain homogenates the CC antibody identifies Casq2 (**Figure 1B**), the Purkinje cell isoform is a *bona fide* Casq2. The molecular layer area was further analyzed by confocal microscopy at higher magnification, confirming the differential localization of Casq1 and Casq2 proteins (see **Supplementary Figures S3, S4**). On the contrary, double immunofluorescence with MC and Pvalb antibodies showed an overall separation of the two signals (**Figures 6D–F**), indicating that the MC antibody decorated granule cells only. Using double immunofluoresence with specific antibodies for Casq1 (MC) and NeuroD1, a specific nuclear marker of granule cells in adult zebrafish (Takeuchi et al., 2015), **Figures 6G–I** shows that the NeuroD1-positive cells in the GCL are also clearly stained by the MC antibody in the perinuclear area where the ER is densely packed. A similar cellular co-localization of the immune signal was detectable at the granular cells of the optic tectum (PVZ) and the torus longitudinalis (not shown). Since the MC antibody does not recognize Casq2, our interpretation is that the protein identified in the granule cells is a *bona fide* Casq1.

Identification of Casqs and Other Ca^{2+} Store Markers by Mass Spectrometry

Quantitative MS-based proteomics on subfractions P4 and S4 provided 24, 396 peptides corresponding to 3, 966 proteins. The corresponding MS data files were deposited in the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD015577. The list of proteins identified by more than one unique peptide (2, 886 proteins) is provided in **Supplementary Table S2**. General neuronal markers such as NSE (eno2) expressed in mature neurons and glia cells (Bai et al., 2007), MAP2 microtubule-associated protein, calbindin 2, and neurofilament light polypeptide b (Neflb) were identified. Moreover, neuronal type-specific markers such as Grid2 and Ca8 (carbonic anhydrase 8), proteins known to be selectively expressed in the Purkinje cells of the cerebellum (Huang et al., 2014), calbindin 2, marker of dendrites from Purkinje and granule cells, slc17a6a and slc17a6b (vesicular glutamate transporter 2.1 and 2.2) and slc17a7a (Vglut1) markers of granular layer, Lca (locus caudalis cerebelli), and torus longitudinalis glutamatergic neurons. Finally, a marker for Bergmann glia, Slc1a3b (Bae et al., 2009), was also identified. Trans-golgi network proteins Lman1 (ERGIC53) and EMC3 were present, whereas abundant proteins from mitochondrial membranes, such as cytochrome c oxidase I and II, were not detected in P4 and S4, suggesting a negligible contamination of the mitochondria.

Overall Protein Distribution in P4 Membrane Pellet vs. S4 Supernatant

A manually curated list of informative proteins identified in P4, S4, or both is presented in **Supplementary Table S3**. Three protein groups display a different partition between P4 and S4: the first group is comprised of soluble cytoplasmic proteins 95–100% enriched in S4 (for example, enolases), the second group consists mostly of trans-membrane proteins (belonging to pre- and post-synaptic membranes, ER or Golgi membranes, and plasma membrane) 95–100% enriched in P4, and the third group of proteins is more homogeneously distributed between P4 and S4, such as ER/Golgi resident luminal proteins Grp94, PDI, and calreticulin, which was found both in P4 (53%) and in S4 (47%) according to western blot analysis (**Figure 2**). Mass spectrometry identified another ER resident protein calnexin (91% in P4). Calnexin and calreticulin are two ER chaperon proteins that share a common sequence and structure, but calnexin is membrane-bound and calreticulin is soluble according to their differential compartmentation between P4 and S4. Proteins belonging to various vesicles were also identified; in particular, clathrin-coated vesicle components were restricted to P4 fraction and COPI vesicle components were restricted to S4. Several synaptic proteins were found exclusively in the P4 fraction, among them, peptides belonging to vesicular glutamate trasporter 1, synaptotagmin Vb, VAMP2, and ionotropic glutamate receptor. In addition, MAP2, microtubule-

associated protein 2 (E7FBI2), described as a component of dendritic spines (Izant and McIntosh, 1980), was identified in the P4 fraction by 16 peptides. Two important calcium store markers, the IP3-sensitive Ca^{2+} channel type 1 (ITPR1) and an isoform of sarco-endoplasmic reticulum Ca^{2+} pump (ATP2a2a), were identified in fraction P4. No mitochondria membrane protein was detected both in P4 and in S4, further suggesting the lack of substantial contamination by mitochondria. Global Gene Ontology enrichment analysis (see **Supplementary Figure S6**) confirmed that the majority of either membrane or membrane-associated proteins were enriched in the P4 fraction.

DISCUSSION

In this article, we studied Casq expression and distribution in the adult brain of zebrafish with particular focus on the cerebellum and the optic tectum. Our aim was to determine how many Casq isoforms are expressed and to study their cellular distribution and subcellular localization. Due to the scarcity (in comparison with mammals) of immunomarkers for adult zebrafish tissues, we used multiple approaches to integrate a different set of data. Thus, we *in situ* hybridization with Casq-specific designed primers, single or double immunofluorescence experiments, with commercially available antibodies which recognize zebrafish skeletal muscle Casq1 and Casq2, and mass spectrometry.

The major findings are as follows: (1) Casq1a and Casq2 are expressed in zebrafish brain especially in the cerebellum and the optic tectum; (2) Casq1b is not detectable; (3) Casq1a is expressed in granule cells of the cerebellum and the optic tectum; and (4) Casq2 is concentrated in Purkinje cells at cell bodies, axons, proximal and distal dendritic shafts.

Identification and Localization of Casqs

Comparison With Other Teleostei and Mammals

Among teleostei, in *Solea senegalensis*, four mRNAs with different organ specificity were identified: *casq1a* and *casq1b* mainly expressed in the skeletal muscle, *casq2a* in the heart, and *casq2b* in the brain of juvenile fishes (Infante et al., 2011). Solea Casq2b presents 69.9% identity with zebrafish Casq2 (protein ID: Q6DI16). In mammalian brain (mouse), *Casq2* RNA is expressed in the Purkinje cells of the cerebellum (Pavlidis and Noble, 2001; Rong et al., 2004). In this article, we show for the first time that Casq1 mRNA and protein are expressed in the granular cells of the cerebellum and the optic tectum. *In situ* hybridization showed an isoform-specific pattern of Casq1a mRNA expression in the cerebellum, in the GCL of corpus and valvula cerebelli, and, interestingly, in the optic tectum–torus longitudinalis granular cells which belong to specific neuronal circuits defined as cerebellum-like structures. Optic tectum and torus longitudinalis are cerebellum-like structures consisting of a molecular layer (OTML), a principal cell layer (type I neurons), and a granular structure (TL) composed of densely packed glutamatergic neuronal cell bodies. The TL cells were positive with MC antibody as PVZ, while the type I neurons of the SFGS stratum were negative. Another cerebellum-like structure, medial octavolateral nucleus, crossed by projections of eminentia granularis cells which project their dendrites in the molecular layer of crista cerebellaris (Bell et al., 2008; Robra and Thirumalai, 2016), has not been analyzed. The localization of Casq1 protein is in agreement with *in situ* mRNA localization. The MC antibody is the commercially available polyclonal antibody that detects Casq1 in zebrafish skeletal muscle by both western blot and immunofluorescence (see Furlan et al., 2016). Validation of the antibody was performed previously in the skeletal muscle, where Casq1 is extremely abundant, by correlating the characteristic chemical properties of Casq1 (Stains's all staining and Ca^{2+}-induced shift in SDS-PAGE) with immunodetection. The reactivity of the antibody was weak in western blot but reliable and specific for the Casq1 isoform compared with Casq2 in zebrafish muscle. For these reasons, we found a low intensity of the western blot signal in the brain by MC antibody but with good isoform specificity and high signal-to-noise ratio, as shown in **Figure 1**. The possibility that the MC antibody cross-reacted with a brain-specific protein similar to Casq1 but different from it cannot be excluded, but it is unlikely since Casq1a was unequivocally identified in brain homogenates by mass spectrometry.

By *in situ* hybridization and immunofluorescence, we show for the first time that Casq2 mRNA and protein are expressed in zebrafish cerebellum Purkinje cells of both corpus and valvula cerebelli. In particular, CC antibody reactivity implies that the expression of Casq2 extends from the cell body to the dendrites and the axons. It is ruled out that the signal refers to cross-reactivity with Casq1 because it is not detectable in Purkinje cell dendrites by Western blot and immunofluorescence. The strong *in situ* hybridization and immunofluorescence signals in Purkinje cells indicate a high concentration of the protein. A similar concentration in Purkinje cells has been observed in chicken (Villa et al., 1991; Takei et al., 1992), where Casq is a component of specialized ER sub-domains distributed along all Purkinje regions, except the majority of the dendritic spines.

Differential Cellular Localization of Casqs: Physiological Implications

In situ hybridization and immunofluorescence experiments show that Casq1 and Casq2 are differentially localized in zebrafish brain with virtually no overlap. We found two main differences: (a) the high concentration of both Casq2 protein and mRNA in Purkinje cells as compared to other neurons; and (b) the significant expression of Casq1 in the granular cell layer of the cerebellum and the optic tectum.

Here, we show for the first time the identification and the localization of the skeletal Casq1 isoform (both RNA and protein) in the granular cells of a vertebrate cerebellum. A characteristic of this species is the continuous renewal of some neurons such as granule cells, Bergmann glia, and inhibitory interneurons (Grandel et al., 2006; Kani et al., 2010; Jászai et al., 2013; Kaslin et al., 2013). These cells regenerate in specific areas and, during cell differentiation, migrate to the final functional region (Zupanc et al., 2005). We found the highly positive Casq1 cells to be sparse in the molecular layer of adult cerebellum where migrating granules transit (see also **Supplementary Figure S5E**). It appears that the neuronal migration and the proliferation of granule cells is regulated by

Ca^{2+} release from ER stores *via* IP3- and/or ryanodine-sensitive channels (Kumada and Komuro, 2004; Komuro et al., 2015; Horigane et al., 2019). It is plausible that Casq1 plays a role in such a mechanism in fish, especially in shaping either Ca^{2+} transients or spikes, as occurs in the skeletal muscle (Tomasi et al., 2012). Preliminary evidence of Ryr1 positive cells in the molecular layer are presented in **Supplementary Figure S5F**. In this perspective, Casq1 could be a functional marker of regenerating and/or migrating neuronal progenitors in adult and possibly developing zebrafish.

Neuronal plasticity mechanisms, such as LTD, have not been described in zebrafish. Little is known on the molecular composition of Ca^{2+} stores in the Purkinje cell of zebrafish cerebellum. As for ER Ca^{2+} channels, the RNA of five ryanodine receptor genes is expressed in zebrafish brain (Darbandi and Franck, 2009, and **Supplementary Figure S5F**); in Purkinje cells, the ITPR1 protein has been identified (Koulen et al., 2000). We found that Casq2 is concentrated in all cellular compartments (axon, cell body, and dendrites) of Purkinje cells (as previously shown for chicken cerebellum; Volpe et al., 1991) similarly to what happens for ITPR1 (Koulen et al., 2000), differently to cell homogeneous distribution of Calreticulin and SERCA (**Supplementary Figures S5A,B,G,H**). In zebrafish, like in mammals, a high concentration of cytoplasmic Ca^{2+}-binding proteins in Purkinje cells implicate a high buffering power in the cytosol (**Supplementary Figure S5D**): zebrafish Casq2 could be essential in maintaining the high-capacity and strictly localized Ca^{2+} stores in the ER despite cytoplasmic buffering. Store operating calcium entry (SOCE) is the Ca^{2+} refilling mechanism of ER in the granular and the Purkinje cells of mammals (Hartmann et al., 2014; Ryu et al., 2017; Wegierski and Kuznicki, 2018). The acidic C-terminal of mammal Casq1 is involved in the mechanism of SOCE (Wang et al., 2015; Zhang et al., 2016). In mammalian brain, stromal interaction molecule 1 (STIM1) has been shown to link mGluRs and IPTR1 signals and to play a critical role in cerebellar Purkinje cells (Hartmann and Konnerth, 2008); moreover, STIM and Orai have been identified in several neuronal compartments (Segal and Korkotian, 2014). Nothing is known about SOCE in zebrafish brain except for the positive expression of STIM1 in the neuronal progenitor cells (Tse et al., 2018). We obtained preliminary evidence of STIM1 expression in Purkinje cells and in granule cells, although at lower levels (see **Supplementary Figure S5C**), suggesting that SOCE could occur and that both Casq1 and Casq2, having a highly acidic C-terminal tail, could be involved in its regulation.

Given that the different Casq isoforms are preferentially expressed in distinct cell types, our results suggest that they might have very different functions in the zebrafish brain. This might, at least in part, be referable to the long acidic tail at the C terminus of zebrafish Casq1a. Structural studies on mammal Casq show that the C-terminal tail in domain III is an intrinsically disordered region involved in the polymerization and the binding of cations and transition metals (Bal et al., 2011). It is plausible to speculate that these protein domains could bind neurotossic transition metals in particular environment conditions. Thus, in aquatic organisms, the maintenance of a Casq isoform with a very long acidic tail could have conferred an evolutive advantage.

DATA AVAILABILITY STATEMENT

The datasets generated for this study have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD015577.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Padova Ethical Committee on Animal Experimentation and Ministero della salute (Project Number D2784.N.BGL).

AUTHOR CONTRIBUTIONS

AN and SF contributed to the conception, the design of the study and drafted and wrote the manuscript. MC, MM, SF, and SM performed the experiments and analyzed the data. FA and PV critically revised the manuscript. All the authors contributed to manuscript revision and read and approved the submitted version.

FUNDING

This work was supported by research funds from the University of Padova (BIRD 2016).

AKNOWLEDGMENTS

We thank Matthias Mann for his support in mass spectrometry data generation and analysis as well as Martina Milanetto, Igor Paron, and Christian Deiml for their technical assistance. We thank Sara Schiavon (Light Microscopy Facility) and the Zebrafish Facility Department of Biology, University of Padova.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnana.2020.00015/full#supplementary-material.

REFERENCES

Alonso, J. R., Arèvalo, R., Briñòn, J. G., Lara, J., Weruaga, E., and Aijòn, J. (1992). Parvalbumin immunoreactive neurons and fibres in the teleost cerebellum. *Anat. Embryol.* 185, 355–361. doi: 10.1007/bf00188547

Bae, Y. K., Kani, S., Shimizu, T., Tanabe, K., Nojima, H., Kimura, Y., et al. (2009). Anatomy of zebrafish cerebellum and screen for mutations affecting its development. *Dev. Biol.* 330, 406–426. doi: 10.1016/j.ydbio.2009.04.013

Bai, Q., Garver, J. A., Hukriede, N. A., and Burton, E. A. (2007). Generation of a transgenic zebrafish model of Tauopathy using a novel promoter element

derived from the zebrafish eno2 gene. *Nucleic Acids Res.* 35, 6501–6516. doi: 10.1093/nar/gkm608

Bal, N. C., Jena, N., Sopariwala, D., Balaraju, T., Shaikh, S., Bal, C., et al. (2011). Probing cationic selectivity of cardiac Casq and its CPVT mutants. *Biochem J.* 435, 391–399. doi: 10.1042/bj20101771

Bell, C. C., Han, V., and Sawtell, N. B. (2008). Cerebellum-like structures and their implications for cerebellar function. *Annu. Rev. Neurosci.* 31, 1–24. doi: 10.1146/annurev.neuro.30.051606.094225

Berg, E. M., Bertuzzi, M., and Ampatzis, K. (2018). Complementary expression of calcium binding proteins delineates the functional organization of the locomotor network. *Brain Struct. Funct.* 223, 2181–2196. doi: 10.1007/s00429-018-1622-4

Brini, M., Calì, T., Ottolini, D., and Carafoli, E. (2014). Neuronal calcium signalling: function and dysfunction. *Cell. Mol. Life Sci.* 71, 2787–2814. doi: 10.1007/s00018-013-1550-7

Castro, A., Becerra, M., Manso, M. J., and Anadón, R. J. (2006). Calretinin immunoreactivity in the brain of the zebrafish, *Danio rerio*: distribution and comparison with some neuropeptides and neurotransmitter-synthesizing enzymes. II. Midbrain, hindbrain and rostral spinal cord. *Comp. Neurol.* 494, 792–814. doi: 10.1002/cne.20843

Darbandi, S., and Franck, J. P. (2009). A comparative study of ryanodine receptor (RyR) gene expression levels in a basal ray-finned fish, bichir (Polypterus ornatipinnis) and the derived euteleost zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 154, 443–448. doi: 10.1016/j.cbpb.2009.09.003

Dieteren, C. E. J., Willems, P. H. G. M., Vogel, R. O., Swarts, H. G., Fransen, J., Roepman, R., et al. (2008). Subunits of mitochondrial complex I exist as part of matrix- and membrane-associated subcomplexes in living cells. *J. Biol. Chem.* 283, 34753–34761. doi: 10.1074/jbc.m807323200

Furlan, S., Mosole, S., Murgia, M., Nagaraj, N., Argenton, F., Volpe, P., et al. (2016). Casqs in skeletal and cardiac muscle from adult *Danio rerio*. *J. Muscle Res. Cell Motil.* 37, 27–39. doi: 10.1007/s10974-015-9432-2

Germanà, A., Marino, F., Guerrera, M. C., Campo, S., de Girolamo, P., Montalbano, G., et al. (2008). Expression and distribution of S100 protein in the nervous system of the adult zebrafish (*Danio rerio*). *Microsc. Res. Tech.* 71, 248–255. doi: 10.1002/jemt.20544

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I., and Brand, M. (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. *Developmental Biology.* 295, 263–277. doi: 10.1016/j.ydbio.2006.03.040

Hartmann, J., and Konnerth, A. (2008). Mechanisms of metabotropic glutamate receptor-mediated synaptic signalling in cerebellar Purkinje cells. *Acta Physiol.* 195, 79–90. doi: 10.1111/j.1748-1716.2008.01923.x

Hartmann, J., Karl, R. M., Alexander, R. P., Adelsberger, H., Brill, M. S., Rühlmann, C., et al. (2014). STIM1 controls neuronal Ca^{2+} signaling, mGluR1-dependent synaptic transmission, and cerebellar motor behavior. *Neuron* 82, 635–644. doi: 10.1016/j.neuron.2014.03.027

Holaska, J. M., Black, B. E., Love, D. C., Hanover, J. A., Leszyk, J., and Paschal, B. M. (2001). Calreticulin is a receptor for nuclear export. *J. Cell Biol.* 152, 127–140. doi: 10.1083/jcb.152.1.127

Holland, E. B., Goldstone, J. V., Pessah, I. N., Whitehead, A., Reid, N. M., Karchner, S. I., et al. (2017). Ryanodine receptor and FK506 binding protein 1 in the Atlantic killifish (Fundulus heteroclitus): a phylogenetic and population-based comparison. *Aquat. Toxicol.* 192, 105–115. doi: 10.1016/j.aquatox.2017.09.002

Horigane, S. I., Ozawa, Y., Yamada, H., and Takemoto-Kimura, S. (2019). Calcium signalling: a key regulator of neuronal migration. *J. Biochem.* 165, 401–409. doi: 10.1093/jb/mvz012

Huang, M. S., Wang, T. K., Liu, Y. W., Li, Y. T., Chi, T. H., Chou, C. W., et al. (2014). Roles of carbonic anhydrase 8 in neuronal cells and zebrafish. *Biochim. Biophys. Acta* 1840, 2829–2842. doi: 10.1016/j.bbagen.2014.04.017

Infante, C., Ponce, M., and Manchado, M. (2011). Duplication of Casq genes in teleosts: molecular characterization in the Senegalese solea (*Solea senegalensis*). *Comp. Biochem. Physiol. Part B* 158, 304–314. doi: 10.1016/j.cbpb.2011.01.002

Inoue, T., Kato, K., Kohda, K., and Mikoshiba, K. (1998). Type 1 inositol 1,4,5-trisphosphate receptor is required for induction of long-term depression in cerebellar Purkinje neurons. *J. Neurosci.* 18, 5366–5373. doi: 10.1523/JNEUROSCI.18-14-05366.1998

Izant, J., and McIntosh, J. (1980). Microtubule-associated proteins: a monoclonal antibody to MAP2 binds to differentiated neurons. *Proc. Natl. Acad. Sci. U S A* 77, 4741–4745. doi: 10.1073/pnas.77.8.4741

Jászai, J., Graupner, S., Tanaka, E. M., Funk, R. H., Huttner, W. B., Brand, M., et al. (2013). Spatial distribution of prominin-1 (CD133)-positive cells within germinative zones of the vertebrate brain. *PLoS One* 8:e63457. doi: 10.1371/journal.pone.0063457

Kani, S., Bae, Y. K., Shimizu, T., Tanabe, K., Satou, C., Parsons, M. J., et al. (2010). Proneural gene-linked neurogenesis in zebrafish cerebellum. *Dev. Biol.* 343, 1–17. doi: 10.1016/j.ydbio.2010.03.024

Kaslin, J., Kroehne, V., Benato, F., Argenton, F., and Brand, M. (2013). Development and specification of cerebellar stem and progenitor cells in zebrafish: from embryo to adult. *Neural Dev.* 8:9. doi: 10.1186/1749-8104-8-9

Komuro, Y., Galas, L., Lebon, A., Raoult, E., Fahrion, J. K., Tilot, A., et al. (2015). The role of calcium and cyclic nucleotide signalling in cerebellar granule cell migration under normal and pathological conditions. *Dev. Neurobiol.* 75, 369–387. doi: 10.1002/dneu.22219

Koulen, P., Janowitz, T., Johnston, L. D., and Ehrlich, B. E. (2000). Conservation of localization patterns of IP_3 receptor type 1 in cerebellar Purkinje cells across vertebrate species. *J. Neurosci. Res.* 61, 493–499. doi: 10.1002/1097-4547(20000901)61:5<493::aid-jnr3>3.0.co;2-9

Kress, S., Biechl, D., and Wullimann, M. F. (2015). Combinatorial analysis of calcium-binding proteins in larval and adult zebrafish primary olfactory system identifies differential olfactory bulb glomerular projection fields. *Brain Struct. Funct.* 220, 1951–1970. doi: 10.1007/s00429-014-0765-1

Kumada, T., and Komuro, H. (2004). Completion of neuronal migration regulated by loss of Ca^{2+} transients. *Proc. Natl. Acad. Sci. U S A* 101, 8479–8484. doi: 10.1073/pnas.0401000101

Labriola, C. A., Conte, I. L., López Medus, M., Parodi, A. J., and Caramelo, J. J. (2010). Endoplasmic reticulum calcium regulates the retrotranslocation of Trypanosoma cruzi calreticulin to the cytosol. *PLoS One* 5:e13141. doi: 10.1371/journal.pone.0013141

Miyamura, Y., and Nakayasu, H. (2001). Zonal distribution of Purkinje cells in the zebrafish cerebellum: analysis by means of a specific monoclonal antibody. *Cell Tissue Res.* 305, 299–305. doi: 10.1007/s004410100421

Moorman, A. F., Houweling, A. C., de Boer, P. A., and Christoffels, V. M. (2001). Sensitive nonradioactive detection of mRNA in tissue sections: novel application of the whole-mount *in situ* hybridization protocol. *J. Histochem. Cytochem.* 49, 1–8. doi: 10.1177/002215540104900101

Nolte, H., Konzer, A., Ruhs, A., Jungblut, B., Braun, T., and Krüger, M. (2014). Global protein expression profiling of zebrafish organs based on *in vivo* incorporation of stable isotopes. *J. Proteome Res.* 13, 2162–2174. doi: 10.1021/pr5000335

Pavlidis, P., and Noble, W. S. (2001). Analysis of strain and regional variation in gene expression in mouse brain. *Genome Biol.* 2:RESEARCH0042. doi: 10.1186/gb-2001-2-10-research0042

Rappsilber, J., Ishihama, Y., and Mann, M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* 75, 663–670. doi: 10.1021/ac026117i

Reddish, F. N., Miller, C. L., Gorkhali, R., and Yang, J. J. (2017). Calcium dynamics mediated by the endoplasmic/sarcoplasmic reticulum and related diseases. *Int. J. Mol. Sci.* 18:E1024. doi: 10.3390/ijms18051024

Robra, L., and Thirumalai, V. (2016). The intracellular signaling molecule darpp-32 is a marker for principal neurons in the cerebellum and cerebellum-like circuits of zebrafish. *Front. Neuroanat.* 10:81. doi: 10.3389/fnana.2016.00081

Rong, Y., Wang, T., and Morgan, J. I. (2004). Identification of candidate Purkinje cell-specific markers by gene expression profiling in wild-type and pcd^{3J} mice. *Mol. Brain Res.* 132, 128–145. doi: 10.1016/j.molbrainres.2004.10.015

Rose, C. R., and Konnerth, A. (2001). Stores not just for storage: intracellular calcium release and synaptic plasticity. *Neuron* 31, 519–522. doi: 10.1016/s0896-6273(01)00402-0

Ryu, C., Jang, D. C., Jung, D., Kim, Y. G., Shim, H. G., Ryu, H. H., et al. (2017). STIM1 regulates somatic Ca^{2+} signals and intrinsic firing

properties of cerebellar purkinje neurons. *J. Neurosci.* 37, 8876–8894. doi: 10.1523/JNEUROSCI.3973-16.2017

Sacchetto, R., Cliffer, K. D., Podini, P., Villa, A., Christensen, B. N., and Volpe, P. (1995). Intracellular Ca^{2+} stores in chick cerebellum Purkinje neurons: ontogenetic and functional studies. *Am. J. Physiol.* 269, C1219–C1227. doi: 10.1152/ajpcell.1995.269.5.c1219

Saleem, S., and Kannan, R. R. (2018). Zebrafish: an emerging real-time model system to study Alzheimer's disease and neurospecific drug discovery. *Cell Death Discov.* 4:45. doi: 10.1038/s41420-018-0109-7

Salvatori, S., Biral, D., Furlan, S., and Marin, O. (1997). Evidence for localization of the myotonic dystrophy protein kinase to the terminal cisternae of the sarcoplasmic reticulum. *J. Muscle Res. Cell Motil.* 18, 429–440. doi: 10.1023/a:1018694730773

Sanchez, E. J., Lewis, K. M., Danna, B. R., and Kang, C. (2012). High-capacity Ca^{2+} binding of human skeletal Casq. *J. Biol. Chem.* 287, 11592–11601. doi: 10.1074/jbc.m111.335075

Segal, M., and Korkotian, E. (2014). Endoplasmic reticulum calcium stores in dendritic spines. *Front. Neuroanat.* 8:64. doi: 10.3389/fnana.2014.00064

Shin, D. W., Ma, J., and Kim, D. H. (2000). The asp-rich region at the carboxyl-terminus of Casq binds to Ca^{2+} and interacts with triadin. *FEBS Lett.* 486, 178–182. doi: 10.1016/s0014-5793(00)02246-8

Smidak, R., Aradska, J., Kirchberger, S., Distel, M., Sialana, F. J., Wackerlig, J., et al. (2016). A detailed proteomic profiling of plasma membrane from zebrafish brain. *Proteomics Clin. Appl.* 10, 1264–1268. doi: 10.1002/prca.201600081

Takei, H., Stukenbrok, A., Metcalf, G. A., Mignery, T. C., Sudhof, P., Volpe, P., et al. (1992). Ca^{2+} stores in Purkinje neurons: endoplasmic reticulum subcompartments demonstrated by the heterogeneous distribution of the InsP3 receptor, Ca^{2+}-ATPase, and Casq. *J. Neurosci.* 12, 489–505. doi: 10.1523/JNEUROSCI.12-02-00489.1992

Takeuchi, M., Matsuda, K., Yamaguchi, S., Asakawa, K., Miyasaka, N., Lal, P., et al. (2015). Establishment of Gal4 transgenic zebrafish lines for analysis of development of cerebellar neural circuitry. *Dev. Biol.* 397, 1–17. doi: 10.1016/j.ydbio.2014.09.030

Takeuchi, M., Yamaguchi, S., Sakakibara, Y., Hayashi, T., Matsuda, K., Hara, Y., et al. (2017). Gene expression profiling of granule cells and Purkinje cells in the zebrafish cerebellum. *J. Comp. Neurol.* 525, 1558–1585. doi: 10.1002/cne.24114

Tomasi, M., Canato, M., Paolini, C., Dainese, M., Reggiani, C., Volpe, P., et al. (2012). Casq (CASQ1) rescues function and structure of calcium release units in skeletal muscles of CASQ1-null mice. *Am. J. Physiol. Cell Physiol.* 302, C575–C586. doi: 10.1152/ajpcell.00119.2011

Tse, M. K., Hung, T. S., Chan, C. M., Wong, T., Dorothea, M., Leclerc, C., et al. (2018). Identification of Ca^{2+} signaling components in neural stem/progenitor cells during differentiation into neurons and glia in intact and dissociated zebrafish neurospheres. *Sci. China Life Sci.* 61, 1352–1368. doi: 10.1007/s11427-018-9315-6

Ullmann, J. F. P., Cowin, G., Kurniawan, N. D., and Collin, S. P. (2010). A three-dimensional digital atlas of the zebrafish brain. *NeuroImage* 51, 76–82. doi: 10.1016/j.neuroimage.2010.01.086

Verkhratsky, A. (2005). Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. *Physiol. Rev.* 85, 201–279. doi: 10.1152/physrev.00004.2004

Villa, A., Podini, P., Clegg, D. O., Pozzan, T., and Meldolesi, J. (1991). Intracellular Ca^{2+} stores in chicken Purkinje neurons: differential distribution of the low affinity-high capacity Ca^{2+} binding protein, Casq, of Ca^{2+} ATPase and of the ER lumenal protein, Bip. *J. Cell Biol.* 113, 779–791. doi: 10.1083/jcb.113.4.779

Volpe, P., Alderson-Lang, B. H., Madeddu, L., Damiani, E., Collins, J. H., and Margreth, A. (1990). Casq, a component of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of chicken cerebellum. *Neuron* 5, 713–721. doi: 10.1074/jbc.274.46.32881

Volpe, P., Villa, A., Damiani, E., Sharp, A. H., Podini, P., Snyder, S. H., et al. (1991). Heterogeneity of microsomal Ca^{2+} stores in chicken Purkinje neurons. *EMBO J.* 10, 3183–3189. doi: 10.1002/j.1460-2075.1991.tb04880.x

Wang, Q., Groenendyk, J., Paskevicius, T., Qin, W., Kor, K. C., Liu, Y., et al. (2019). Two pools of IRE1α in cardiac and skeletal muscle cells. *FASEB J.* 33, 8892–8904. doi: 10.1096/fj.201802626R

Wang, L., Zhang, L., Li, S., Zheng, Y., Yan, X., Chen, M., et al. (2015). Retrograde regulation of STIM1-Orai1 interaction and store-operated Ca^{2+} entry by calsequestrin. *Sci. Rep.* 5:11349. doi: 10.1038/srep11349

Wegierski, T., and Kuznicki, J. (2018). Neuronal calcium signalling *via* store-operated channels in health and disease. *J. Cell Calcium.* 74, 102–111. doi: 10.1016/j.ceca.2018.07.001

Wu, H. H., Brennan, C., and Ashworth, R. (2011). Ryanodine receptors, a family of intracellular calcium ion channels, are expressed throughout early vertebrate development. *BMC Res. Notes* 4:541. doi: 10.1186/1756-0500-4-541

Zhang, L., Wang, L., Li, S., Xue, J., and Luo, D. (2016). Casq-1 regulates store-operated Ca^{2+} entry by inhibiting STIM1 aggregation. *Cell. Physiol. Biochem.* 38, 2183–2193. doi: 10.1159/000445574

Zündorf, G., and Reiser, G. (2011). Calcium dysregulation and homeostasis of neural calcium in the molecular mechanisms of neurodegenerative diseases provide multiple targets for neuroprotection. *Antioxid. Redox Signal.* 14, 1275–1288. doi: 10.1089/ars.2010.3359

Zupanc, G. K., Hinsch, K., and Gage, F. H. (2005). Proliferation, migration, neuronal differentiation, and long-term survival of new cells in the adult zebrafish brain. *J. Comp. Neurol.* 488, 290–319. doi: 10.1002/cne.20571

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroanatomy

ORIGINAL RESEARCH
published: 21 August 2020
doi: 10.3389/fnana.2020.00048

The Melanocortin System in Atlantic Salmon (*Salmo salar* L.) and Its Role in Appetite Control

Tharmini Kalananthan[1†], Floriana Lai[1†], Ana S. Gomes[1], Koji Murashita[1,2], Sigurd Handeland[1,3] and Ivar Rønnestad[1*]*

[1] Department of Biological Sciences, University of Bergen, Bergen, Norway, [2] Research Center for Aquaculture Systems, National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, Tamaki, Japan, [3] Norwegian Research Center, NORCE Environment, Bergen, Norway

The melanocortin system is a key neuroendocrine network involved in the control of food intake and energy homeostasis in vertebrates. Within the hypothalamus, the system comprises two main distinct neuronal cell populations that express the neuropeptides proopiomelanocortin (POMC; anorexigenic) or agouti-related protein (AGRP; orexigenic). Both bind to the melanocortin-4 receptor (MC4R) in higher order neurons that control both food intake and energy expenditure. This system is relatively well-conserved among vertebrates. However, in Atlantic salmon (*Salmo salar* L.), the salmonid-specific fourth round whole-genome duplication led to the presence of several paralog genes which might result in divergent functions of the duplicated genes. In the current study, we report the first comprehensive comparative identification and characterization of Mc4r and extend the knowledge of Pomc and Agrp in appetite control in Atlantic salmon. *In silico* analysis revealed multiple paralogs for *mc4r* (*a1*, *a2*, *b1*, and *b2*) in the Atlantic salmon genome and confirmed the paralogs previously described for *pomc* (*a1*, *a2*, and *b*) and *agrp* (*1* and *2*). All Mc4r paralogs are relatively well-conserved with the human homolog, sharing at least 63% amino acid sequence identity. We analyzed the mRNA expression of *mc4r*, *pomc*, and *agrp* genes in eight brain regions of Atlantic salmon post-smolt under two feeding states: normally fed and fasted for 4 days. The *mc4ra2* and *b1* mRNAs were predominantly and equally abundant in the hypothalamus and telencephalon, the *mc4rb2* in the hypothalamus, and *a1* in the telencephalon. All *pomc* genes were highly expressed in the pituitary, followed by the hypothalamus and saccus vasculosus. The *agrp* genes showed a completely different expression pattern from each other, with prevalent expression of the *agrp1* in the hypothalamus and *agrp2* in the telencephalon. Fasting did not induce any significant changes in the mRNA level of *mc4r*, *agrp*, or *pomc* paralogs in the hypothalamus or in other highly expressed regions between fed and fasted states. The identification and wide distribution of multiple paralogs of *mc4r*, *pomc*, and *agrp* in Atlantic salmon brain provide new insights and give rise to new questions of the melanocortin system in the appetite regulation in Atlantic salmon.

Keywords: melanocortin system, Atlantic salmon, *melanocortin-4 receptor* (*mc4r*), *proopiomelanocortin* (*pomc*), *agouti-related protein* (*agrp*), food intake, brain, appetite control centers

OPEN ACCESS

Edited by:
Livia D'Angelo,
University of Naples Federico II, Italy

Reviewed by:
Sebastiano Vilella,
University of Salento, Italy
Flavio S. J. De Souza,
Molecular Biology and Neurosciences (IFIBYNE), Argentina

****Correspondence:***
Tharmini Kalananthan
Tharmini.Kalananthan@uib.no
Ivar Rønnestad
Ivar.Ronnestad@uib.no

† These authors share first authorship

Received: *14 February 2020*
Accepted: *09 July 2020*
Published: *21 August 2020*

Citation:
Kalananthan T, Lai F, Gomes AS, Murashita K, Handeland S and Rønnestad I (2020) The Melanocortin System in Atlantic Salmon (Salmo salar L.) and Its Role in Appetite Control. Front. Neuroanat. 14:48. doi: 10.3389/fnana.2020.00048

INTRODUCTION

In vertebrates, food intake is controlled by the synergic actions of central and peripheral signaling pathways which provide information on ingestion and presence of food in the digestive tract and on the nutritional status (Volkoff, 2016; Rønnestad et al., 2017). In mammals, the melanocortin system is a key neuroendocrine network playing a pivotal role in regulating appetite and energy homeostasis. This system is mainly located within the hypothalamus where neurons expressing the melanocortin-4 receptor (MC4R) mediates either anorexigenic or orexigenic signals, thereby controlling the energy homeostasis of the animal (Nuzzaci et al., 2015). The MC4R neurons receive dual innervation from neurons expressing MCR agonists as melanocortin-stimulating hormones (α-, β-, γ-MSHs) and adrenocorticotropic hormone (ACTH), which derive from the posttranslational cleavage of proopiomelanocortin (POMC) peptide and from neurons expressing the antagonist agouti-related peptide (AGRP) (Ghamari-Langroudi et al., 2011; Kim et al., 2014; Nuzzaci et al., 2015). Furthermore, both POMC and AGRP neurons integrate peripheral endocrine signals and information on nutrient levels received through blood circulation or vagal afferent projections. In mammals, leptin was shown to play an anorexigenic role by increasing the excitability of POMC neurons and decreasing AGRP neuron action (Cowley et al., 2001; Baver et al., 2014); on the contrary, ghrelin plays an orexigenic (appetite stimulator) role by directly stimulating AGRP neurons and inhibiting POMC neurons (Riediger et al., 2003). This complex network has been well-described in mammals; however, in other vertebrates, such as teleost, little knowledge still exists.

Many of the neuropeptides and endocrine signals involved in appetite control in mammals have also been identified in teleost, although only a few have been functionally described.

The involvement of the Mc4r receptor in teleost energy balance was demonstrated in salmonids, and modulation of the Mc4r activity with the receptor antagonist (HS024 or SHU9119) or the agonist (MTII) increased or decreased, respectively, food intake in rainbow trout (*Oncorhynchus mykiss*) (Schjolden et al., 2009). In common carp (*Cyprinus carpio*), brain *mc4r* expression declined with fasting, while it surged with refeeding (Wan et al., 2012). Similarly, a winter fasting state also induced a lower expression of *mc4r* in the hypothalamus in Arctic charr (*Salvelinus alpinus*) (Striberny et al., 2015). In an extended study in spotted sea bass (*Lateolabrax maculatus*), incubation of isolated brain cells with α-MSH showed changes at *npy* and *agrp* levels and a downregulation of *mc4r* transcript levels during both short- and long-term fasting (Zhang et al., 2019). Both *in vitro* and *in vivo* experiments demonstrated that a naturally mutated Mc4r in Mexican cave fish (*Astyanax mexicanus*) is responsible for elevated appetite, growth, and starvation resistance as an adaptation to an environment with poor nutrient conditions (Aspiras et al., 2015). In contrast, food deprivation in barfin flounder (*Verasper moseri*) did not induce any changes of *mc4r* transcripts in the brain (Kobayashi et al., 2008).

Several studies have explored the involvement of Pomc in appetite control in teleost, and the results have suggested that its role may be species-specific. Intracerebroventricular administration (ICV) of the Mc4r agonist MTII downregulated *pomc* mRNA levels (Kojima et al., 2010), whereas intraperitoneal injection of cholecystokinin octapeptide (Kang et al., 2010) and leptin (Yan et al., 2016) upregulated *pomc* expression in the diencephalon, favoring enhanced anorexigenic action of Pomc. Starvation of zebrafish (*Danio rerio*) larvae resulted in a decrease in *pomc* expression levels, and refeeding after 2 days of fasting recovered *pomc* to the level of the control group (Liu et al., 2016). Under hyperglycemic conditions, rainbow trout resulted in increased hypothalamic *pomc* mRNA levels (Conde-Sieira et al., 2010; Otero-Rodiño et al., 2015), while 28 days of fasting downregulated *pomc* expression in the same species (Leder and Silverstein, 2006).

In Atlantic salmon (*Salmo salar*), brain *pomc* levels declined from 3 to 6 h of post-feeding of a single meal (Valen et al., 2011). In fully fed growth hormone (GH) transgenic coho salmon (*Oncorhynchus kisutch*), hypothalamic *pomc* mRNA decreased 4 h post-feeding, while there was no difference in the non-transgenic group (Kim et al., 2015). Similar results were also reported in GH transgenic zebrafish fasted for 2 days (Dalmolin et al., 2015). No changes in the *pomc* mRNA expression were observed in zebrafish (Opazo et al., 2019), barfin flounder (Takahashi et al., 2005), and goldfish (*Carassius auratus*) (Cerdá-Reverter et al., 2003b) under fasting regimes. In Atlantic halibut (*Hippoglossus hippoglossus*) larvae, the response of *pomc* to food deprivation and refeeding did not show a consistent expression pattern to explain their contribution to appetite control (Gomes et al., 2015).

The AGRP-mediated action on food intake seen in mammals appears to be conserved in teleost species. Ablation of Agrp1-expressing neurons and knockout of the *agrp1* gene showed that Agrp stimulates food consumption in zebrafish larvae (Shainer et al., 2019) or induces obesity in transgenic zebrafish overexpressing *agrp* (Song and Cone, 2007). An upregulation of *agrp* transcript was described in the larvae of the same species under fasting conditions (Song et al., 2003). GH transgenic common carp (Zhong et al., 2013) and coho salmon (Kim et al., 2015) showed increased hypothalamic *agrp1* mRNA and elevated food intake compared to the wild type. Moreover, fasting upregulated the hypothalamic *agrp* mRNA in goldfish (Cerdá-Reverter and Peter, 2003), coho salmon (Kim et al., 2015), sea bass (Agulleiro et al., 2014), mouth brooding African cichlid (*Astatotilapia burtoni*) (Porter et al., 2017), rainbow trout (Comesaña et al., 2017), seabream (*Sparus aurata*) larvae (Koch et al., 2019), Atlantic salmon (Kalananthan et al., 2020), and Ya-fish embryo (*Schizothorax prenanti*) (Wei et al., 2013). In contrast, an opposite action was described in the brain of common carp (Wan et al., 2012) and Atlantic salmon (Murashita et al., 2009; Valen et al., 2011).

The extensively described variations in the role of Mc4r receptor and neuropeptides Pomc and Agrp in teleost compared to mammals may be due to major physiological and environmental adaptation. Moreover, salmonids went through a salmonid-specific fourth round whole-genome duplication (Ss 4R WGD) around 80 million years ago (mya),

leading to large genomic rearrangements and the presence of several paralog genes, which may result in divergent functions for the different paralogs (Takahashi and Kawauchi, 2006; Warren et al., 2014; Lien et al., 2016).

Atlantic salmon is an economically important species of aquaculture industry in Norway. Periods of 2–4 days' fasting is a common practice during transport, handling, vaccination, and harvest of salmon to ensure a proper evacuation of the gut (Waagbø et al., 2017). Studying the impact of fasting on fish biology is essential to optimize the Atlantic salmon aquaculture practices with regard to the period of recovery, fish welfare, and feed utilization. In this study, we investigated the spatial gene expression of *mc4r, pomc*, and *agrp* genes and their paralogs in the brain of Atlantic salmon post smolts at fed and fasting (4 days) states. Our study provides a foundation for new insights on the role of *mc4r*, *pomc*, and *agrp* genes in appetite, feed intake, and fasting in Atlantic salmon.

MATERIALS AND METHODS

Ethics Statement

The animal experiments were carried out in accordance with Norwegian Animal Research Authority regulations and approved by the local representative of Animal Welfare at the Department of Biological Sciences, University of Bergen, Norway.

Experimental Design

In this study, Atlantic salmon post smolt of ca. 250 g were obtained from Engesund fish farm (Fitjar, Norway) and randomly distributed into two 2,000-L tanks (48 fish per tank) at the Industrial Lab (ILAB) in Bergen (Norway). Fish were reared in tanks supplied with flow through seawater (27 ppt; 16 L/min) at 10°C and oxygen saturation above 80%. Constant light (LD 24:0) was provided in accordance to common practice in commercial aquaculture to promote optimal growth and to inhibit unwanted sexual maturation (Hansen et al., 1992; Endal et al., 2000; Nordgarden et al., 2003; Fjelldal et al., 2012). Fish were fed continuously with commercial dry feed pellets (Biomar intro 75 HH 50 mg Q) using an automatic feeder. Oxygen saturation, temperature, and salinity were measured daily, and the fish were acclimatized for 3 weeks. After the acclimation period, the two tanks were randomly labeled into two experimental groups, fed, and fasted. Thereafter, 21 fish per tank (263 ± 13.06 g and 275.7 ± 15.68 g) were sampled as a baseline control. Next, one tank was kept under continuous feeding (fed group) with the same commercial dry pellet, whereas the other tank was fasted for 4 days (fasted group). After the 4 days, 27 fish were sampled from the fed group (280 ± 12.69 g) and 26 from the fasted group (246 ± 12.88 g). One fish was excluded from this group due to previous mild winter sore mark. Fish from the fed and the fasted group were collected and euthanized using an overdose of 200 mg/L of MS222 (Tricaine methanesulfonate, Scan-Vacc, Hvam, Norway) before and after the 4 days fed/fasted, respectively. Length and weight were recorded. The whole brains were rapidly collected and transferred into RNAlater solution (Invitrogen, Carlsbad, CA, United States), kept at 4°C overnight, and then stored in −80°C until further analysis.

Condition Factor (*K*) Calculation

Condition factor (*K*) was used to analyze the fitness of the fish before (fed $n = 21$; fasted $n = 21$) and after (fed $n = 27$; fasted $n = 26$) the feeding experiment by using weight and length of the fish in the following equation:

$$K = 100\frac{W}{L^3}$$

where W is the weight (g) and L is the length of the fish (cm) (Froese, 2006).

Structural Analysis and Phylogenetic Comparison of Mc4r and Pomc in the Salmonidae Family

Mc4r and Pomc peptide sequences of 17 species representatives of ray-finned fishes (Actinopterygiian) were retrieved from NCBI GenBank[1] and Ensembl[2]: the Lepisosteidae spotted gar (*Lepisosteus oculatus*) as a species before the teleost specific WGD (Ts WGD) (around 320 mya) (Lien et al., 2016), the Osteoglossidae Asian arowana (*Scleropages formosus*) as one of the oldest teleost groups; three Cyprinidae, including goldfish and common carp as species that went through a very recent 4R WGD and zebrafish which did not; one Characidae, cave fish; seven Salmonidae species, including Atlantic salmon, rainbow trout, chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), coho salmon, arctic char, and brown trout (*Salmo trutta*); the Esocidae northern pike (*Esox lucius*) as a sister group of salmonids that diverged before the Ss 4R WGD; and three Neoteleostei Atlantic cod (*Gadus morhua*), medaka (*Oryzias latipes*), and stickleback (*Gasterosteus aculeatus*) (**Supplementary Figures 2** and **3**). The human amino acid (AA) sequences were also included in the analysis. Multiple alignments were generated using MUSCLE from MEGAX (Hall, 2013) and edited using GeneDoc 2.7 software (Nicholas et al., 1997).

Mc4r transmembrane domains/helices (TMHI-TMHVII), extracellular loops (ECL1-ECL3), and intracellular loops (ICL1-ICL3) were retrieved from UniProt[3] database. Potential cleavage sites of Pomc precursor were acquired using UniProt and ProP 1.0[4] by using the full-length AA sequences.

The phylogenetic trees were predicted using the maximum likelihood (ML) method and based on the predicted full-length AA sequence of Mc4r and Pomc. The substitution model used in the phylogenetic analysis was determined by using the best-fit substitution model suggested by MEGAX. A Jones Taylor Thornton (JTT) and gamma distributed (G)

[1]https://www.ncbi.nlm.nih.gov/

[2]http://www.ensembl.org/index.html

[3]https://www.uniprot.org/

[4]http://www.cbs.dtu.dk/services/ProP/

matrix-based model was used to produce the phylogenetic tree for Mc4r, while a JTT and G with invariant sites (I) matrix-based model was used for Pomc (Hall, 2013). Tertiary protein structures of Atlantic salmon Mc4r were predicted using the IntFOLD5 (McGuffin et al., 2019) and human MC4R structure with AGRP (PDB entry 2IQV) was retrieved from UniProt. The images were edited, and disulfide bonds were predicted by PyMOL Molecular Graphics System v 2.3.[5] Searches for Agrp in Atlantic salmon genomic database did not identify any novel paralogs in addition to the ones previously published by Murashita et al. (2009).

Brain Dissection

The Atlantic salmon brain of fed (n = 6) and fasted group (n = 6) was randomly selected and dissected into eight regions for appetite gene expression analysis: olfactory bulb, telencephalon, midbrain, cerebellum, hypothalamus, saccus vasculosus, pituitary, and medulla oblongata/brain stem. To ensure high RNA yield and quality, the brain was placed on an ice block during dissection under a zoom stereomicroscope (Olympus SZ51) and cleaned from blood vessels. The pineal gland, olfactory bulb, telencephalon, brain stem, cerebellum, saccus vasculosus, hypothalamus, and midbrain were separated in this order (**Figure 4A**).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from each section of the brains by using TRI Reagent (Sigma-Aldrich, MO, United States) following the manufacturer's protocol. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, United States) and a 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, CA, United States) were used to assess the quantity and the quality of the extracted total RNA, respectively. To avoid any remnants of genomic DNA, 5 or 10 μg of total RNA was treated with TURBO DNase-free Kit (Ambion Applied Biosystems, CA, United States) with 1 μl of DNase (2 Units/μl) in 10 or 30 μl reaction volume. The amount of total RNA and the reaction volume for DNase treatment was adjusted depending on the amount of total RNA availability per region. First-strand cDNA was synthesized from 2 μg of the total RNA sample using SuperScript III Reverse Transcriptase (Invitrogen, CA, United States) and Oligo$(dT)_{20}$ (50 μM) primers in a total reaction volume of 20 μl.

Quantitative RT-PCR Setup and Primer Design

The salmon mRNA of *mc4r* (*a1*, *a2*, *b1*, and *b2*), *pomc* (*a1*, *a2*, and *b*), and *agrp* (*1* and *2*) was quantified by real-time quantitative RT-PCR (qPCR). The qPCR primers were designed from Atlantic salmon gene sequences retrieved from GenBank database (**Table 1** for accession numbers information). For each gene paralog, primer pairs were designed using Primer3[6] and/or NCBI primer designing tool and synthesized by Sigma-Aldrich (St. Louis, MO, United States). The specific primers were designed spanning exon–exon junctions when possible. All primers were analyzed for quantitation cycle (Cq), primers efficiency (E), and melting peaks. All qPCR products were analyzed in a 2% agarose gel, purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and cloned into a pCR4-TOPO vector (Thermo fisher, Scientific, Waltham, MA, United States). Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway), and their identity was confirmed using blastn analysis against the Atlantic salmon genome database.

Quantitative RT-PCR

To quantify the absolute mRNA abundance for each gene, qPCR products were purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and used to generate a standard curve using a 10-fold dilution series (initial concentration 10^{10} number of copies).

qPCR was carried out using 10 μl of iTaq Universal SYBR Green supermix (Bio-Rad, CA, United States), 0.6 μl of forward and 0.6 μl of reverse primers each (10 μM), 6.8 ultrapure water (Biochrom, Berlin, Germany), and 2 μl cDNA template (40 or 50 ng/reaction).

All reactions were run in duplicate, and a non-template control, no-reverse transcriptase control, and a positive between plate controls were always included. The following RT-PCR protocol was performed: (1) 95°C for 30 s, (2) 95°C for 5 s, (3) 60°C for 25 s, (4) repeating steps 2–3 for 39 more times. Melting curve analysis over a range of 65°C–95°C (increment of 0.5°C for 2 s) allowed the detection of non-specific products and/or primer dimers. The qPCR was performed using CFX96 Real-Time System (Bio-Rad Laboratories, CA, United States) in connection to CFX Manager Software version 3.1 (Bio-Rad, Laboratories, CA, United States).

Subsequently, the absolute mRNA expression level for each gene was determined based on the respective standard curve using the following equation:

$$Copy\ number = 10^{\left(\frac{Cq-intercept}{slope}\right)}$$

The copy number was normalized using the total ng of RNA used for each target gene.

Statistical Analysis

All statistical analyses were performed using GraphPad (GraphPad Software, version 8). Data related to the K were analyzed by two-way ANOVA followed by Sidak posttest. Equal variances and normality of distribution of gene expression were assessed using F-test and Shapiro–Wilk normality test. To achieve normal distribution, data were log-transformed and the analysis of differential expression between the fed and fasted groups was performed with two-tailed t-test. When either the F-test or the normality test failed, the no-parametric Mann–Whitney test was performed. Two-way analysis of variance (ANOVA) followed by the Sidak *post hoc* test was used to examine differences in the expression within the brain regions and the two treatment groups. A $p < 0.05$ was considered

[5]https://pymol.org/2/

[6]http://primer3.ut.ee/

TABLE 1 | Primers sequences used for quantitative RT-PCR (qPCR) mRNA expression analysis in Atlantic salmon.

Gene	Gene Bank ID	Primer Sequence (5′ → 3′)	Amplicon size (bp)	R^2	Efficiency %
pomca1	NM_001198575.1	ATACTTTTGAAACAGCGTGACGA	108	0.9985	103
		CAACGAGGATTCTCCCAGCA			
pomca2	NM_001198576.1	TTTGGCGACAGGCGAAGATG	91	0.999	98
	AB462420.1	TCCCAGCACTGACCTTTCAC			
pomcb	NM_001128604.1	CAGAGGACAAGATCCTGGAGTG	182	0.995	89
		TTTGTCGCTGTGGGACTCAG			
agrp1	NM_001146677.1	ATGGTCATCTCAGTATTCCCAT	152	0.9998	96
	XM_014182676.1	AGAGAGCCTTTACCGATATCTG			
	XM_014182677.1				
agrp2	NM_001146678.1	TGTTTCGCCGAAGACCTGAA	142	0.9986	101
		GTTTCTGAAATGCAACGTGGTG			
mc4ra1	XM_014140480.1	GTCATCGCCGCCATCATTAAG	152	0.9997	95
	XM_014140481.1	CCAATCCCCAGATTTCCGTC			
	XM_014140482.1				
mc4ra2	XM_014190362.1	TGGCAACTTGGGTATCGGC	170	0.9995	98
		GGCGCACGGTCATAATGTTG			
mc4rb1	XM_014157590.1	GGCGGTAATCGTGTGCATCT	185	0.9997	95
		GCACGGCGATCCTCTTTATG			
mc4rb2	XM_014180569.1	GAGCTCCCCGGGAAATAGTG	153	0.9996	97
		AGTGCAAATCAGTCCTCACCA			

Primer sequences, amplicon sizes (bp), R^2, and qPCR efficiency (in %) are indicated for each primer pair. agrp, agouti-related protein; mc4r, melanocortin-4 receptor; pomc, proopiomelanocortin.

significant. All data are presented as mean ± SEM, unless otherwise stated.

RESULTS

Characterization of Mc4r in Atlantic Salmon and Phylogenetic Analysis

In salmonids, four Mc4r protein paralogs (**Supplementary Figure 2**) were identified, showing well-conserved domains with respect to other ortholog sequences within teleost. In Atlantic salmon, Mc4r paralogs were found to be encoded by genes located on chromosomes ss03 (Mc4ra2), ssa14 (Mc4ra1), ssa19 (Mc4rb1), and ssa29 (Mc4rb2). The predicted AA sequence of Atlantic salmon Mc4r varied from 333 to 339 AA in length, and protein weighed from 37.37 to 37.99 kDa (data retrieved from UniProt). The paralogs Mc4ra1 and a2, and paralogs Mc4rb1 and b2, shared 89% identity at the AA level, whereas Mc4ra and Mc4rb shared at least 73% of identity. All four paralogs are relatively well-conserved with the human homolog, sharing from 63 to 68% of AA sequence identity. Atlantic salmon Mc4r paralogs shared from 73 to 90% AA identity with northern pike Mc4r and 73 to 95% of identity with other salmonid species (**Supplementary Table 3**). In the phylogenetic analysis, the teleost Mc4r divided into two clades and the Atlantic salmon Mc4r paralogs clustered into four different groups (**Figure 1**), each containing species belonging to the Salmonidae family. Each cluster, except for the Mc4rb1, branches from the northern pike. According to our phylogenetic analysis, two Mc4r duplicates (Mc4ra and Mc4rb) are present in salmonids and northern pike. In addition, salmonids have two copies of Mc4ra (Mc4ra1, Mc4ra2) and two copies of Mc4rb (Mc4rb1 and Mc4rb2) possibly as a result of the Ss 4R WGD. The alignment of human MC4R and Atlantic salmon Mc4r showed well-conserved seven transmembrane domains with divergent AA residues at N-terminus, ECL1, and C-terminus (**Figures 2**, **3**). Two of three N-terminal asparagine (N) N-glycosylated sites (NxS/T) are also conserved in Atlantic salmon (**Figure 2**). Further, the C-terminal palmitoylation site cysteine (Cys) residue Cys318 in human MC4R is conserved in the Atlantic salmon Mc4ra1 and a2. Importantly, motif DPxIY and C-terminal motif $E(x)_7LL$ for G Protein-Coupled Receptors (GPCRs) [reviewed in Rodrigues et al. (2013)] are conserved in all salmon paralogs. There are two putative disulfide bonds in human MC4R, one between Cys271 (TMHVI) and Cys277 (ECL3) and another between Cys40 (N-terminus) and Cys271 (ECL3). Our predicted model showed one disulfide bond within the ECL3 for Mc4ra1 (Cys274 and Cys280) and Mc4rb2 (Cys275 and Cys281) in Atlantic salmon (**Supplementary Figure 5**).

Characterization of Mature Pomc in Atlantic Salmon and Phylogenetic Analysis

In Atlantic salmon, three previously identified Pomc protein paralogs were located in chromosomes ssa01 (Pomca2), ssa06 (Pomcb), and ssa09 (Pomca1). The predicted AA sequence length of Atlantic salmon Pomc varied from 225 to 232 AA and protein weight from 24.7 to 25.9 kDa (data retrieved from UniProt). Pomca1 and a2 shared 84% of AA identity

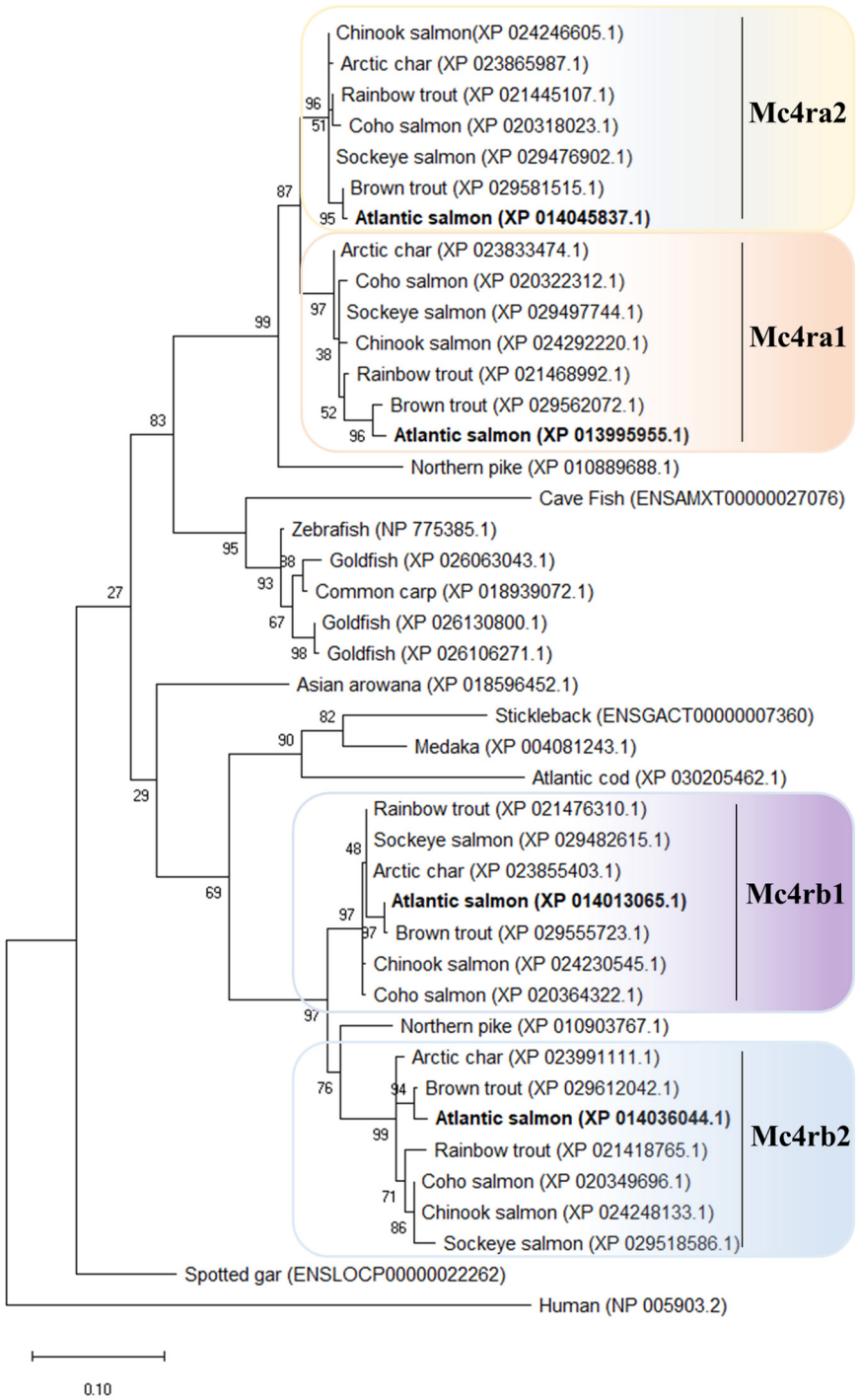


FIGURE 1 | Phylogenetic relationship of melanocortin-4 receptor (Mc4r) in Salmonidae family. The phylogenetic tree was constructed based on the predicted full-length peptide sequences using the maximum likelihood (ML) method, 1,000 bootstraps replicates, and JTT + G matrix-based model in MEGA X. The tree with the highest log likelihood (–3598.40) is shown. Protein ID accession numbers are shown after the species name. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Phylogenetic tree is rooted to the human MC4R sequence. For additional information related to the protein sequence alignment, please refer to **Supplementary Figure 2**.

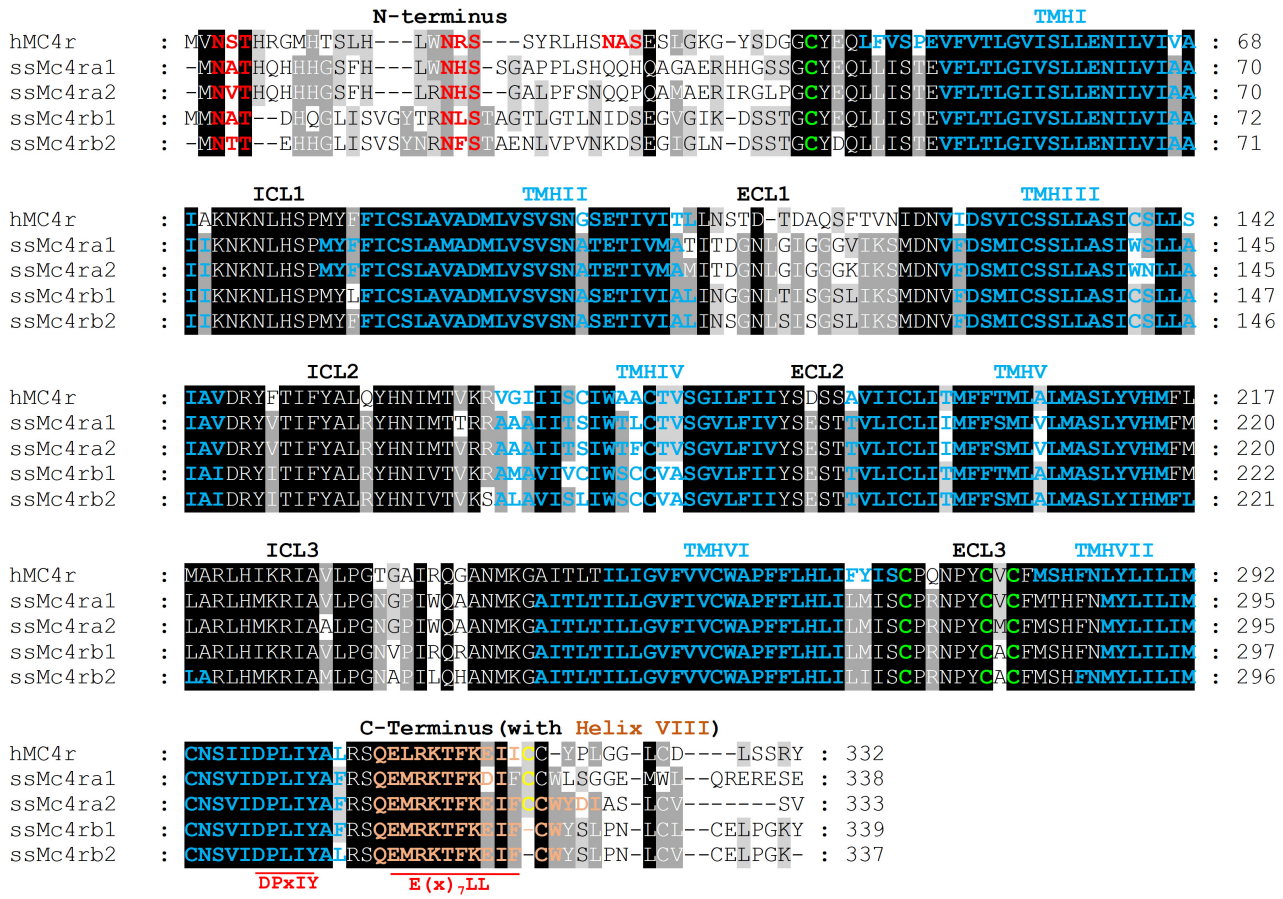


FIGURE 2 | Primary protein sequence alignment of the human melanocortin-4 receptor (hMC4R) and the Atlantic salmon paralogs (ssMc4ra1, a2, b1, and b2). The transmembrane domains for hMC4R (as reviewed in UniProt) and ssMc4r (as predicted in UniProt) are marked in blue. The N-terminal, extracellular loops (ECLs) 1–3, intracellular loops (ICLs) 1 to 3, and C-terminus (with helix VIII) are also shown. The N-terminal glycosylated amino acid residues and the important conserved motifs of GPCRs are marked in red (Rodrigues et al., 2013). C-terminal palmitoylation Cys is shown in yellow. The Cys involved in the disulfide bonds in hMC4R and those conserved in ssMc4r are in green.

(**Supplementary Table 4**). Pomcb AA sequence shared 36% of identity with Pomca1 and a2. Salmon Pomc paralogs shared 27–37% AA sequence identity with the human Pomc and 31–51% with northern pike Pomc. Atlantic salmon Pomc shared from 32 to 98% of AA identity with other species from the Salmonidae family. Atlantic salmon Pomc, as the human homolog, has a signal peptide of 26 AA, with the exception of Pomcb, which has a 21-AA signal peptide (**Supplementary Figure 3**). As expected, the phylogenetic analyses divided the salmonid Pomc peptide sequences in two main clusters Pomca and Pomcb (**Supplementary Figure 4**). The salmonid Pomca and Pomcb clustered with the northern pike and the Neoteleostei Pomca and Pomcb sequences, respectively. The salmonids have two copies of Pomca, whereas common carp and goldfish have duplicate Pomcs that belong to each of two separate clades. The phylogenetic tree suggests that the duplicated *pomc* would have evolved from Ts WGD.

The predicted posttranslational cleavages sites in Atlantic salmon were determined taking a comparative homology approach using the human homolog protein (**Supplementary Figure 4**). The human KR, KRR, and KK cleavage sites lead Pomc into mature peptide hormones: α-, β-, and γ-MSH, ACTH, corticotropin-like intermediate peptide (CLIP), β- and γ-LPH (lipotropin), β-endorphin, and INN (Met-enkephalin). In the teleost species analyzed, the same potential KR, KK, and RR cleavage sites were present in Pomca1 and a2, while in Pomcb, the last KK cleavage site was not present. Moreover, the alignment confirmed the lack of γ-MSH in teleost compared to human.

Brain Distribution of Atlantic Salmon *mc4r*, *pomc*, and *agrp* mRNA

Both the melanocortin receptor *mc4r* and the neuropeptides *pomc* and *agrp* mRNA analyzed in this study showed a wide distribution in the eight brain regions (**Figures 4**, **5**). All Atlantic salmon *mc4r* genes showed high mRNA expression levels in the hypothalamus, whereas *mc4ra1* was more abundant in the telencephalon (**Figure 4**). Interestingly, *mc4ra2* and *mc4rb1* showed a predominant mRNA abundance in the

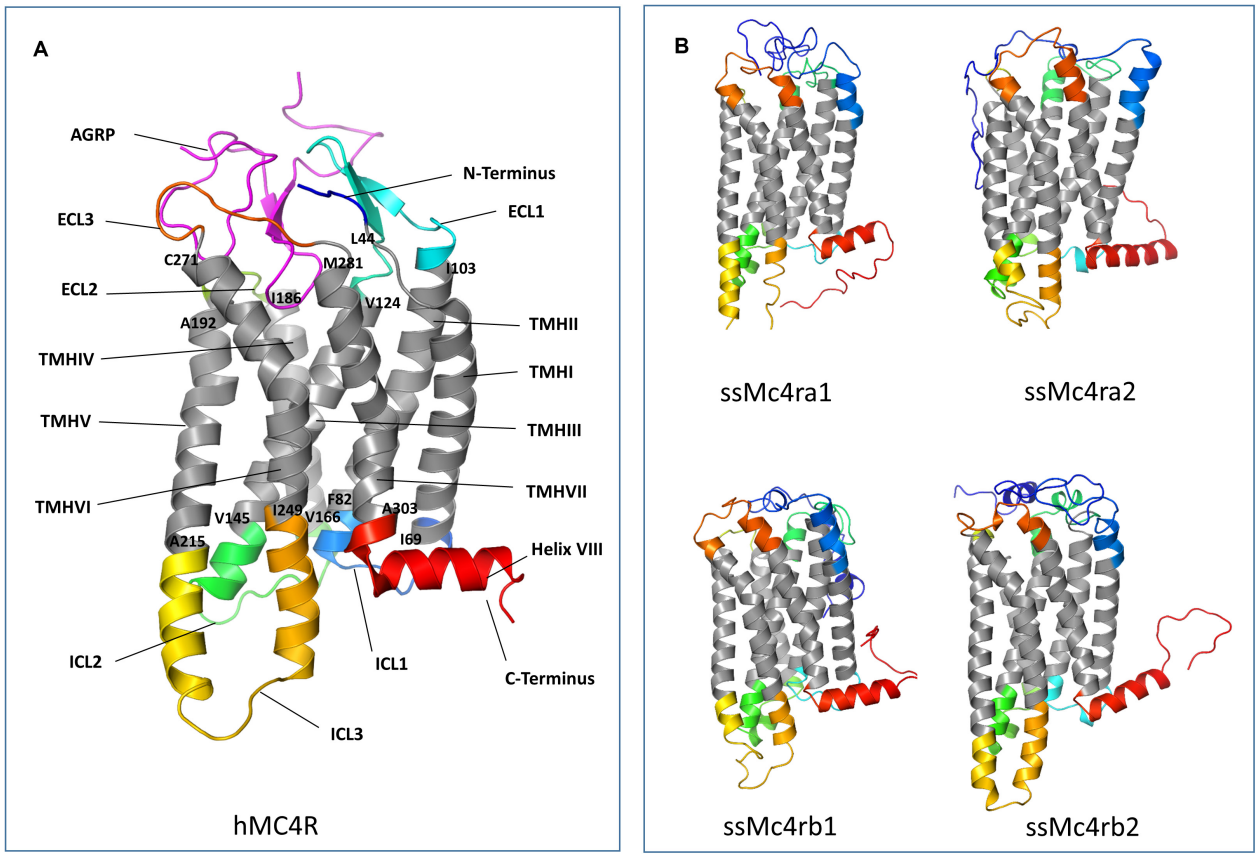


FIGURE 3 | Tertiary structure of human melanocortin-4 receptor (MC4R) and Atlantic salmon Mc4r paralogs. Three-dimensional (3D) protein structures were obtained from IntFOLD and edited in the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC. **(A)** Human MC4R 3D structure with agouti-related protein (AGRP) (PDB entry 2IQV). **(B)** Atlantic salmon Mc4r paralogs 3D structure. N-terminal domains are colored in dark blue, C-terminal domains in red, the seven transmembrane helices (TMH) in gray. The extracellular (ECL) and intracellular (ICL) loops are represented by different colors. The boundary amino acids of TMH are labeled according to the protein sequence alignment.

telencephalon and hypothalamus, and *mc4rb2* was high in the hypothalamus and similar expression level in other regions. The *mc4rb1* was the most abundant paralog in the Atlantic salmon brain. All *mc4r* paralogs show low mRNA expression levels in the olfactory bulb, cerebellum, and saccus vasculosus.

The *pomca1*, *pomca2*, and *pomcb* were predominantly expressed in the pituitary, followed by hypothalamus and saccus vasculosus, but low levels of expression were also found in other brain regions (**Figure 5**). *Agrp1* showed a prevalence gene expression in the hypothalamus, pituitary, and saccus vasculosus (**Figure 5**). On the contrary, *agrp2* was mainly expressed in the telencephalon, saccus vasculosus, and olfactory bulb.

Effects of 4 Days of Fasting in Atlantic Salmon

The *K* factor of fed and fasted Atlantic salmon was significantly different (two-way ANOVA $p = 0.0010$) (**Supplementary Figure 1**). After 4 days of fasting, fish showed a significantly lower *K* factor (1.035 ± 0.011) than the fed group (1.095 ± 0.015) (Sidak posttest $p = 0.0027$) (**Supplementary Table 1**).

No significant differences in the mRNA expressions of *mc4r*, *pomc*, and *agrp* paralogs were observed between the fed and fasted groups (**Figures 4**, **5**) in any highly expressed brain regions, i.e., hypothalamus, telencephalon, pituitary, and saccus vasculosus. On the other side, 4 days of fasting had a significant effect on the expression of *pomca1*, *pomca2*, and *pomcb* (**Figure 5**) in very low expressed regions (**Supplementary Table 2**). A significant decrease was found in the expression of *pomca1* in the olfactory bulb (*t*-test $p = 0.0057$), of *pomca2* in the telencephalon (*t*-test $p = 0.0233$) and cerebellum (*t*-test $p = 0.0340$), and on *pomcb* in the telencephalon (Mann–Whitney $p = 0.0303$). *pomca1* showed a decreased tendency in the telencephalon of the fasted group, although not statistically significant (*t*-test $p = 0.0873$). Both *agrp1* and *agrp2* did not show any significant difference between the fed and fasted groups (**Figure 5**). A high individual variation was observed in the mRNA expression levels of the target genes analyzed.

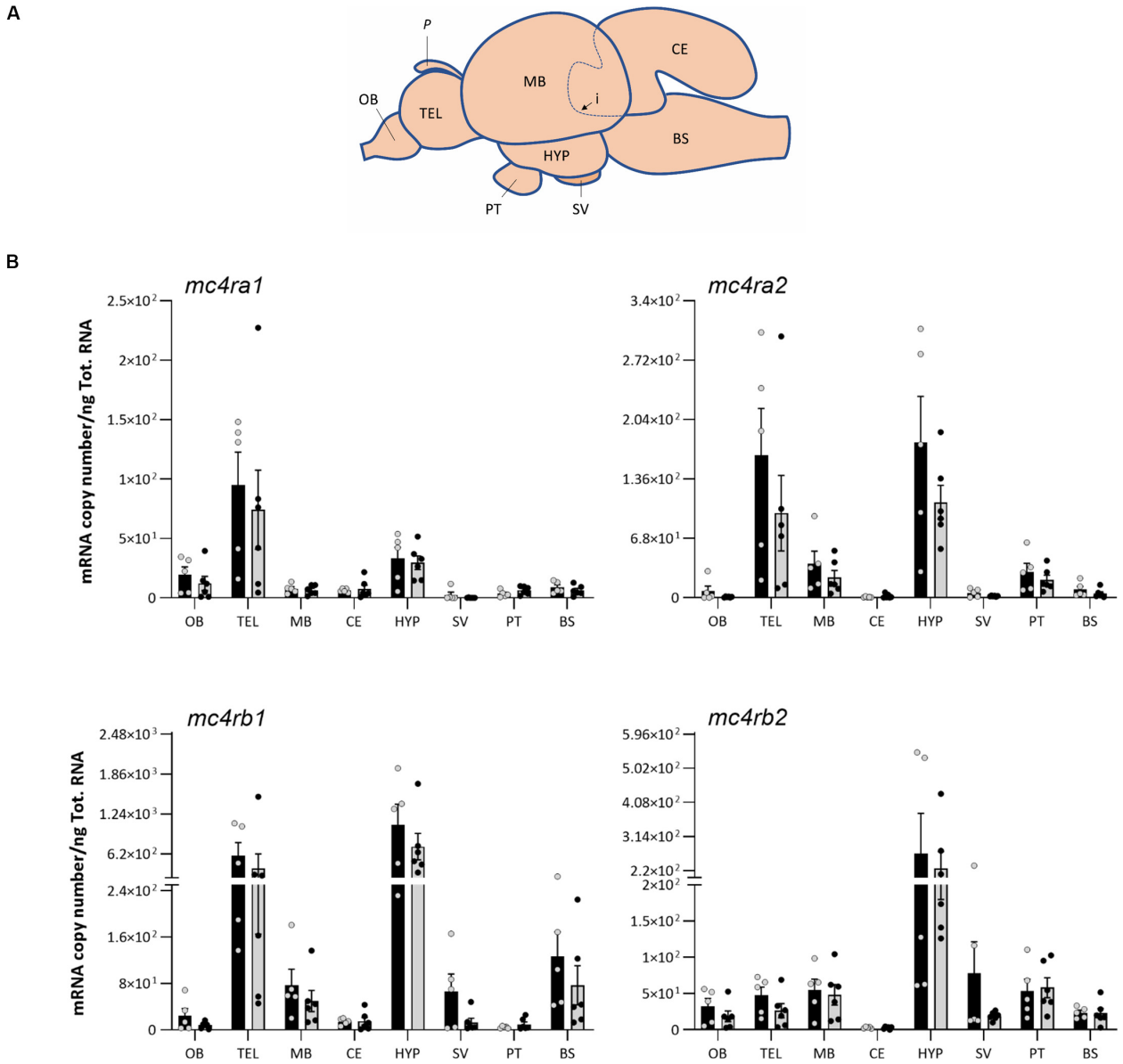


FIGURE 4 | (A) Schematic representation of Atlantic salmon post smolt brain, showing dissection of the eight brain regions for gene expression analysis: olfactory bulb (OB), telencephalon (TEL), midbrain (MB), hypothalamus (HYP), cerebellum (CE), saccus vasculosus (SV), pituitary (PT), and brain stem (BS). Dashed line (i) represents the dissected area of the CE inside the MB, and P represents the pineal gland. **(B)** Effects of 4 days of fasting on the mRNA expression levels of *melanocortin-4 receptor* (*mc4r*) paralog genes in eight regions of Atlantic salmon brain. Black and gray columns represent fed (n = 6) and fasted (n = 6) fish, respectively. Values are expressed as copy number per total RNA used in the reaction. The dots represent the individual fish, and bars represent mean ± SEM. Two-tailed t-test was performed to assess the statistically significant differences between the two groups. Interaction between the brain region response and the treatment was analyzed with two-way ANOVA (**Supplementary Table 1** for detailed information).

DISCUSSION

The role of the melanocortin system on appetite and energy homeostasis in Atlantic salmon is still poorly understood. In addition, the presence of several paralog genes as a result of the Ss 4R WGD has led to possible divergent functions of the key genes involved in this system.

In the current study, we report for the first time the identification and characterization of the Atlantic salmon receptor Mc4r. Our *in silico* analysis revealed the presence of four paralog genes *mc4ra1*, *mc4ra2*, *mc4rb1*, and *mc4rb2*, clustered into four different groups in all salmonid species analyzed. The presence of four Mc4r in Atlantic salmon (and other salmonids) appears to be the result of the Ss 4R WGD. The presence of *mc4ra* and *mc4rb* homologs in Northern pike suggests that the origin of

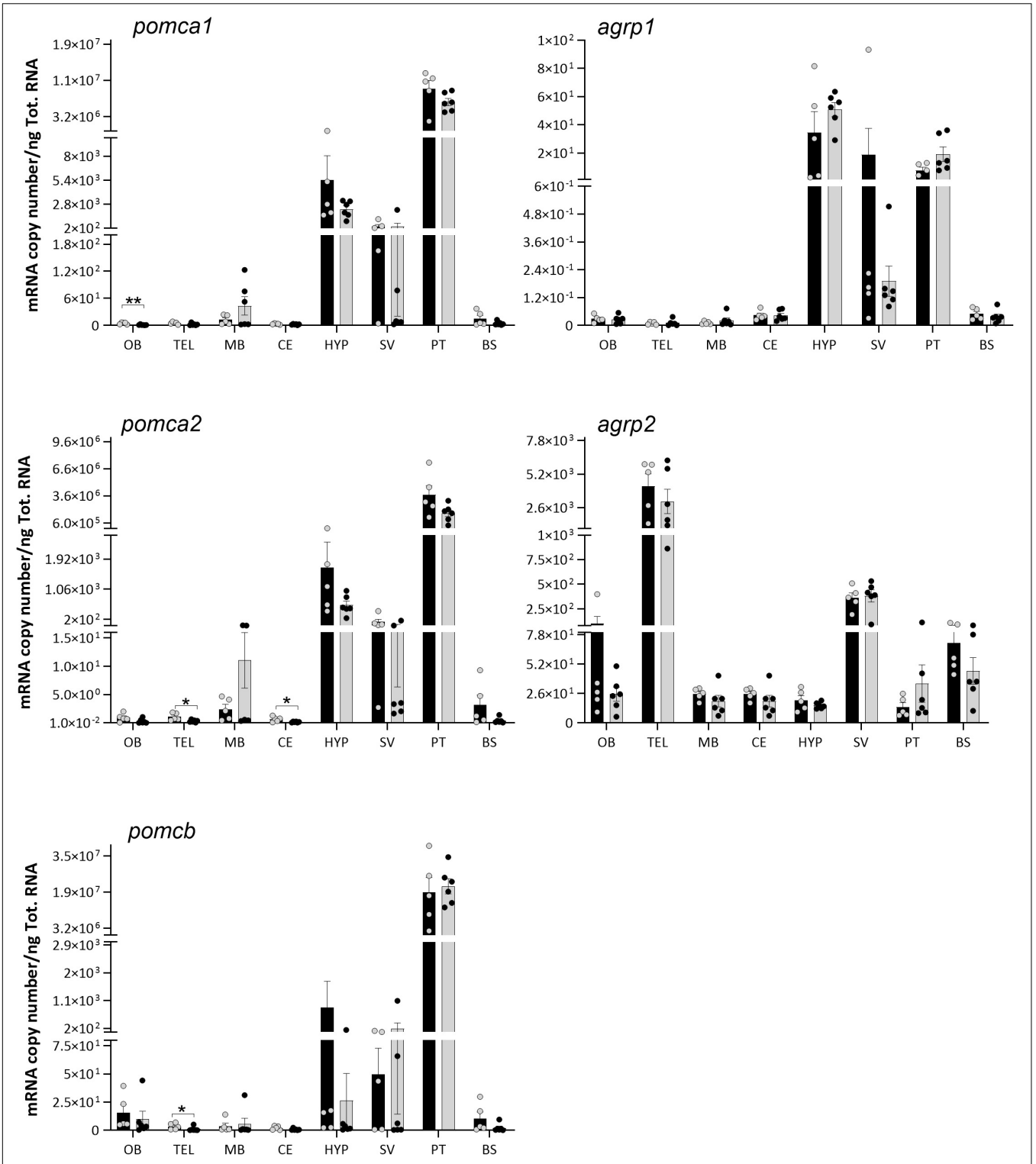


FIGURE 5 | Effects of 4 days of fasting on the mRNA expression levels of *proopiomelanocortin* (*pomc*) and *agouti-related protein* (*agrp*) paralog genes in eight regions of Atlantic salmon brain. Black and gray columns represent fed (n = 6) and fasted (n = 6) fish, respectively. Values are expressed as copy number per total RNA used in the reaction. The dots represent individual fish, bars represent mean ± SEM, and asterisks show the significant degree (*$p < 0.05$, **$p < 0.01$). Two-tailed t-test was performed to assess the statistically significant differences between the two groups. Interaction between the brain region response and the treatment was analyzed with two-way ANOVA, followed by Sidak posttest (refer to **Supplementary Table 1** for detailed information).

these genes occurred either just prior to the divergence between Salmoniformes and Esociformes or it is a result of an independent species-specific duplication. Cypriniformes, such as common carp and goldfish, have also experienced additional 4RWGD around 50–16 mya subsequent to the Ts WGD and Ss 4R WGD. Consequently, we found three Mc4r in goldfish but only one Mc4r in common carp (Larhammar and Risinger, 1994; David et al., 2003). Moreover, the Cypriniformes Mc4r is distantly related to the other analyzed teleost, which is reflected in the separated clade and seems to be the Mc4ra type. The confirmed mRNA sequences of the qPCR amplicons indicated that all four salmon *mc4r* genes are not pseudogenes. The Atlantic salmon showed well-conserved seven hydrophobic transmembrane domains as well as one putative disulfide bond, within the ECL3 for Mc4ra1 (Cys274 and Cys280) and Mc4rb2 (Cys275 and Cys281), as described for human MC4R (Cys271 and Cys277) (Chai et al., 2005; Chapman et al., 2010; Heyder et al., 2019). Even though Cys are also present in the primary sequence of Mc4ra2 and Mc4rb1, a disulfide bond was not present in the predicted tertiary structure in PyMOL. Natural mutation occurring in the human Cys271 (C271R and C271Y) have been linked to severe MC4R functional changes, but this AA substitution was not found in the predicted Atlantic salmon Mc4r sequence. Further analysis is needed to investigate these aspects in this species. The human N-glycosylated site (NxS/T) located in the N-terminus is also present in Atlantic salmon Mc4r paralogs. The glycosylated site and the disulfide bonds are important for the receptor structure folding, stability, and target trafficking (Chai et al., 2005; Tao, 2010; Rodrigues et al., 2013). Furthermore, the palmitoylation site at the Cys residue Cys318 of the C-terminus in human MC4R is also present in the Atlantic salmon Mc4ra1 and a2. The conserved C-terminal Cys318 serving as palmitoylation site might possibly lead to a fourth intracellular loop by anchoring the C-terminus to the cell membrane [reviewed in Tao (2010)]. Importantly, GPCR motifs N/DPxIY and $E(x)_7LL$ [reviewed in Rodrigues et al. (2013)] are also present in all Mc4r salmon paralogs. The N/DPxIY motif acts as an on/off switch with two conformational changes according to the active and inactive states (Chapman et al., 2010; Rodrigues et al., 2013), whereas $E(x)_7LL$ motif seems to be important in anterograde trafficking of MCRs (from endoplasmic reticulum to cell surface) [reviewed by Rodrigues et al. (2013)].

In general, for human MC4Rs, the pocket of aspartic acid Asp122/126 in TMHIII and basic histidine (His) 264 residues in TMHVI (Metz et al., 2006; Chapman et al., 2010; Wen et al., 2017; Heyder et al., 2019) along with ECL2 and ECL3 (Tao, 2010) are essential for ligand binding. β-MSH has been shown to the have the highest affinity to human MC4R, followed by α-MSH and ACTH (Tao, 2010). The same pocket seems to be conserved in Atlantic salmon; however, future studies are necessary to explore the ligand–Mc4r interactions in this species.

In the mammalian hypothalamus, numerous interconnecting nuclei, as the arcuate nucleus (ARC), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), and lateral hypothalamus (LH), are organized into a complex neuronal network that plays a crucial role in the central control of appetite (Bouret et al., 2004; Rønnestad et al., 2017; Soengas et al., 2018). The ARC has been described as the location for neurons expressing POMC and AGRP that project to the hypothalamic PVN where the MC4R is located (Ghamari-Langroudi et al., 2011; Hall, 2011). In teleost, the lateral tuberal nucleus (NLT) in the hypothalamus has been described as the homolog of the mammalian ARC (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003a,b). In goldfish and spotted sea bass, *in situ* hybridization showed neurons expressing *mc4r* in the telencephalon, thalamus, preoptic area, and hypothalamus (NLT and hypothalamic inferior lobe) (Cerdá-Reverter et al., 2003a; Zhang et al., 2019). Similarly, *agrp* and *pomc* were found in the NLT and in rostral hypothalamus of gold fish and rainbow trout (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003b; Otero-Rodiño et al., 2019).

Our results showed that all the Atlantic salmon *mc4r* paralogs were predominantly expressed in the hypothalamus and telencephalon, even though to a lesser extent expressed in other regions of the brain. It seems therefore that the hypothalamus and telencephalon are the major functional sites for the central *mc4r* in Atlantic salmon, but their role in appetite regulation is still unclear. These results are in line with the study of Zhang et al. (2019), where high *mc4r* levels were detected in the telencephalon and diencephalon of spotted sea bass. Among the Atlantic salmon *mc4r* paralogs, *mc4rb1* had the highest levels of expression, particularly in the hypothalamus, but its role in appetite regulation it is still unclear.

In this study, we have extended the current knowledge on Atlantic salmon *pomc* and *agrp* on appetite regulation, which was previously based on the analysis of the whole brain (Murashita et al., 2009, 2011) or on the hypothalamus (Kalananthan et al., 2020). Murashita et al. (2011, 2009) identified and characterized three *pomc* gene paralogs (*pomca1*, *a2*, and *b*) and one splice variant (*pomca2s*) and two *agrp* paralogs (*agrp1* and *2*). In goldfish, *in situ* hybridization studies demonstrated *pomc* mRNA cell bodies exclusively expressed within the mediobasal hypothalamus, in the NLT, and in the medial region of the lateral recess nucleus (Cerdá-Reverter et al., 2003b). In our spatial analysis, we found a clear dominant expression of *pomca1, pomca2,* and *pomcb* in the pituitary, followed by the hypothalamus and saccus vasculosus. The pituitary is an important site of *pomc* expression, where it is further post-translated into ACTH and α-MSH, responsible for the biosynthesis of glucocorticoids (e.g., cortisol) from the adrenal cortex (Dunn and Berridge, 1990). On the other hand, α-MSH in the hypothalamus activates the MC4R, leading to reduced food intake and increased energy expenditure (Anderson et al., 2016). The α-MSH has been described as the most well-conserved posttranslated forms of Pomc, underlining the strong functional constraint along the vertebrate lineage (Takahashi and Kawauchi, 2006).

The *agrp* paralogs showed different spatial distributions in the brain of Atlantic salmon. The *agrp1* was mainly expressed in the hypothalamus, as also described in previous studies in the ventral neurons of the NLT and rostral hypothalamus in goldfish (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003a), sea bream (Koch et al., 2019), and rainbow trout (Otero-Rodiño et al., 2019). The *agrp1* mRNA was also detected in other regions of the brain as saccus vasculosus and pituitary.

Agrp2 showed high expression levels in telencephalon and saccus vasculosus. Recently, Agrp1 was reported to be involved in the control of food consumption in zebrafish, while the Agrp2 in the preoptic neurons was suggested to act as a neuroendocrine regulator of stress response by downregulating cortisol secretion (Shainer et al., 2019).

The role of AGRP, POMC, and MC4R on appetite regulation have been suggested to be evolutionarily conserved across vertebrates (Ghamari-Langroudi et al., 2011; Kim et al., 2014; Nuzzaci et al., 2015). However, in the present study, no significant differences in mRNA expression of *mc4r*, *agrp*, or *pomc* paralogs between fed and fasted states were observed in the hypothalamus, which has been described as the central area in the control of appetite in mammals and teleost fishes (Nuzzaci et al., 2015; Volkoff, 2016; Rønnestad et al., 2017). Similar results were also observed in sea bass, where 4 days of food deprivation did not affect the *mc4r* expression in the hypothalamus or *pomc* mRNA expression in the hypothalamus and pituitary (Sánchez et al., 2009). Few studies have described that Mc4r was downregulated when feeding was restricted in teleost species (Wan et al., 2012; Aspiras et al., 2015; Striberny et al., 2015; Zhang et al., 2019). In barfin flounder, in contrast, the *mc4r* was downregulated in the liver, but no changes in expression were detected in the brain by fasting (Kobayashi et al., 2008). However, a previous study in sea bass showed the Mc4r activity to be dependent on the Agrp binding rather than the *mc4*r expression in case of progressive fasting (Sánchez et al., 2009).The importance of Mc4r in the regulation of appetite in fish is emphasized by naturally occurring mutations in Mexican cavefish (Aspiras et al., 2015). In this species, coding mutations in conserved residues reduces the signaling efficiency and basal activity of the Mc4r probably due to adaptation to long-term starvation and sporadic food availability (Aspiras et al., 2015).

On the other hand, 4 days of fasting had a significant effect on the expression of *pomca1*, *pomca2*, and *pomcb* in other regions of the Atlantic salmon brain. A significant decrease was found at the mRNA level of *pomca1* in the olfactory bulb, *pomca2* in the telencephalon and cerebellum, and *pomcb* in the telencephalon. However, it is important to underline that *pomc* was very lowly expressed in these regions, and it is not yet clear if these brain regions actually contribute to the appetite regulation in Atlantic salmon. In coho salmon, the posttranslational Pomc-derived α-Msh exhibits an anorexigenic effect (White et al., 2016). These authors showed that intraperitoneal injections of α-Msh suppressed feed intake, acting as an anorexigenic factor. However, in rainbow trout, 14 days of fasting did not have any effect on the mRNA expression of the three *pomc* paralogs (*pomca1*, *a2*, and *b*), but 28 days of fasting favored the decrease in hypothalamic *pomca1*, but not in *pomca2* or *pomcb* (Leder and Silverstein, 2006). Furthermore, hyperglycemic conditions increased the hypothalamic *pomca1* mRNA expression levels in rainbow trout (Conde-Sieira et al., 2010; Otero-Rodiño et al., 2015). In the whole-brain analysis of Atlantic salmon, the upregulation of *pomca1* (3 h post-feeding) and *pomcb* (0.5 and 6 h post-feeding) was suggested to represent a role in short-term feeding regulation (Valen et al., 2011).

In teleost, Agrp shows different functions depending on the region of the brain where it is expressed. In zebrafish, hypothalamic *agrp1* was proposed to have a similar function in the control of appetite and food intake as in mammals, whereas *agrp2* in the preoptic region acted as a stress regulator (Shainer et al., 2017, 2019). These authors also found *agrp2* to be expressed in the pineal and proposed that it may have a novel function rather than a neuroendocrine role involved in the regulation of the stress axis. An upregulation of Agrp was observed in short-term fasting of early larvae of Ya-fish (Wei et al., 2013). This was supported by Song et al. (2003) in zebrafish and Koch et al. (2019) in gilthead sea bream larvae, where starving increased *agrp1* expression. In another study, Agulleiro et al. (2014) reported the involvement of both *agrp* paralogs in appetite regulation by showing an increase of hypothalamic *agrp1* and decrease of *agrp2* in sea bass when subject to progressive fasting. In rainbow trout, Comesaña et al. (2017) showed that ICV of leucine decreased feed intake with a decrease in mRNA abundance of *agrp*. In Atlantic salmon, Murashita et al. (2009) reported that whole-brain *agrp1* was downregulated after 6 days of fasting, while *agrp2* was not affected, indicating that *agrp2* may not play a role on the control of appetite. A similar effect was described in common carp (Wan et al., 2012). In contrast, in our recent study, hypothalamic *agrp1* was upregulated after 3 days of fasting in Atlantic salmon (Kalananthan et al., 2020). However, in the current study, no differences were observed in the hypothalamus or other regions between the fed and fasted groups for either *agrp1* or *agrp2*.

The identification and basic characterization of the multiple paralogs of the appetite-regulating genes provide an essential groundwork to elucidate their functional role in the central control of food intake in Atlantic salmon. Minimal or no effects of fasting on the mRNA expression of the investigated genes suggest that they play a minor role in the central control of appetite in the short 4 days' fasting. However, a high individual variability was observed in both fed and fasted experimental groups, which might have led to a possible deviation in the results from our recent study on hypothalamic *agrp1* in Atlantic salmon (Kalananthan et al., 2020). Among the factors that contribute to the differences in feed intake, the feeding rate, frequency and time, and social relationships between conspecifics are the result of stimulating competition for a feed resource among individuals (Attia et al., 2012). Differences in physiology, life stages, feeding requirements, living environments, and individual variability might be at the base of the species-specific responses (Volkoff, 2016; Soengas et al., 2018). In salmonids as in other teleost species, the presence of dominant individuals can increase aggression and inhibition and limit food viability toward subordinate fishes, leading to differences in feeding behavior (Gilmour et al., 2005). Moreover, the difference in the sampling protocols and methodology used increases the complexity and variability of the data when comparing across and in between species.

The genome of teleost, compared to mammals, is the result of a third round (3R) or 4R (e.g., Salmonidae) of WGD. This evolutionary duplication make the teleost a potentially more complex model to study the function of feed-regulating factors

in comparison to the mammalian homologs (Volkoff, 2016). This fact should be taken into consideration when comparing studies in teleost *versus* mammals.

In conclusion, the present study provides new understanding of the still limited information available on the appetite regulation in Atlantic salmon. The identification of the multiple paralogs of *mc4r*, *pomc*, and *agrp* and their wide distribution in Atlantic salmon brain provide novel insights and lay the groundwork for experimental studies. Fasting did not affect the mRNA expression levels of melanocortin system players in the hypothalamus compared with fed fish. Further studies exploring the mRNA and/or protein localization within the brain areas and functional characterization are needed to elucidate the role of the melanocortin system in the central control of food intake in Atlantic salmon.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by local representative of Animal Welfare (at Department of Biological Sciences, University of Bergen, Norway) in accordance with Norwegian Animal Research Authority regulations.

AUTHOR CONTRIBUTIONS

All authors conceived and designed the study, contributed to the writing of the manuscript, and read and approved the submitted version. TK, SH, IR, and FL contributed to the sampling. KM, FL, and AG did the preparatory lab work. TK and FL performed the qPCR analysis. IR made the schematic illustration of Atlantic salmon brain. TK performed the tertiary protein structures. FL did the statistical and phylogeny analyses.

FUNDING

This study was supported by the Regional Research Fund West (Grant No. 259183) and the Research Council of Norway (Grant Nos. 267626 and 311627).

ACKNOWLEDGMENTS

We are grateful for technical assistance in the lab provided by Dr. Ann-Elise Olderbakk Jordal and assistance during sampling by Dr. Pablo Balseiro Vigo, Enrique Pino Martinez, Muhammad Rahmad Royan, and Silei Xia. A special thanks to Dr. Pablo Balseiro Vigo for the assistance during the transport of the fish to the ILAB facility. We thank Prof. Naoyuki Yamamoto for the precious advice on the brain dissections and localization of feeding centers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnana.2020.00048/full#supplementary-material

REFERENCES

Agulleiro, M. J., Cortés, R., Leal, E., Ríos, D., Sánchez, E., and Cerdá-Reverter, J. M. (2014). Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (Dicentrarchus labrax). *Gen. Comp. Endocrinol.* 205, 251–259. doi: 10.1016/j.ygcen.2014.02.009

Anderson, E. J. P., Carrington, S. J., Cone, R. D., Gillyard, T., Gimenez, L. E., and Litt, M. J. (2016). 60 years of POMC: regulation of feeding and energy homeostasis by α-MSH. *J. Mol. Endocrinol.* 56, 1–30. doi: 10.1530/JME-16-0014.60

Aspiras, A. C., Rohner, N., Martineau, B., Borowsky, R. L., and Tabin, C. J. (2015). Melanocortin 4 receptor mutations contribute to the adaptation of cavefish to nutrient-poor conditions. *Proc. Natl. Acad. Sci. U.S.A.* 112, 9668–9673. doi: 10.1073/pnas.1510802112

Attia, J., Millot, S., Di-Poï, C., Bégout, M. L., Noble, C., Sanchez-Vazquez, F. J., et al. (2012). Demand feeding and welfare in farmed fish. *Fish Physiol. Biochem.* 38, 107–118. doi: 10.1007/s10695-011-9538-9534

Baver, S. B., Hope, K., Guyot, S., Bjørbaek, C., Kaczorowski, C., and O'Connell, K. M. S. (2014). Leptin modulates the intrinsic excitability of AgRP/NPY neurons in the arcuate nucleus of the hypothalamus. *J. Neurosci.* 34, 5486–5496. doi: 10.1523/JNEUROSCI.4861-12.2014

Bouret, S. G., Draper, S. J., and Simerly, R. B. (2004). Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice. *J. Neurosci.* 24, 2797–2805. doi: 10.1523/JNEUROSCI.5369-03.2004

Cerdá-Reverter, J. M., and Peter, R. E. (2003). Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. *Endocrinology* 144, 4552–4561. doi: 10.1210/en.2003-2453

Cerdá-Reverter, J. M., Ringholm, A., Schiöth, H. B., and Peter, R. E. (2003a). Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: involvement in the control of food intake. *Endocrinology* 144, 2336–2349. doi: 10.1210/en.2002-2213

Cerdá-Reverter, J. M., Schiöth, H. B., and Peter, R. E. (2003b). The central melanocortin system regulates food intake in goldfish. *Regul. Pept.* 115, 101–113. doi: 10.1016/S0167-0115(03)00144-147

Chai, B. X., Pogozheva, I. D., Lai, Y. M., Li, J. Y., Neubig, R. R., Mosberg, H. I., et al. (2005). Receptor-antagonist interactions in the complexes of agouti and agouti-related protein with human melanocortin 1 and 4 receptors. *Biochemistry* 44, 3418–3431. doi: 10.1021/bi0478704

Chapman, K. L., Kinsella, G. K., Cox, A., Donnelly, D., and Findlay, J. B. C. (2010). Interactions of the melanocortin-4 receptor with the peptide agonist NDP-MSH. *J. Mol. Biol.* 401, 433–450. doi: 10.1016/j.jmb.2010.06.028

Comesaña, S., Velasco, C., Ceinos, R. M., López-Patiño, M. A., Míguez, J. M., Morais, S., et al. (2017). Evidence for the presence in rainbow trout brain of amino acid-sensing systems involved in the control of food intake. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 314, 201–215. doi: 10.1152/ajpregu.00283.2017

Conde-Sieira, M., Agulleiro, M. J., Aguilar, A. J., Míguez, J. M., Cerdá-Reverter, J. M., and Soengas, J. L. (2010). Effect of different glycaemic conditions on gene

expression of neuropeptides involved in control of food intake in rainbow trout. Interaction with stress. *J. Exp. Biol.* 213, 3858–3865. doi: 10.1242/jeb.048439

Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdán, M. G., Diano, S., Horvath, T. L., et al. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411, 480–484. doi: 10.1038/35078085

Dalmolin, C., Almeida, D. V., Figueiredo, M. A., and Marins, L. F. (2015). Food intake and appetite control in a GH-transgenic zebrafish. *Fish Physiol. Biochem.* 41, 1131–1141. doi: 10.1007/s10695-015-0074-75

David, L., Blum, S., Feldman, M. W., Lavi, U., and Hillel, J. (2003). Recent duplication of the common carp (*Cyprinus carpio* L.) Genome as revealed by analyses of microsatellite loci. *Mol. Biol. Evol.* 20, 1425–1434. doi: 10.1093/molbev/msg173

Dunn, A. J., and Berridge, C. W. (1990). Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Res. Rev.* 15, 71–100. doi: 10.1016/0165-0173(90)90012-D

Endal, H. P., Taranger, G. L., Stefansson, S. O., and Hansen, T. (2000). Effects of continuous additional light on growth and sexual maturity in Atlantic salmon, Salmo salar, reared in sea cages. *Aquaculture* 191, 337–349. doi: 10.1016/S0044-8486(00)00444-440

Fjelldal, P. G., Lock, E. J., Hansen, T., Waagbø, R., Wargelius, A., Gil Martens, L., et al. (2012). Continuous light induces bone resorption and affects vertebral morphology in Atlantic salmon (*Salmo salar* L.) fed a phosphorous deficient diet. *Aquac. Nutr.* 18, 610–619. doi: 10.1111/j.1365-2095.2011.00918.x

Froese, B. R. (2006). Cube law, condition factor and weight – length relationships: history, meta-analysis and recommendations. *J. Appl. Ichthyol.* 22, 241–253. doi: 10.1111/j.1439-0426.2006.00805.x

Ghamari-Langroudi, M., Srisai, D., and Cone, R. D. (2011). Multinodal regulation of the arcuate/paraventricular nucleus circuit by leptin. *Proc. Natl. Acad. Sci. U.S.A.* 108, 355–360. doi: 10.1073/pnas.1016785108

Gilmour, K. M., DiBattista, J. D., and Thomas, J. B. (2005). Physiological causes and consequences of social status in salmonid fish. *Integr. Comp. Biol.* 45, 263–273. doi: 10.1093/icb/45.2.263

Gomes, A. S., Jordal, A. E. O., Olsen, K., Harboe, T., Power, D. M., and Rønnestad, I. (2015). Neuroendocrine control of appetite in Atlantic halibut (*Hippoglossus hippoglossus*): changes during metamorphosis and effects of feeding. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 183, 116–125. doi: 10.1016/j.cbpa.2015.01.009

Hall, B. G. (2013). Building phylogenetic trees from molecular data with MEGA. *Mol. Biol. Evol.* 30, 1229–1235. doi: 10.1093/molbev/mst012

Hall, J. E. (2011). "Dietary balances; regulation of feeding; obesity and starvation; vitamins and minerals," in *Guyton and Hall Textbook of Medical Physiology*, ed. Saunders (Philadelphia: Elsevier Inc), 843–855.

Hansen, T., Stefansson, S., and Taranger, G. L. (1992). Growth and sexual maturation in Atlantic salmon, *Salmo salar* L., reared in sea cages at two different light regimes. *Aquac. Fish. Manag.* 23, 275–280. doi: 10.1111/j.1365-2109.1992.tb00770.x

Heyder, N., Kleinau, G., Szczepek, M., Kwiatkowski, D., Speck, D., Soletto, L., et al. (2019). Signal transduction and pathogenic modifications at the melanocortin-4 receptor: a structural perspective. *Front. Endocrinol.* 10:515. doi: 10.3389/fendo.2019.00515

Kalananthan, T., Murashita, K., Rønnestad, I., Ishigaki, M., Takahashi, K., Silva, M. S., et al. (2020). Hypothalamic agrp and pomc mRNA responses to gastrointestinal fullness and fasting in Atlantic salmon (*Salmo salar*. L.). *Front. Physiol.* 11:61. doi: 10.3389/fphys.2020.00061

Kang, K. S., Yahashi, S., Azuma, M., and Matsuda, K. (2010). The anorexigenic effect of cholecystokinin octapeptide in a goldfish model is mediated by the vagal afferent and subsequently through the melanocortin- and corticotropin-releasing hormone-signaling pathways. *Peptides* 31, 2130–2134. doi: 10.1016/j.peptides.2010.07.019

Kim, J. D., Leyva, S., and Diano, S. (2014). Hormonal regulation of the hypothalamic melanocortin system. *Front. Physiol.* 5:480. doi: 10.3389/fphys.2014.00480

Kim, J. H., Leggatt, R. A., Chan, M., Volkoff, H., and Devlin, R. H. (2015). Effects of chronic growth hormone overexpression on appetite-regulating brain gene expression in coho salmon. *Mol. Cell. Endocrinol.* 413, 178–188. doi: 10.1016/j.mce.2015.06.024

Kobayashi, Y., Tsuchiya, K., Yamanome, T., Schiöth, H. B., Kawauchi, H., and Takahashi, A. (2008). Food deprivation increases the expression of melanocortin-4 receptor in the liver of barfin flounder. *Verasper moseri*. *Gen. Comp. Endocrinol.* 155, 280–287. doi: 10.1016/j.ygcen.2007.05.010

Koch, L., Shainer, I., Gurevich, T., and Holzman, R. (2019). The expression of agrp1, a hypothalamic appetite-stimulating neuropeptide, reveals hydrodynamic-induced starvation in a larval fish. *Integr. Org. Biol.* 1, 1–10. doi: 10.1093/iob/oby003

Kojima, K., Amiya, N., Kamijo, M., Kageyama, H., Uchiyama, M., Shioda, S., et al. (2010). Relationship between α-melanocyte-stimulating hormone- and neuropeptide Y-containing neurons in the goldfish hypothalamus. *Gen. Comp. Endocrinol.* 167, 366–372. doi: 10.1016/j.ygcen.2009.12.004

Larhammar, D., and Risinger, C. (1994). Molecular genetic aspects of tetraploidy in the Common carp Cyprinus carpio. *Mol. Phylogenet. Evol.* 3, 59–68. doi: 10.1006/mpev.1994.1007

Leder, E. H., and Silverstein, J. T. (2006). The pro-opiomelanocortin genes in rainbow trout (*Oncorhynchus mykiss*): duplications, splice variants, and differential expression. *J. Endocrinol.* 188, 355–363. doi: 10.1677/joe.1.06283

Lien, S., Koop, B. F., Sandve, S. R., Miller, J. R., Kent, M. P., Nome, T., et al. (2016). The Atlantic salmon genome provides insights into rediploidization. *Nature* 533, 200–205. doi: 10.1038/nature17164

Liu, S., Zhang, C. Z., and Peng, G. (2016). Effects of starvation on the expression of feeding related neuropeptides in the larval zebrafish hypothalamus. *Yi Chuan* 38, 821–830. doi: 10.16288/j.yczz.16-087

McGuffin, L. J., Adiyaman, R., Maghrabi, A. H. A., Shuid, A. N., Brackenridge, D. A., Nealon, J. O., et al. (2019). IntFOLD: an integrated web resource for high performance protein structure and function prediction. *Nucleic Acids Res.* 47, 408–413. doi: 10.1093/nar/gkz322

Metz, J. R., Peters, J. J. M., and Flik, G. (2006). Molecular biology and physiology of the melanocortin system in fish: a review. *Gen. Comp. Endocrinol.* 148, 150–162. doi: 10.1016/j.ygcen.2006.03.001

Murashita, K., Jordal, A. E. O., Nilsen, T. O., Stefansson, S. O., Kurokawa, T., Björnsson, B. T., et al. (2011). Leptin reduces Atlantic salmon growth through the central pro-opiomelanocortin pathway. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 158, 79–86. doi: 10.1016/j.cbpa.2010.09.001

Murashita, K., Kurokawa, T., Ebbesson, L. O. E., Stefansson, S. O., and Rønnestad, I. (2009). Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (*Salmo salar*). *Gen. Comp. Endocrinol.* 162, 160–171. doi: 10.1016/j.ygcen.2009.03.015

Nicholas, K. B., Nicholas, H. B. J., and Deerfield, D. W. (1997). GeneDoc: analysis and visualization of genetic variation. *Embnew. News* 4:14.

Nordgarden, U., Oppedal, F., Taranger, G. L., Hemre, G. I., and Hansen, T. (2003). Seasonally changing metabolism in Atlantic salmon (*Salmo salar* L.) I - Growth and feed conversion ratio. *Aquac. Nutr.* 9, 287–293. doi: 10.1046/j.1365-2095.2003.00256.x

Nuzzaci, D., Laderrière, A., Lemoine, A., Nédélec, E., Pénicaud, L., Rigault, C., et al. (2015). Plasticity of the melanocortin system: determinants and possible consequences on food intake. *Front. Endocrinol.* 6:143. doi: 10.3389/fendo.2015.00143

Opazo, R., Plaza-Parrochia, F., Cardoso Dos Santos, G. R., Carneiro, G. R. A., Sardela, V. F., Romero, J., et al. (2019). Fasting Upregulates npy, agrp, and ghsr without Increasing Ghrelin Levels in Zebrafish (*Danio rerio*) larvae. *Front. Physiol.* 10:1901. doi: 10.3389/fphys.2018.01901

Otero-Rodiño, C., Librán-Pérez, M., Velasco, C., López-Patiño, M. A., Míguez, J. M., and Soengas, J. L. (2015). Evidence for the presence of glucosensor mechanisms not dependent on glucokinase in hypothalamus and hindbrain of rainbow trout (*Oncorhynchus mykiss*). *PLoS One* 10:e0128603. doi: 10.1371/journal.pone.0128603

Otero-Rodiño, C., Rocha, A., Sánchez, E., Álvarez-Otero, R., Soengas, J. L., and Cerdá-Reverter, J. M. (2019). Sensing glucose in the central melanocortin circuits of rainbow trout: a morphological study. *Front. Endocrinol.* 10:254. doi: 10.3389/fendo.2019.00254

Porter, D. T., Roberts, D. A., and Maruska, K. P. (2017). Distribution and female reproductive state differences in orexigenic and anorexigenic neurons in the brain of the mouth brooding African cichlid fish, *Astatotilapia burtoni*. *J. Comp. Neurol.* 525, 3126–3157. doi: 10.1002/cne.24268

Riediger, T., Traebert, M., Schmid, H. A., Scheel, C., Lutz, T. A., and Scharrer, E. (2003). Site-specific effects of ghrelin on the neuronal activity in the hypothalamic arcuate nucleus. *Neurosci. Lett.* 341, 151–155. doi: 10.1016/S0304-3940(02)01381-1382

Rodrigues, A. R., Sousa, D., Almeida, H., and Gouveia, A. M. (2013). Structural determinants regulating cell surface targeting of melanocortin receptors. *J. Mol. Endocrinol.* 51, 23–32. doi: 10.1530/JME-13-0055

Rønnestad, I., Gomes, A. S., Murashita, K., Angotzi, R., Jönsson, E., and Volkoff, H. (2017). Appetite-controlling endocrine systems in teleosts. *Front. Endocrinol.* 8:73. doi: 10.3389/fendo.2017.00073

Sánchez, E., Rubio, V. C., Thompson, D., Metz, J., Flik, G., Millhauser, G. L., et al. (2009). Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (*Dicentrarchus labrax*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 296, 1293–1306. doi: 10.1152/ajpregu.90948.2008

Schjolden, J., Schiöth, H. B., Larhammar, D., Winberg, S., and Larson, E. T. (2009). Melanocortin peptides affect the motivation to feed in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 160, 134–138. doi: 10.1016/j.ygcen.2008.11.003

Shainer, I., Buchshtab, A., Hawkins, T. A., Wilson, S. W., Cone, R. D., and Gothilf, Y. (2017). Novel hypophysiotropic AgRP2 neurons and pineal cells revealed by BAC transgenesis in zebrafish. *Sci. Rep.* 7, 1–10. doi: 10.1038/srep44777

Shainer, I., Michel, M., Marquart, G. D., Bhandiwad, A. A., Zmora, N., Ben-Moshe Livne, Z., et al. (2019). Agouti-related protein 2 is a new player in the teleost stress response system. *Curr. Biol.* 29:2009-2019.e7. doi: 10.1016/j.cub.2019.05.021

Soengas, J. L., Cerdá-Reverter, J. M., and Delgado, M. J. (2018). Central regulation of food intake in fish: an evolutionary perspective. *J. Mol. Endocrinol.* 60, R171–R199. doi: 10.1530/JME-17-0320

Song, Y., and Cone, R. D. (2007). Creation of a genetic model of obesity in a teleost. *FASEB J.* 21, 2042–2049. doi: 10.1096/fj.06-7503com

Song, Y., Golling, G., Thacker, T. L., and Cone, R. D. (2003). Agouti-Related Protein (AGRP) Is Conserved and Regulated by Metabolic State in the Zebrafish, *Danio rerio*. *Endocrine* 22, 257–265.

Striberny, A., Ravuri, C. S., Jobling, M., Jørgensen, E. H., and Fuentes, J. (2015). Seasonal differences in relative gene expression of putative central appetite regulators in arctic charr (*Salvelinus alpinus*) do not reflect its annual feeding cycle. *PLoS One* 10:e0138857. doi: 10.1371/journal.pone.0138857

Takahashi, A., Amano, M., Itoh, T., Yasuda, A., Yamanome, T., Amemiya, Y., et al. (2005). Nucleotide sequence and expression of three subtypes of proopiomelanocortin mRNA in barfin flounder. *Gen. Comp. Endocrinol.* 141, 291–303. doi: 10.1016/j.ygcen.2005.01.010

Takahashi, A., and Kawauchi, H. (2006). Evolution of melanocortin systems in fish. *Gen. Comp. Endocrinol.* 148, 85–94. doi: 10.1016/j.ygcen.2005.09.020

Tao, Y. X. (2010). The melanocortin-4 receptor: physiology, pharmacology, and pathophysiology. *Endocr. Rev.* 31, 506–543. doi: 10.1210/er.2009-2037

Valen, R., Jordal, A. E. O., Murashita, K., and Rønnestad, I. (2011). Postprandial effects on appetite-related neuropeptide expression in the brain of Atlantic salmon, *Salmo salar*. *Gen. Comp. Endocrinol.* 171, 359–366. doi: 10.1016/j.ygcen.2011.02.027

Volkoff, H. (2016). The neuroendocrine regulation of food intake in fish: a review of current knowledge. *Front. Neurosci.* 10:540. doi: 10.3389/fnins.2016.00540

Waagbø, R., Jørgensen, S. M., Timmerhaus, G., Breck, O., and Olsvik, P. A. (2017). Short-term starvation at low temperature prior to harvest does not impact the health and acute stress response of adult Atlantic salmon. *PeerJ* 5:e3273. doi: 10.7717/peerj.3273

Wan, Y., Zhang, Y., Ji, P., Li, Y., Xu, P., and Sun, X. (2012). Molecular characterization of CART, AgRP, and MC4R genes and their expression with fasting and re-feeding in common carp (*Cyprinus carpio*). *Mol. Biol. Rep.* 39, 2215–2223. doi: 10.1007/s11033-011-0970-974

Warren, I. A., Ciborowski, K. L., Casadei, E., Hazlerigg, D. G., Martin, S., Jordan, W. C., et al. (2014). Extensive local gene duplication and functional divergence among paralogs in Atlantic Salmon. *Genome Biol. Evol.* 6, 1790–1805. doi: 10.1093/gbe/evu131

Wei, R. Bin, Yuan, D. Y., Wang, T., Zhou, C. W., Lin, F. J., Chen, H., et al. (2013). Characterization, tissue distribution and regulation of agouti-related protein (AgRP) in a cyprinid fish (*Schizothorax prenanti*). *Gene* 527, 193–200. doi: 10.1016/j.gene.2013.06.003

Wen, Z.-Y., Zou, Y.-C., Qin, C.-J., Yuan, D.-Y., and Li, R. (2017). International journal of agricultural science and food technology DOI CC By Melanocortin-4 receptor in fish: a review. *Int. J. Agri. Sci. Food Technol.* 3, 1–4. doi: 10.17352/2455-815X.000014

White, S. L., Volkoff, H., and Devlin, R. H. (2016). Regulation of feeding behavior and food intake by appetite-regulating peptides in wild-type and growth hormone-transgenic coho salmon. *Horm. Behav.* 84, 18–28. doi: 10.1016/j.yhbeh.2016.04.005

Yan, A. F., Chen, T., Chen, S., Ren, C. H., Hu, C. Q., Cai, Y. M., et al. (2016). Goldfish leptin-AI and leptin-AII: function and central mechanism in feeding control. *Int. J. Mol. Sci.* 17:783. doi: 10.3390/ijms17060783

Zhang, K. Q., Hou, Z. S., Wen, H. S., Li, Y., Qi, X., Li, W. J., et al. (2019). Melanocortin-4 receptor in spotted sea bass, lateolabrax maculatus: cloning, tissue distribution, physiology, and pharmacology. *Front. Endocrinol.* 10:705. doi: 10.3389/fendo.2019.00705

Zhong, C., Song, Y., Wang, Y., Zhang, T., Duan, M., Li, Y., et al. (2013). Increased food intake in growth hormone-transgenic common carp (*Cyprinus carpio* L.) may be mediated by upregulating Agouti-related protein (AgRP). *Gen. Comp. Endocrinol.* 192, 81–88. doi: 10.1016/j.ygcen.2013.03.024

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroanatomy

ORIGINAL RESEARCH
published: 02 February 2021
doi: 10.3389/fnana.2020.565109

Cerebrotypes in Cephalopods: Brain Diversity and Its Correlation With Species Habits, Life History, and Physiological Adaptations

Giovanna Ponte[1†], *Morag Taite*[2†], *Luciana Borrelli*[1], *Andrea Tarallo*[3], *A. Louise Allcock*[2] *and Graziano Fiorito*[1*]

[1] Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Naples, Italy, [2] Department of Zoology, Ryan Institute, National University of Ireland Galway, Galway, Ireland, [3] Department of Research Infrastructures for Marine Biological Resources (RIMAR), Stazione Zoologica Anton Dohrn, Naples, Italy

Here we analyze existing quantitative data available for cephalopod brains based on classical contributions by J.Z. Young and colleagues, to cite some. We relate the relative brain size of selected regions (area and/or lobe), with behavior, life history, ecology and distribution of several cephalopod species here considered. After hierarchical clustering we identify and describe ten clusters grouping 52 cephalopod species. This allows us to describe cerebrotypes, i.e., differences of brain composition in different species, as a sign of their adaptation to specific niches and/or clades in cephalopod molluscs for the first time. Similarity reflecting niche type has been found in vertebrates, and it is reasonable to assume that it could also occur in Cephalopoda. We also attempted a phylogenetic PCA using data by Lindgren et al. (2012) as input tree. However, due to the limited overlap in species considered, the final analysis was carried out on <30 species, thus reducing the impact of this approach. Nevertheless, our analysis suggests that the phylogenetic signal alone cannot be a justification for the grouping of species, although biased by the limited set of data available to us. Based on these preliminary findings, we can only hypothesize that brains evolved in cephalopods on the basis of different factors including phylogeny, possible development, and the third factor, i.e., life-style adaptations. Our results support the working hypothesis that the taxon evolved different sensorial and computational strategies to cope with the various environments (niches) occupied in the oceans. This study is novel for invertebrates, to the best of our knowledge.

Keywords: neuroecology, cephalopods, brain diversity, adaptation, evolution

OPEN ACCESS

Edited by:
Jean-Pierre Bellier, Shiga University of Medical Science, Japan

Reviewed by:
Christian Marcelo Ibáñez, Andres Bello University, Chile
Jennifer Ann Mather, University of Lethbridge, Canada
Simon G. Sprecher, Université de Fribourg, Switzerland

***Correspondence:**
Graziano Fiorito
graziano.fiorito@szn.it

[†]These authors have contributed equally to this work

Received: *23 May 2020*
Accepted: *30 December 2020*
Published: *02 February 2021*

Citation:
Ponte G, Taite M, Borrelli L, Tarallo A, Allcock AL and Fiorito G (2021) Cerebrotypes in Cephalopods: Brain Diversity and Its Correlation With Species Habits, Life History, and Physiological Adaptations. Front. Neuroanat. 14:565109. doi: 10.3389/fnana.2020.565109

INTRODUCTION

Cephalopoda is the most charismatic class of the phylum Mollusca. The richness of their behavioral repertoire inspired many aspects of human life including contemporary art (as provided by camouflage and body patterns, see Nakajima, 2018) and robotics (e.g., Cianchetti et al., 2012; Xie et al., 2020; as inspired by the study of soft, flexible, and muscular body). In recent years, increased interest for their commercial value inspired gastronomy (Mouritsen and Styrbæk, 2018; see also:

Sörensen and Mouritsen, 2019 and Cephs & Chefs: https://www.cephsandchefs.com/). In addition, social media provided access to specialized information and growing interest in interdisciplinary academic fields, and images and videos where cephalopods represent a great example (Nakajima et al., 2018; McClain, 2019). Together with fishes, images of cephalopods have been "liked" more than other organisms (including sharks) on social media platforms (McClain, 2019).

Cephalopods are an ancient taxon that diverged from a monoplacophoran ancestor about 500 million years ago, during the late Cambrian (see also Allcock et al., 2015). The early Devonian saw the rise of the ammonites and nautiloids (Kröger et al., 2011), both still with chambered shells. The greatest structural innovation, the internalization of the cephalopod shell, likely occurred in the Permian or Carboniferous (Smith and Caron, 2010; Kröger et al., 2011; Tanner et al., 2017; Klug et al., 2019), exposing the mantle for the first time and providing a possible significant boost to their evolution, including cognitive abilities (Packard, 1972; Amodio et al., 2019a,b).

Cephalopods evolved several innovations, the most intriguing perhaps being their capability of exhibiting rapid and neurally-controlled changes in their body patterning (Packard and Hochberg, 1977; Packard, 1988; Messenger, 2001; Borrelli et al., 2006), and a large and complex nervous system (review in: Nixon and Young, 2003; Shigeno et al., 2018).

During their evolution the brain of cephalopods increased its complexity reaching the maximum agglomeration of the neural masses, as exemplified by comparing the outline of the "central nervous system" of *Nautilus* and that of *Octopus vulgaris* (Young, 1965, 1971). This resulted after the addition or loss of ganglia (molluscan origin and plan) that brought about the change in position and relative volume, achieving features considered to be unusual to molluscan, and invertebrate or even vertebrate, standards, but allowing significant functional analogies with vertebrates (see: Bullock, 1965; Young, 1971, 1974, 1976, 1977b, 1979; Messenger, 1979; Budelmann B., 1995; Nixon and Young, 2003; Shigeno et al., 2018; see also **Supplementary Information**).

These animals have been the preeminent "model" for cephalopod developmental (Naef, 1928), neurophysiological (e.g., Keynes, 1989; Pozzo-Miller et al., 1998; Brown and Piscopo, 2013) and behavioral studies (review in e.g., Huffard, 2013), including an early systematic attempt to develop a model of the brain (Young, 1964; review in Marini et al., 2017). Furthermore, cephalopods (and maybe octopuses especially) exhibit advanced cognitive faculties paralleling mammalian capabilities (Edelman and Seth, 2009; Amodio et al., 2019b). Cephalopods also provide a very interesting case study for the evolution of novelties/innovations in Metazoa (for review see for example: Shigeno et al., 2018; Zarrella et al., 2019; Albertin and Simakov, 2020). These innovations originated through an increase in genome complexity linked to polyploidy, differential arrangements of key genes, exceptional RNA editing capacities, and expansion of transposable elements, to cite some (e.g., Packard and Albergoni, 1970; De Marianis et al., 1979; Lee et al., 2003; Liscovitch-Brauer et al., 2017; Zarrella et al., 2019; Albertin and Simakov, 2020).

Cephalopods have the largest and most complex invertebrate nervous system. During evolution, the "brain" was assembled through the fusion of a number of molluscan ganglia to form lobes connected to the periphery by many nerve trunks regulating the arms, viscera and other parts of the animal's body. Although their nervous system is confined to the basic molluscan form comprising a set of (five to) six pairs of ganglia, it has a complexity akin to that of lower vertebrates (Bullock, 1965; Budelmann B. U., 1995), but with important functional analogies when compared with higher vertebrates (Shigeno et al., 2018). In addition, cephalopods are known for a brain-to-body weight ratio that exceeds that of fishes and reptiles (Packard, 1972). These features correlate with the sophisticated sensory equipment and complex behavior that cephalopods display (for review see for example: Budelmann et al., 1997; Williamson and Chrachri, 2004; Hanlon and Messenger, 2018).

The central nervous system of cephalopods is characterized by a high level of organization and is therefore considered to be a "proper" brain, which is unusual by molluscan, invertebrate, and even vertebrate standards (Young, 1967; Budelmann B. U., 1995; Hochner et al., 2006; Shigeno et al., 2018) for: (i) the highest degree of centralization compared with any other mollusc or invertebrate (insects excluded), achieved by the shortening of the connectives; (ii) the presence of very small neurons (3–5 micron of nuclear size) acting as local interneurons (Young, 1971, 1991); (iii) the reported absence of somatotopy (except for the chromatophore lobes) contrary to what appears to be the case for the insect or vertebrate brain (Plän, 1987; Zullo, 2004; Zullo et al., 2009); (iv) a blood-brain barrier, an exception for molluscs (Abbott and Pichon, 1987); (v) compound field potentials, similar to those of vertebrate brains (e.g., Bullock and Budelmann, 1991; Williamson and Chrachri, 2004; for review see Brown and Piscopo, 2013); (vi) an elevated efferent innervation of the receptors (e.g., the retina, the equilibrium receptor organs); (vii) peripheral first order afferent neurons (see: Young, 1971, 1991; Brown and Piscopo, 2013); (viii) a large variety of putative transmitters (review in Messenger, 1996).

The greatest centralization among cephalopods is found in the octopodiforms. It is achieved by the shortening of the connectives between the superior buccal and brachial lobes (Nixon and Young, 2003). In contrast, *Nautilus* has the simplest central nervous system characterized by three broad bands that are joined laterally, one dorsal (i.e., cerebral ganglia and commissure) and two ventral (i.e., pedal, anterior; palliovisceral, posterior) to the esophagus (Owen, 1832; Young, 1965).

During its evolution, the cephalopod brain increased in complexity and, in Coleoidea, became completely surrounded by a cartilaginous capsule. It attained maximum aggregation of the neural masses by fusing the supra– and suboesophageal regions, enclosed in a cartilaginous cranium, alongside expansion of the two large optic lobes (positioned behind the eyes) which extend laterally from the supraoesophageal mass. The change in position and relative volume of the different sections of the brain occurred as a result of the addition or loss of ganglia.

The neural mass forming the "brain" is subdivided into varying numbers of lobes in different species (from 12 in nautiluses to 24 in octopods, excluding the optic lobes).

Neuroanatomically the central nervous system varies between different cephalopod genera. Previous authors show that distinct differences exist between octopods and decapods, which correlate well with the different anatomies. The grades of complexity of the brain parallel the complexity of the sensory inputs received and the different behaviors controlled and exhibited (Young, 1977a; Maddock and Young, 1987; Budelmann B. U., 1995). Overall, the octopod brain is more centralized that the decapod brain i.e., its brachial and pedal lodes are joined, and the superior buccal lobe is united with the inferior frontal lobes. In addition, the brachial and pedal lobes of octopods, and their inferior frontal lobe system, are larger, reflecting the sophisticated use of their arms and tactile learning. Decapods, in contrast, have larger basal lobes and a larger, unfolded, vertical lobe. Their inferior frontal lobe system is simpler, and they have no suprabrachial commissure. Decapods also possess a ventral magnocellular commissure which is not found in octopods.

At the beginning of the twenty-first century, Clark et al. (2001) introduced the concept of a cerebrotype, defining it as a species-by-species measure of brain composition. This despite the fact that a number of studies, antecedent to Clark and coworkers, recognized that different groups of vertebrates possess specific patterns of brain composition that vary among clades and ecological niches.

Cerebrotypes have been shown, in one form or another, across a range of mammals and birds (e.g., Clark et al., 2001; Lundmark, 2001; Burish et al., 2004; Iwaniuk and Hurd, 2005; Willemet, 2012; Lewitus et al., 2014; Hamodeh et al., 2017), amphibians and fish (e.g., Charvet et al., 2010; Sylvester et al., 2010; Yopak, 2012). The degree to which phylogeny and ecology relate to species-specific cerebrotypes varies among studies and taxa examined. Despite the demonstration of cerebrotypes in vertebrates, no such analyses have been performed in invertebrates, to the best of our knowledge.

Here we attempt to explore such a possibility.

Several studies have provided a considerable amount of quantitative data on the brains of cephalopod molluscs (Wirz, 1959; Frösch, 1971; Maddock and Young, 1987). Nixon and Young's effort (lasting 30 years) to collect and compare the "brains and lives" of cephalopods further stimulated interest in this field of research (Nixon and Young, 2003).

Wirz, however, was the first to compare quantitative data of the brain of 34 species of cephalopods although her pioneering study was restricted to sub-adult and adult individuals from the Mediterranean Sea (Wirz, 1959). Frösch (1971) extended Wirz's work by calculating the volumes of the brain lobes in "Schlüpfstadien" (i.e., hatchlings) of ten species of Mediterranean cephalopods. Finally, Maddock and Young (1987) assembled the largest data set available on quantitative information of the brain in cephalopods, determining the volumes of the lobes of the brain for 63 cephalopod species. Like Wirz (1959) and Frösch (1971), the values were expressed as percentages of brain volume, but in addition to the two previous studies, Maddock and Young utilized species from more varied locations, including several deep-sea forms.

In our view, cerebrotypes are identifiable in this taxon. Their evolution could be related to a number of factors. First, phylogenetic constraints could largely dictate brain composition. In this case, closely related species should have a similar brain composition or architecture. Second, developmental constraints could exert the strongest influence on cephalopod brain composition. Constraining factors could include the developmental state of hatchlings, whether or not the species undergoes metamorphosis, the habitat that the eggs are deposited on, to mention some. Third, behavior and ecology could be instrumental in determining cephalopod "cerebrotypes" such that species occupying similar niches exhibit similar brain composition. Similarity reflecting niche type has been found in vertebrates (e.g., Gonzalez-Voyer et al., 2009b; Schuppli et al., 2016; Hamodeh et al., 2017; Kamhi et al., 2019) and it is reasonable to assume that it could also occur in Cephalopoda, the invertebrates with the highest degree of brain centralization.

In this study we focus on the third factor, behavior and ecology. We aim to relate the cephalopod cerebrotypes to their "adaptive" characters and niches that they occupy. Although we recognize that the other two constraints, phylogenetic, and developmental, could play an important role in the "evolution" of cerebrotypes, these aspects are not addressed in our study. Here we analyzed existing data available for cephalopod brain organization and considered the relative size of five major brain "functional" areas in relation to ecological variables in different species.

MATERIALS AND METHODS

Data Set

Quantitative data of the brain of various cephalopod species were obtained from the three aforementioned studies (Wirz, 1959; Frösch, 1971; Maddock and Young, 1987; see **Table 1**) and compiled to build the "brain" dataset (see **Supplementary Table 3**). *Nautilus* was excluded *a priori* because of the differences in the nervous system with respect to that of coleoids (Young, 1965; for review see also: Budelmann B. U., 1995; Nixon and Young, 2003). *Sepiola* sp. and *Sepietta petersi* (included in Frösch, 1971) were also excluded as the data for these two species were based on juvenile specimens.

The final data set comprised 78 species, grouped in 33 families and six orders.

The main issue with volumetric analysis in cephalopod brains is the variation in the volume of the brain, and of the single lobes within it, with the size and age of the individual (e.g., Packard and Albergoni, 1970; Frösch, 1971; Shigeno et al., 2001a). In addition, there is a general consensus that there is not a cephalopod "reference" or "type" body size at maturity, as occurs in many vertebrate species (see also Discussion). To circumvent these problems, we utilized only the a-dimensional measurements (percentages) of the different sections of the brain from Wirz (1959) and Maddock and Young (1987).

Maddock and Young (1987) grouped single brain lobes into functional sets, allowing for comparisons between taxa with different numbers of lobes. Wirz (1959) however did not consider the lobes of the brain in terms of functional sets.

In order to combine the data included in the two papers we (i) searched for correspondence between lobes, (ii) grouped each

TABLE 1 | List of species (✓, $N = 81$) included in the three published reports including quantitative data of brains in cephalopods (Wirz, 1959; Frösch, 1971; Maddock and Young, 1987).

Order	Suborder	Family	Subfamily	Current Species name	Wirz (1959)	Frösch (1971)	Maddock and Young (1987)	Nixon and Young (2003)	Lindgren et al. (2012)
Nautilida		Nautilidae		*Nautilus pompilius*			✓		
Spirulida		Spirulidae		*Spirula spirula*			✓		Y
Sepiida		Sepiidae		*Sepia officinalis*	✓	✓	✓		Y
Sepiida		Sepiidae		*Sepia elegans*	✓				
Sepiida		Sepiidae		*Sepia orbignyana*	✓				
Sepiida		Sepiolidae	Sepiolinae	*Sepiola* sp.		✓			
Sepiida		Sepiolidae	Sepiolinae	*Sepiola rondeletii*	✓		✓		
Sepiida		Sepiolidae	Sepiolinae	*Sepiola affinis*	✓				Y
Sepiida		Sepiolidae	Sepiolinae	*Sepiola robusta*	✓				Y
Sepiida		Sepiolidae	Sepiolinae	*Sepietta oweniana*	✓	✓			
Sepiida		Sepiolidae	Sepiolinae	***Sepietta petersi*[a]**		✓			
Sepiida		Sepiolidae	Rossiinae	*Rossia macrosoma*	✓				Y
Sepiida		Sepiolidae	Rossiinae	*Neorossia caroli*		✓	✓		
Sepiida		Sepiolidae	Heteroteuthinae	*Heteroteuthis (Heteroteuthis) dispar*	✓		✓		
Myopsida		Loliginidae		*Loligo vulgaris*	✓	✓			Y
Myopsida		Loliginidae		***Loligo (Alloteuthis) media*[c]**	✓	✓	✓		
Myopsida		Loliginidae		*Alloteuthis subulata*	✓				
Myopsida		Loliginidae		*Lolliguncula (Lolliguncula) brevis*			✓		Y
Myopsida		Loliginidae		*Sepioteuthis sepioidea*			✓		
Myopsida		Loliginidae		*Pickfordiateuthis pulchella*			✓		
Myopsida		Loliginidae		*Loligo forbesii*			✓		Y
[unassigned][b]		Bathyteuthidae		*Bathyteuthis* sp.			✓		
[unassigned][b]		Chtenopterygidae		*Chtenopteryx sicula*	✓		✓		Y
Oegopsida		Architeuthidae		*Architeuthis dux*			✓		Y
Oegopsida		Brachioteuthidae		*Brachioteuthis riisei*	✓				
Oegopsida		Chiroteuthidae		*Chiroteuthis veranii veranii*			✓		Y
Oegopsida		Chiroteuthidae		*Grimalditeuthis bonplandii*			✓		Y
Oegopsida		Cranchiidae	Cranchiinae	*Cranchia scabra*			✓		Y
Oegopsida		Cranchiidae	Cranchiinae	*Leachia pacifica*			✓		
Oegopsida		Cranchiidae	Taoniinae	*Taonius pavo*			✓		Y
Oegopsida		Cranchiidae	Taoniinae	*Galiteuthis glacialis*			✓	✓	
Oegopsida		Cranchiidae	Taoniinae	*Helicocranchia papillata*			✓		
Oegopsida		Cranchiidae	Taoniinae	*Bathothauma lyromma*			✓	✓	
Oegopsida		Cranchiidae	Taoniinae	*Sandalops melancholicus*			✓		
Oegopsida		Cranchiidae	Taoniinae	*Egea inermis*			✓		
Oegopsida		Cranchiidae	Taoniinae	*Megalocranchia* sp.			✓		
Oegopsida		Cranchiidae	Taoniinae	*Teuthowenia megalops*			✓		Y
Oegopsida		Cycloteuthidae		*Discoteuthis laciniosa*			✓		Y
Oegopsida		Enoploteuthidae		*Abralia (Asteroteuthis) veranyi*	✓				Y
Oegopsida		Enoploteuthidae		*Abraliopsis (Abraliopsis) morisii*	✓		✓	✓	
Oegopsida		Gonatidae		*Gonatus (Gonatus) fabricii*			✓		Y
Oegopsida		Histioteuthidae		*Histioteuthis miranda*			✓		Y
Oegopsida		Joubiniteuthidae		*Joubiniteuthis portieri*			✓		Y
Oegopsida		Lycoteuthidae	Lycoteuthinae	*Lycoteuthis lorigera*			✓		Y
Oegopsida		Mastigoteuthidae		*Mastigoteuthis schmidti*			✓		

(Continued)

TABLE 1 | Continued

Order	Suborder	Family	Subfamily	Current Species name	Wirz (1959)	Frösch (1971)	Maddock and Young (1987)	Nixon and Young (2003)	Lindgren et al. (2012)
Oegopsida		Neoteuthidae		*Neoteuthis thielei*			✓		**Y**
Oegopsida		Octopoteuthidae		*Octopoteuthis sicula*	✓				**Y**
Oegopsida		Octopoteuthidae		*Octopoteuthis danae*			✓		
Oegopsida		Ommastrephidae	Illicinae	*Illex illecebrosus*			✓		
Oegopsida		Ommastrephidae	Illicinae	*Illex coindetii*	✓				**Y**
Oegopsida		Ommastrephidae	Todarodinae	*Todarodes sagittatus*	✓				
Oegopsida		Ommastrephidae	Todarodinae	*Todaropsis eblanae*	✓				**Y**
Oegopsida		Onychoteuthidae		*Onychoteuthis banksii*	✓		✓		**Y**
Oegopsida		Onychoteuthidae		*Ancistroteuthis lichtensteinii*	✓				**Y**
Oegopsida		Pyroteuthidae		*Pyroteuthis margaritifera*	✓		✓		
Oegopsida		Pyroteuthidae		*Pterygioteuthis giardi*			✓		**Y**
Octopoda	Cirrata	Cirroteuthidae		*Cirroteuthis* sp.			✓		
Octopoda	Cirrata	Cirroteuthidae		*Cirrothauma murrayi*			✓		**Y**
Octopoda	Cirrata	Opisthoteuthidae		*Opisthoteuthis* sp.			✓		
Octopoda	Cirrata	Opisthoteuthidae		*Grimpoteuthis* sp.			✓		
Octopoda	Incirrata	Argonautidae		*Argonauta argo*	✓	✓	✓		
Octopoda	Incirrata	Alloposidae		*Haliphron atlanticus*			✓		**Y**
Octopoda	Incirrata	Tremoctopodidae		*Tremoctopus violaceus*	✓		✓		**Y**
Octopoda	Incirrata	Ocythoidae		*Ocythoe tuberculata*	✓		✓		
Octopoda	Incirrata	Eledonidae		*Eledone moschata*	✓		✓		
Octopoda	Incirrata	Eledonidae		*Eledone cirrhosa*	✓	✓			**Y**
Octopoda	Incirrata	Octopodidae		*Octopus vulgaris*	✓	✓	✓		**Y**
Octopoda	Incirrata	Octopodidae		*Octopus bimaculatus*			✓		
Octopoda	Incirrata	Octopodidae		*Octopus salutii*	✓		✓		
Octopoda	Incirrata	Octopodidae		*Macrotritopus defilippi*	✓		✓		
Octopoda	Incirrata	Octopodidae		*Callistoctopus macropus*			✓		
Octopoda	Incirrata	Octopodidae		*Pteroctopus tetracirrhus*	✓		✓		
Octopoda	Incirrata	Octopodidae		*Scaeurgus unicirrhus*	✓		✓		
Octopoda	Incirrata	Enteroctopodidae		*Enteroctopus dofleini*			✓		**Y**
Octopoda	Incirrata	Amphitretidae	Amphitretinae	*Amphitretus* sp.			✓		
Octopoda	Incirrata	Amphitretidae	Bolitaeninae	*Japetella* sp.			✓		Y
Octopoda	Incirrata	Amphitretidae	Bolitaeninae	***Eledonella sp.***[d]			✓		
Octopoda	Incirrata	Amphitretidae	Vitreledonellinae	*Vitreledonella richardi*			✓		**Y**
Octopoda	Incirrata	Bathypolypodidae		*Bathypolypus sponsalis*	✓		✓		
Octopoda	Incirrata	Bathypolypodidae		***Benthoctopus piscatorum***[e]			✓		
Vampyromorpha		Vampyroteuthidae		*Vampyroteuthis infernalis*			✓		**Y**

An orange tick (✓) marks the work considered in the final data set (n = 78). Updates (✓) on the measurements of the proportions of some lobes are provided by Nixon and Young (2003). Information on the taxonomy and current species nomenclature is given (WoRMS Editorial Board, 2020). Whenever the original species has been reassigned this is indicated in a footnote to the table, and the original species names are in boldface.

*The last column marks (**Y**) species that resulted included by Lindgren et al. (2012) for obtaining the maximum-likelihood tree for cephalopods based on multiple loci. To be conservative we considered correspondence between Japetella sp. (Maddock and Young, 1987) and Japetella diaphana (Lindgren et al., 2012).*

[a] *Originally attributed to Sepietta petersii by Frösch (1971), the species is currently "unaccepted" and considered synonym of Sepietta oweniana (source: WoRMS Editorial Board, 2020).*

[b] *The Order for these species is reported as "unassigned" and attributed to the Superorder Decapodiformes, but these are closely related to oegopsid squid.*

[c] *Originally attributed to Loligo (Alloteuthis) media by Authors, the species is currently "unaccepted" with accepted name Alloteuthis subulata (source: WoRMS Editorial Board, 2020).*

[d] *Originally attributed to Eledonella sp. by Nixon and Young (2003), the genus and species included is currently "unaccepted" with accepted name Bolitaena (source: WoRMS Editorial Board, 2020).*

[e] *Originally attributed to Benthoctopus piscatorum by Nixon and Young (2003), the species is currently "unaccepted" with accepted name Bathypolypus bairdii (source: WoRMS Editorial Board, 2020). However the actual species to which this name was likely attributed is Muusoctopus normani (see Allcock et al., 2006).*

lobe in its functional set, and (iii) summed-up the values of the lobes per functional set.

Eight functional sets and their corresponding relative brain sizes were identified (**Supplementary Table 2**).

The values of the different functional sets were recalculated as proportions relative to the volume of the whole brain (i.e., the sum of supra- and sub-esophageal masses and optic lobes), without altering the order of magnitude of the data within and between functional sets and providing values that prevented overemphasizing certain values, such as the volume of the optic lobes, in the standardization procedures required by the assumptions of the clustering technique (Everitt, 1993; Everitt et al., 2001).

In order to circumvent the intrinsic differences in the brain size values of the two data sets, we arbitrarily chose the data of Maddock and Young (1987) for species in common to both papers rather than calculating the average of the percentages given in the two works.

Ecological Variables

In order to relate the relative size of the different brain areas to ecological and life-history of different cephalopod species, we collated information available on the ecology, distribution, life history, behavior, morphological adaptations and reproductive strategies (see **Supplementary Information** for details) of the 78 taxa for which we had brain size data. In particular, we considered:

i. ***Method of locomotion***: as indicator of the potential of a species to spread and adapt to new environments;
ii. ***Feeding habits***: whether a species has adapted to become a generalist or a specialist;
iii. ***Development***: as a potential indicator or the relative dispersal of hatchlings following spawning;
iv. ***Reproduction***: i.e., mating/spawning;
v. ***Habitat***: i.e., vertical and horizontal distribution, that potentially affects gene flow and dispersal.

In total, more than 15 categories of data counting a total of 130 variables constituted the final matrix of life-habits data. These data were utilized as life-adaptation descriptors of the species considered (See **Supplementary Information** and **Supplementary Table 3**).

Analyses of Data

The combination of relative brain size data and "ecological variables" for each of the 78 cephalopod species herein considered (**Supplementary Table 3**), represents our database, i.e., a multi-dimensional matrix including data on the diversity of brain lobe' size and life history attributes of 78 species representing different cephalopod families.

Data analysis followed approaches included in Zar (1999) and Everitt et al. (2001). In brief, we utilized principal component analysis (PCA) to reduce the dimensions of the anatomical data (i.e., eight functional sets), followed by Varimax rotation. The resulting factor scores (regression method, see Gorsuch, 1983) and the ecological variables (see list above and **Supplementary Information**) were analyzed through a hierarchical cluster analysis using Ward's method (Ward, 1963).

In analogy to similar studies carried out in vertebrates (e.g., Iwaniuk and Hurd, 2005), we selected PCA to help reduce the number of variables into "components" thus exploring internal structure of the data and possibly the variance. This is considered a useful method for examining cerebrotypes (see for example discussion in Iwaniuk and Hurd, 2005). As mentioned above, we also performed a cluster analysis. We selected Ward's Hierarchical method (Ward, 1963) as a general agglomerative hierarchical clustering procedure, where the criterion for choosing the pair of clusters to merge at each step is based on the optimal value of an objective function (*sensu* Ward, 1963). This is a method utilized in several "social" science and behavioral studies, and has the advantage of not being related to any "phylogeny" (as in our case), but linked to the variability/characteristic and structure of data.

Non parametric analysis of variance (Kruskal Wallis test) followed by *post hoc* pairwise comparisons (Dunn, 1964; Zar, 1999) was utilized to compare mean proportions of functional brain sets belonging to species attributed to different clusters.

All statistical analyses were carried out using SPSS (rel. 18.0 PASW—Predictive Analytics SoftWare, IBM, 2010), except for the hierarchical cluster analysis (CLUSTAN, Wishart, 1987).

Phylogenetic PCA and Further Analysis of Data

This study is not primarily aimed at finding a direct link between brain diversity in cephalopods by means of a phylogenetic analysis. However, to attempt to control for phylogenetic dependence/independence of traits here considered (brains' diversity) and possibly ruling out bias in detecting relationships and inaccurate estimates of correlations (Rezende and Diniz-Filho, 2012; see also Adams and Collyer, 2017) we ran phylogenetic principal component analysis (pPCA, Revell, 2009). This aims to explore association between brain volume proportions and species by taking into account the phylogenetic relationship between species.

We utilized as reference tree (source of "phylogenetic signal" for our data) the multigene phylogeny based on maximum likelihood analysis by Lindgren et al. (2012); this is considered one of the most complete cephalopod phylogeny published so far. The original tree comprises 188 taxa (see Figure 1 in Lindgren et al., 2012).

Despite the large number of species included by Lindgren et al. (2012) only 38 species overlapped with the list of 78 cephalopods considered in our dataset (see **Table 1** and **Supplementary Table 6**).

The phylogenetic PCA was carried out by pruning the original tree by Lindgren et al. (2012) to obtain one including exclusively the 38 species common to both datasets (**Supplementary Figure 1**). The pruned tree was utilized as input phylogeny for "phyloPCA" included in the phytools package (https://CRAN.R-project.org/package=phytools; September, 2020). For phyloPCA we set: method = "lambda," mode = "corr," rotate = "varimax." Data for the brain functional sets were included, for corresponding species.

After pPCA, scores for the first three components were extracted, followed by a further cluster analysis (see **Supplementary Information** section: "Considerations taken for the phylogenetic PCA and subsequent analysis").

RESULTS

Cephalopod Relative Brain Size

Figure 1 presents an overview of the different proportions of brain areas (i.e., functional sets; see also **Supplementary Table 2**) in the 78 species of cephalopods considered. We also include a stacked bar summary of the relative proportions of brain functional sets in different species, excluding the contribution of the optic lobes (**Figure 1B**).

From these data PCA allowed three components to be identified, which together account for 87.3% of the total variance (**Table 2**). The first component (62.3% variance) is positively correlated with "inferior frontal" (INFF), "brachial" (BRAC), "pallial" (PALL) and "pedal" (PEDAL) lobe functional sets, but negatively correlated with the optic lobe. The second component accounts for roughly 13% of the variance and is correlated with "basal" (BASAL) and "chromatophore" (CHRF) lobes. The vertical lobe system (VERT) appears in the third component, which explains 12% of the total variance.

These results confirm the view that cephalopods possess largely diversified brains; a diversity particularly marked when the PCA scores were plotted against the six orders included in the data set (**Figure 2**; see also **Table 3**). Myopsid and oegopsid species included in this study are best separated by the second component (BASAL and CHRF), possibly due to the large contribution of the basal lobe system and chromatophore and fin lobes to the relative proportion of brain areas in these species. For similar reasons, octopods are widely distributed along the first component (correlated with the inferior frontal, brachial, pallial and pedal lobes, and negatively with the optic lobes), with the exception of two species (*Cirroteuthis* sp. and *Cirrothauma murrayi*) which are also widely separated by the second component, and therefore may be considered as outliers. This result may be linked to the fact that the two species had (in our data set, and to the best of our knowledge) the smallest optic lobes (38.6 and 13.5% of the brain, respectively; **Supplementary Table 3**; see also Maddock and Young, 1987). We can speculate that the vertical lobe system occupies a distinct component (the third) because of the large variability in relative proportions, within orders, of this structure: its size varies "more than four times among the decapods and six times in octopods" (Maddock and Young, 1987, p. 749).

Correlating Cephalopod Cerebrotypes Their Life-Styles and Other Adaptations

To search for any possible relationship between the components extracted from the PCA depicting brain diversity in cephalopod and cephalopod' life adaptation descriptors (the "ecological" variables; see **Supplementary Information**) of the various organisms we considered, we carried out a hierarchical cluster analysis as an attempt to summarize patterns of similarity/dissimilarity among species.

The clustering was carried out only on 52 species out of the original list ($n = 78$): 26 species were excluded for missing data (233 null values, 2.3% of the whole dataset) in 32 of the 130 total number of variables/states included as indicators of cephalopod "life-style/adaptations." We cannot ignore that the 26 species not included in the cluster analysis might have provided a different grouping and/or the identification of additional clusters (see discussion around missing data in **Supplementary Information** "Number of species included in the final clustering and reasons for exclusions").

The cluster analysis yielded a dendrogram with ten distinct clusters (labeled from 1 to 10 in **Figure 3**). **Table 3** summarizes mean values of the proportions for each of the brain regions belonging to the groups of species identified through hierarchical clustering to illustrate differences on the resulting "cerebrotype."

A short description for each cluster follows in the following paragraphs. Hereunder, the species are referred according to original names as indicated by Authors (Wirz, 1959; Maddock and Young, 1987); for current valid taxonomy refer to **Table 1**.

The first group of species (*Cluster 1)* consists of a very diverse assemblage of species (i.e., *Spirula spirula*, *Heteroteuthis dispar*, *Brachioteuthis riisei*, *Abralia veranyi*, *Abraliopsis morisi*), belonging to three distinct orders of cephalopods (Spirulida, Sepiolida, Teuthida). The supraoesophageal mass of these species is characterized by a very small "inferior frontal" (INFF), medium-sized VERT, and relatively large "basal" (BASAL) lobes. The functional brain sets pertaining to the suboesophageal mass are, on average, very similar within the species included in this cluster. The chromatophore lobe (the fin lobe is the major contributor to CHRF in most of these species) is almost absent.

Species in this cluster possess relatively large optic lobes representing about 65% of total brain size.

All five species are known to be oceanic (mesopelagic), achieving buoyancy by active swimming (dynamic lift) with the aid of broad fins, with the exception of *Spirula* which has near-neutral buoyancy with a chambered shell and short, subterminal fins. Their geographic distribution is variable, ranging from 3 to 10 Large Marine Ecosystems (LME; see Sherman and Duda, 1999 see also **Supplementary Information**), and, as they are reported to feed almost exclusively on pelagic crustaceans, the species should be considered diet specialists, although the diet is slightly richer in *H. dispar* (in *H. dispar*, the lower beak is characterized by a medium-narrow rostrum, curved hood, shallow or absent notch, and obtuse or right jaw angle). Finally, this group clusters the only species (from the 78 analyzed) with a "*Sthenoteuthis* type" reproductive strategy (sensu Nigmatullin and Laptikhovsky, 1994; see also Nesis, 2002) and multiple spawners *sensu* Rocha et al. (2001). The eggs are small, single, unencapsulated and pelagic (laid at or near the surface).

Cluster 2 groups the three *Sepia* species: *S. officinalis*, *S. elegans,* and *S. orbignyana*. In this case, the supraoesophageal mass is characterized by a very small inferior lobe system, and large vertical and basal lobes. Typical of the decapod subesophageal mass, the brachial lobe is not particularly developed (PEDAL and PALL being on average similar to the other clusters). However, in these species prominent

chromatophore (about 2%) and fin (3%) lobes exist, accompanied by moderately large optic lobes (about 60% of brain size). All three species are reported as having "near-neutral buoyancy" via a chambered shell, with fringed fins. They live in coastal, benthic habitats (mainly littoral and continental shelf), and are quite widely distributed (occurring in 10 LMEs on average). Cuttlefish are known to have a relatively broad diet breadth (i.e., generalists), and the lower beaks are characterized by a long and broad rostrum, curved hood, shallow notch, and curved jaw angle. All the three species have an "*Illex*-type" reproductive strategy (*sensu* Nesis, 2002) and are intermittent terminal spawners (*sensu* Rocha et al., 2001). The eggs are mostly intermediate in size, single, encapsulated and laid in batches on the bottom.

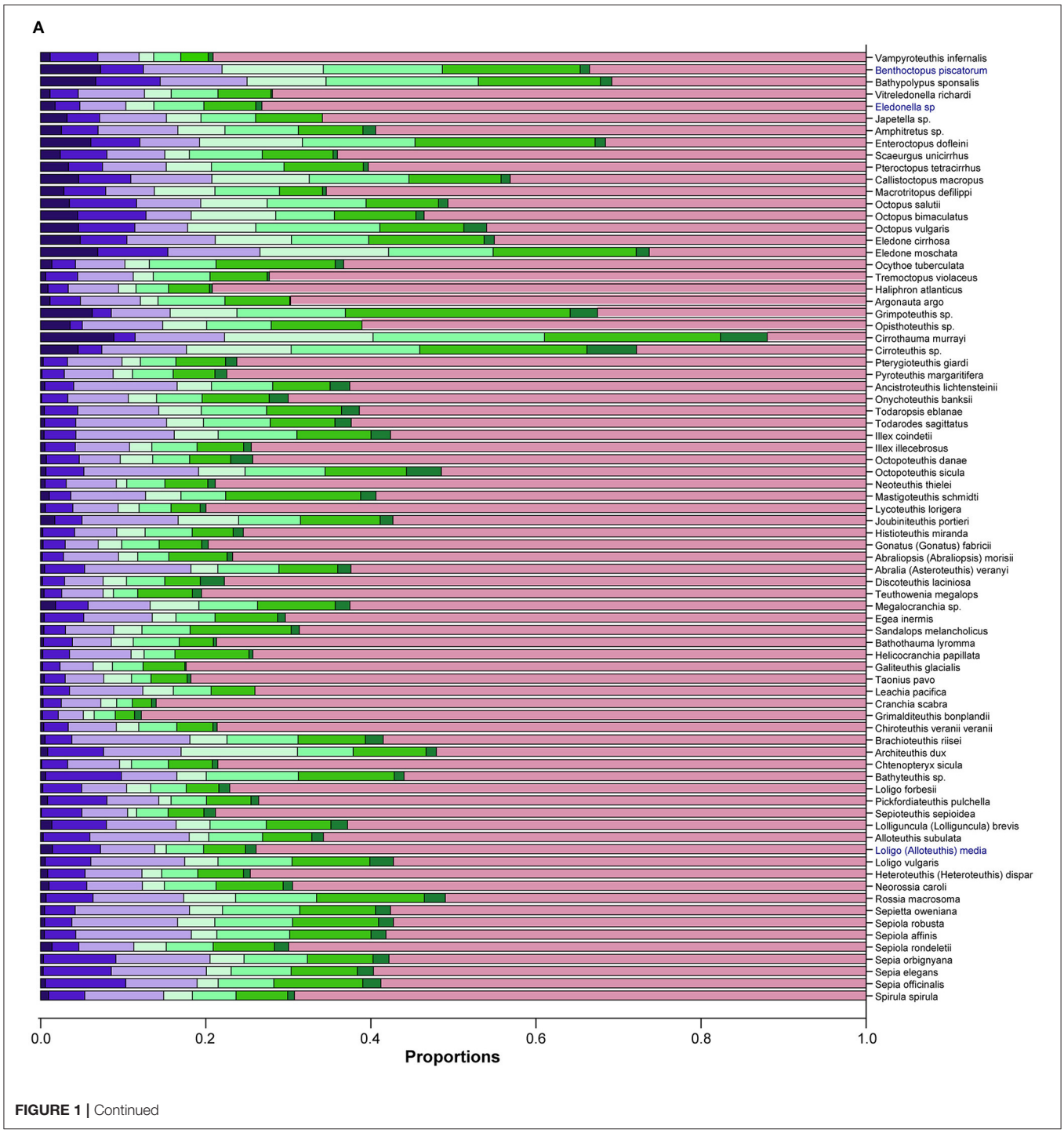


FIGURE 1 | Continued

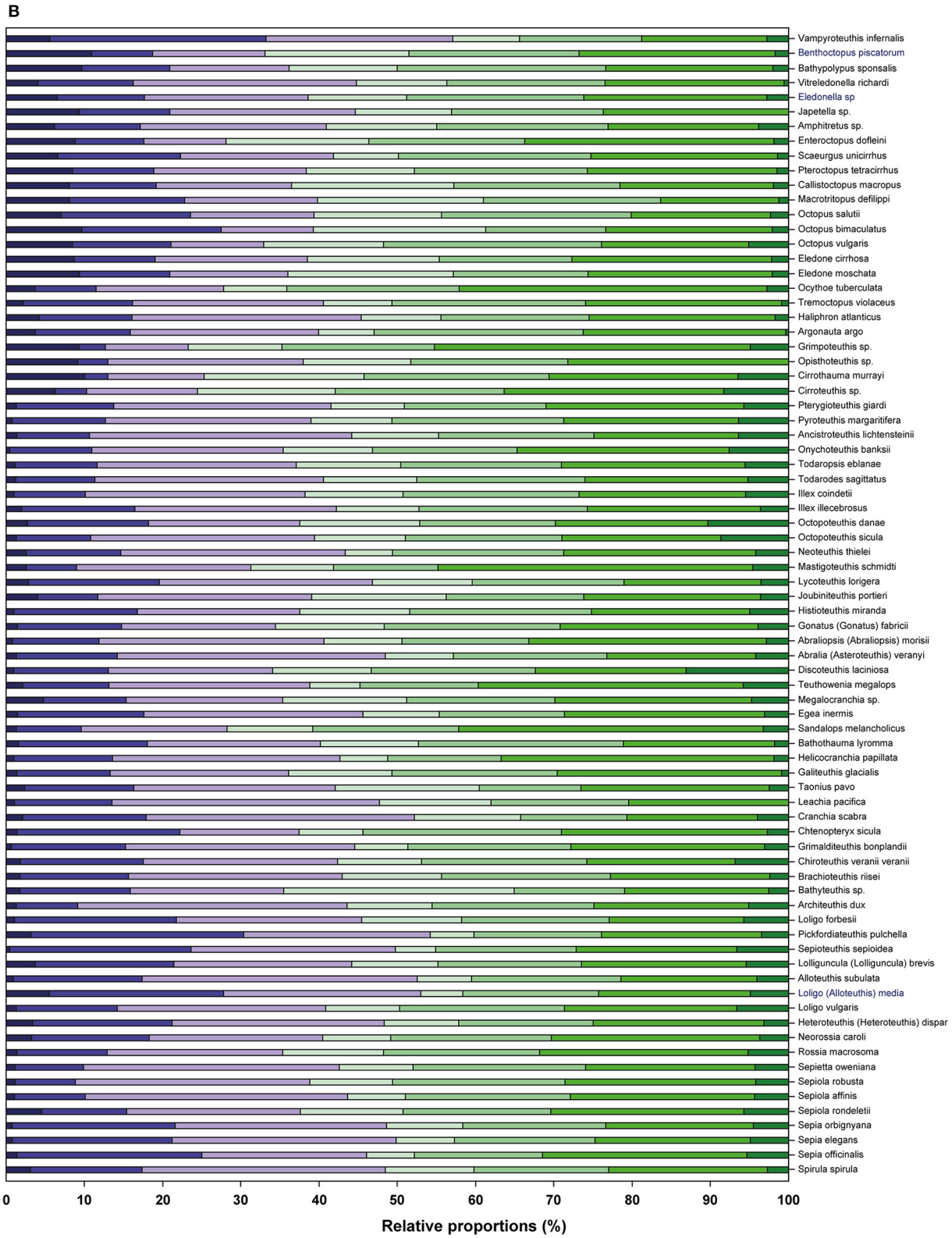


FIGURE 1 | Proportions of the eight functional sets on the whole brain **(A)** in the 78 species of cephalopods considered in this study (see also **Supplementary Table 3** for values). The functional sets (see **Supplementary Table 2** for details) are color coded. For supraoesophageal mass: INFF (■), VERT (■), BASAL (■); for suboesophageal mass: BRAC (■), PEDAL (■), PALL (■), CHRF (■); for optic lobes (OPTIC, ■). **(B)** 100% Stack Bar graph of the relative proportions of the functional brain sets excluding OPTIC, to illustrate variability of brain areas identified in supra- and suboesophageal masses between species.

TABLE 2 | Principal components analysis for the relative proportions of the eight brain-functional sets (for abbreviations and description see **Supplementary Table 2**) of the 78 species of cephalopods considered in this study (see also Borrelli, 2007).

	Components		
	1	**2**	**3**
INFF	**0.961**	−0.033	0.107
BRAC	**0.881**	0.215	0.158
OPTIC	**−0.849**	−0.468	−0.238
PALL	**0.838**	0.344	−0.039
PEDAL	**0.826**	0.420	0.193
BASAL	0.116	**0.859**	0.265
CHRF	0.362	**0.753**	−0.182
VERT	0.178	0.071	**0.949**
Eigenvalue	4.99	1.03	0.97
% Variance	62.30	12.90	12.10

*For relative proportions of brain sets see also **Figures 1A,B** and **Supplementary Table 3**. Bold type indicates correlations of variables with the principal components >0.50.*

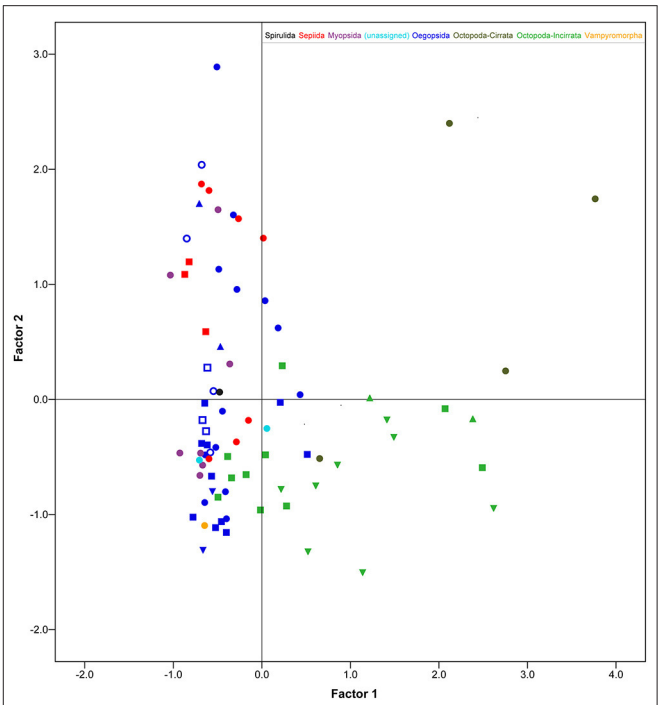


FIGURE 2 | Scatter plot of factor score values (after regression following PCA, Gorsuch, 1983) of the 78 cephalopod species belonging to the six Orders (color-coded) considered in this study. Different symbols group various taxa for a given order, whenever applicable (see **Supplementary Information** and **Supplementary Table 4** for details). Cirrata and Incirrata are coded with different grades of green. Only the first two factor scores are considered here. See text for details and **Table 3** for relative proportions of cephalopod brains between species allocated in different clusters.

The third group (*Cluster 3*; **Figure 3**) includes all benthic bobtail squids in our dataset: *Sepiola rondeletii*, *S. affinis*, *S. robusta*, *Sepietta oweniana*, and *Rossia macrosoma*. In these species the supraesophageal mass is characterized by a relatively small INFF (the exception being *S. rondeleti*), large BASAL and moderately large VERT. Within the suboesophageal mass, the PEDAL and PALL are prominent in comparison to other decapods. The chromatophore and fin lobes are similar in size to those observed in *Sepia* species (see *Cluster 2*). The five species included in *Cluster 3* possess moderately large optic lobes (about 60% of the entire brain). They are considered to be "dense" (see **Supplementary Information** and **Supplementary Table 3**), bottom living, finned species, inhabiting both coastal waters (littoral and continental shelf) and the slope (bathybenthic). *Sepiola rondeletii*, *S. oweniana*, and *R. macrosoma* occupy a moderately extended geographical range while *S. affinis* and *S. robusta* are known to be distributed within a slightly narrower range. Data available to us suggest the diet is restricted to crustaceans (with the exception of *Rossia*); the lower beak being characterized by a long and moderately broad rostrum, curved hood, absent notch, and obtuse jaw angle. The life-style and reproductive strategies (sensu: Nigmatullin and Laptikhovsky, 1994; Nesis, 2002), spawning pattern and egg morphology of the five species is the same as for cuttlefish (as for *Cluster 2*).

Cluster 4 comprises six coastal loliginids (*Loligo vulgaris*, *L. forbesii*, *L.* (*Alloteuthis*) *media*, *L.* (*Alloteuthis*) *subulata*[1], *Lolliguncula brevis*, and *Sepioteuthis sepioidea*). To compensate for their dense body tissues, they swim actively (dynamic lift), by flapping their elongated fins to remain afloat. These squids inhabit neritic and shallow waters (epi-mesopelagic). Species included in this cluster are widely distributed across LMEs; *S. sepioidea* and *L.* (*Alloteuthis*) *media* are reported to have more restricted distributions. The supraoesophageal mass is characterized by a small inferior frontal system, with the exception of *L.* (*Alloteuthis*) *media* and *L. brevis* for which proportions are about four times those of the other species in the group. The species are reported to have large vertical lobes (as compared with other species) and basal lobes of variable relative size. Within this cluster the chromatophore lobes are smaller than the larger fin lobe (together reaching about 6% of the cerebral masses). The optic lobes are comparatively large, ranging between 57 and 78% of the total brain size. Diet is diverse among species, but is generally considered to be intermediate in breadth with *L. vulgaris* and *L. forbesii* having the most diverse diet, and *L. brevis* the most specialist diet. The lower beaks of these species have a long, broad rostrum, curved hood, shallow notch, and obtuse jaw angle. The species reproduce and spawn following similar strategies (see above and **Supplementary Information**) to the other neritic species (sepiids and sepiolids) although the eggs are laid on the bottom in collective capsules rather than singular capsules.

Cluster 5 groups six squids which occupy the same zone (epi-mesopelagic) as loliginids, but which are oceanic. This cluster includes the most voracious cephalopod species, such as

[1]Current valid species: *Alloteuthis media, Alloteuthis subulata.*

TABLE 3 | The mean proportions of the eight brain regions (brain-functional sets) for each of the ten clusters identified after the hierarchical cluster analysis (see **Figure 3**).

Cluster	Taxa	Species	INFF	VERT	BASAL	BRAC	PEDAL	PALL	CHRF	OPTIC
1	Spirulidae	*Spirula spirula*	0.617	3.909	10.061	3.190	5.886	6.827	1.212	68.298
(*n* = 5)	Sepiolidae	*Heteroteuthis (Heteroteuthis) dispar*	(0.243–0.991)	(2.706–5.112)	(5.772–14.349)	(2.094–4.286)	(3.341–8.430)	(5.593–8.061)	(0.427–1.998)	(58.633–77.963)
	Brachioteuthidae	*Brachioteuthis riisei*								
	Enoploteuthidae	*Abralia (Asteroteuthis) veranyi*								
		Abraliopsis (Abraliopsis) morisi								
2	Sepiidae	*Sepia officinalis*	0.414	8.919	10.529	3.214	7.233	8.912	2.025	58.755
(*n* = 3)		*Sepia elegans*	(0.055–0.773)	(7.041–10.797)	(6.501–14.557)	(1.196–5.231)	(6.049–8.417)	(4.911–12.913)	(1.653–2.397)	(56.430–61.080)
		Sepia orbignyana								
3	Sepiolidae	*Sepiola rondeletii*	0.698	3.946	11.658	4.369	8.610	9.985	1.937	58.797
(*n* = 5)		*Sepiola affinis*	(0.211–1.184)	(2.705–5.188)	(7.886–15.429)	(2.879–5.859)	(6.529–10.691)	(7.444–12.526)	(1.509–2.365)	(50.247–67.347)
		Sepiola robusta								
		Sepietta oweniana								
		Rossia macrosoma								
4	Loliginidae	*Loligo vulgaris*	0.678	5.535	8.236	2.646	5.835	6.092	1.706	69.272
(*n* = 6)		*Loligo (Alloteuthis) media*	(0.062–1.2934	(4.827–6.242)	(5.189–11.284)	(1.304–3.987)	(3.762–7.908)	(3.853–8.331)	(1.055–2.357)	(60.224–78.320)
		Alloteuthis subulata								
		Lolliguncula (Lolliguncula) brevis								
		Sepioteuthis sepioidea								
		Loligo forbesii								
5	Architeuthidae	*Architeuthis dux*	0.481	4.217	9.329	5.863	7.219	8.075	1.823	62.992
(*n* = 6)	Ommastrephidae	*Illex illecebrosus*	(0.241–0.721)	(2.872–5.562)	(7.164–11.494)	(1.501–10.226)	(5.553–8.886)	(6.716–9.434)	(1.203–2.444)	(54.435–71.549)
		Illex coindetii								
		Todarodes sagittatus								
		Todaropsis eblanae								
		Onychoteuthis banksii								
6	Chiroteuthidae	*Chiroteuthis veranii veranii*	0.586	2.604	5.011	2.812	3.890	5.355	0.811	78.932
(*n* = 6)	Cranchiidae	*Grimalditeuthis bonplandii*	(-0.049–1.221)[a]	(1.809–3.399)	(3.397–6.626)	(1.003–4.621)	(2.019–5.760)	(2.814–7.896)	(0.206–1.416)	(69.909–87.955)
		Taonius pavo								
		Galiteuthis glacialis								
		Megalocranchia sp.								
		Teuthowenia megalops								
7	Cranchiidae	*Cranchia scabra*	0.339	3.264	6.666	2.688	4.395	6.749	0.561	75.336
(*n* = 6)		*Leachia pacifica*	(0.262–0.417)	(2.336–4.193)	(4.775–8.557)	(1.816–3.559)	(2.888–5.901)	(2.992–10.506)	(0.174–0.947)	(68.749–81.924)
		Helicocranchia papillata								
		Bathothauma lyromma								
		Sandalops melancholicus								
		Egea inermis								
8	Gonatidae	*Gonatus (Gonatus) fabricii*	1.468	3.375	6.107	3.206	6.026	6.609	0.447	72.761
(*n* = 4)	Tremoctopodidae	*Tremoctopus violaceus*	(-0.625–3.561)[b]	(2.374–4.376)	(3.344–8.869)	(1.991–4.422)	(4.377–7.675)	(4.643–8.576)	(-0.166–1.060)[c]	(63.750–81.772)
	Amphitretidae	*Japetella sp.*								
		Eledonella sp.								

(Continued)

TABLE 3 | Continued

Cluster	Taxa	Species	INFF	VERT	BASAL	BRAC	PEDAL	PALL	CHRF	OPTIC
9	Octopodidae	*Octopus vulgaris*	4.212	6.383	7.181	9.089	10.924	10.944	1.187	50.080
(*n* = 7)	Enteroctopodidae	*Octopus bimaculatus* *Octopus salutii* *Octopus defilippi* *Callistoctopus macropus* *Pteroctopus tetracirrhus* *Enteroctopus dofleini*	(3.208–5.216)	(4.979–7.787)	(5.825–8.538)	(6.767–11.411)	(8.109–13.738)	(6.185–15.702)	(0.489–1.885)	(39.674–60.486)
10	Eledonidae	*Eledone moschata*	6.427	6.779	10.478	11.649	13.731	15.695	1.332	33.908
(*n* = 4)	Bathypolypodidae	*Eledone cirrhosa* *Bathypolypus sponsalis* *Benthoctopus piscatorum*	(4.666–8.187)	(4.208–9.349)	(9.382–11.575)	(6.972–16.326)	(7.699–19.763)	(13.179–18.210)	(1.038–1.626)	(21.171–46.645)

For each cluster the table also includes: number of species, taxa represented (attribution to Families, see ***Table 1*** *for reference and current taxonomy and nomenclature), list of species, the 95% Confidence Intervals for each structure (in parentheses). Whenever the lower bound of the 95% Confidence Interval for the mean is negative, we also report as a footnote minimum and maximum values. The mean values are suggested to represent different cerebrotypes for the group of cephalopod species considered. Analysis of differences between mean proportions of cerebrotypes are shown in* ***Supplementary Table 5****. For abbreviations identifying cephalopod brain-functional sets see* ***Supplementary Table 2****.*

[a]*Minimum = 0.222; maximum = 1.807.*

[b]*Minimum = 0.305; maximum = 3.209.*

[c]*Minimum = 0.000; maximum = 0.793.*

the giant squid (*Architeuthis dux*), the muscular, flying squids (*Illex illecebrosus*, *I. coindetii*, *Todarodes sagittatus*, *Todaropsis eblanae*), and the common clubhook squid (*Onychoteuthis banksii*). They occupy moderately wide geographic distributions (*O. banksii* with a particularly broad distribution). Our data sources describe these species as feeding exclusively on pelagic organisms such as crustaceans, fish, and mollusks (including cephalopods). The lower beaks have a long, narrow rostrum (except for *A. dux*), curved hood, and acute (or right) jaw angle. They are fast swimmers (with broad triangular fins) and move using dynamic lift (because they are dense), with the exception of *A. dux* (near-neutral; chemical lift; ammonium). The supraoesophageal mass has a relatively small "inferior frontal" (INFF), moderately variable in size VERT and basal lobes; chromatophore lobes quite reduced when compared with the fin lobe (with the exception of *O. banksii* in which they are equivalent in size), and optic lobes representing about 60% of the total brain size. The reproductive strategy (sensu: Nigmatullin and Laptikhovsky, 1994; Nesis, 2002) of these species is similar to that of loliginid squids (see *Cluster 4*), with the exception of *A. dux*, which like other "ammoniacal" squids, reproduces with a "*Gonatus*-type" strategy (sensu Nigmatullin and Laptikhovsky, 1994; see also Nesis, 2002).

The sixth group of species (*Cluster 6*) includes the chiroteuthid squids (*Chiroteuthis veranyi*, *Grimalditeuthis bonplandi*) and four Cranchiidae (*Taonius pavo*, *Galiteuthis glacialis*, *Megalocranchia* sp., *Teuthowenia megalops*); the other Cranchiidae species here considered grouped in *Cluster 7*. The six species are "near-neutral" and achieve buoyancy by chemical lift (ammonium)[2]. Their fins are short, rounded and subterminal (as in the transparent glass squids) or secondary (chiroteuthids). They are "oceanic" (epi-bathypelagic), with a quite variable geographic distribution, with the exception of *T. pavo*. The supraoesophageal mass has the smallest VERT and BASAL compared with both muscular (see clusters 4 and 5) and glass squids (see *Cluster 7*). The chromatophore lobe is reduced or absent while the fin lobe is relatively developed, when compared with species included in *Cluster 7* (see also: Maddock and Young, 1987; Nixon and Young, 2003). The optic lobes are large, representing about 80% of the entire "brain." Diet breadth (hypothetical for many species) is listed in our data set as relatively wide, restricted to pelagic prey items (i.e., cephalopods, crustaceans, fish). The lower beak is reported as short but broad rostrum, curved hood, broad notch and obtuse jaw angle. All six species are reported to reproduce with an "*Gonatus*-type" strategy (sensu Nigmatullin and Laptikhovsky, 1994; see also Nesis, 2002) and are intermittent terminal spawners (*sensu* Rocha et al., 2001). The eggs are small (the size is hypothetical for many species) and are released as collective capsules in deep layers of the water column.

Cluster 7 groups the six remaining Cranchiidae species (*Cranchia scabra*, *Leachia pacifica*, *Helicocranchia papillata*, *Bathothauma lyromma*, *Sandalops melancholicus*, *Egea inermis*). They occupy the same area of the marine realm as *Cluster*

[2]*Grimalditeuthis* also has flotation devices on its tail; see: http://tolweb.org/Grimalditeuthis_bonplandi/19463.

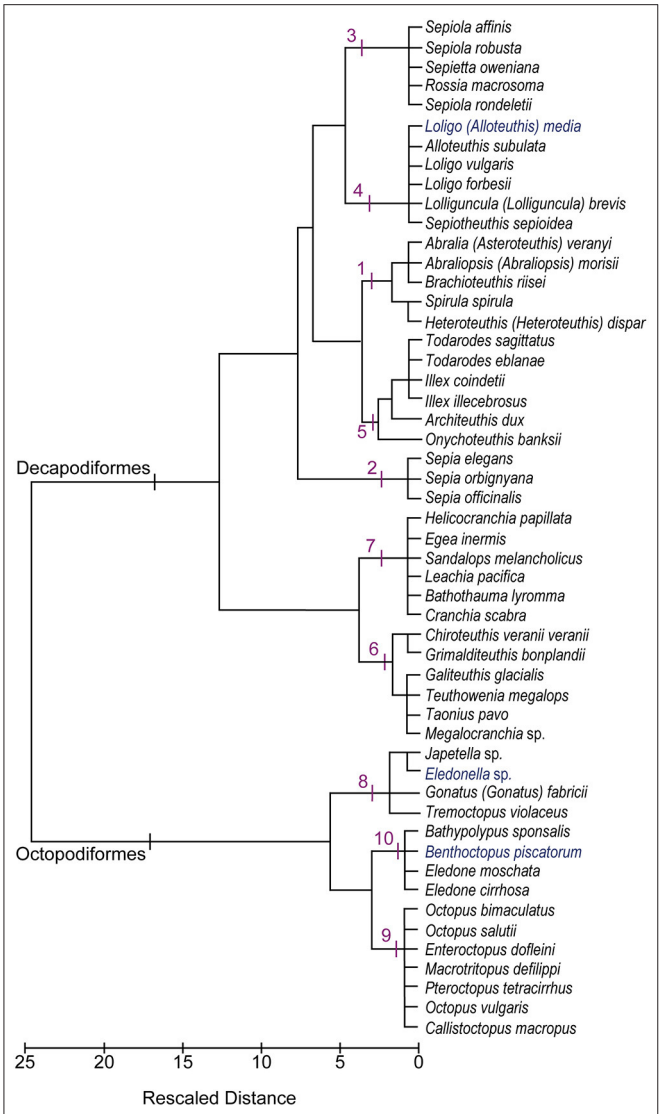


FIGURE 3 | Dendrogram after Ward's hierarchical clustering showing the relationships among cephalopod species ($N = 52$) based upon the data set ($N = 78$). The clusters recognized are numbered from 1 to 10 and described in the text. See also **Table 3** and **Supplementary Table 5**.

6, and have similar modes of locomotion, reproductive and spawning patterns, egg morphology, and site of deposition. The supraoesophageal mass is characterized by the smallest INFF, but larger VERT and basal lobes compared with other cranchiids. The chromatophore lobe is reduced or absent, the fin lobe is relatively small (except in *C. scabra*); the optic lobes are fairly large, accounting for about 75% of the total brain size. The diet is hypothetical although similar lower beak morphology (variable rostrum, curved hood, variable notch, and obtuse jaw angle) to species included in *Cluster 6* would suggest similar diet breadth (see also above).

Armhook squid, *Gonatus fabricii*, the gelatinous octopuses (*Japetella* sp., *Bolitaena* sp.) and the common blanket octopus *Tremoctopus violaceus* are included in *Cluster 8*. These species are finless except for *G. fabricii*, free-swimming (near-neutral by chemical lift via lipids or chlorine) and live-in open waters, mainly occupying surface or intermediate layers (epi-mesopelagic), although bolitaenids are also reported to live in deeper waters (bathypelagic). All species are reported as widely distributed around the world's oceans (7-17 LMEs; but *G. fabricii* is currently known as restricted to the North Atlantic). In our data the diet breadth, similar to glass squids, is reported as relatively broad, with a focus on pelagic organisms, such as pteropods (molluscs), amphipods, copepods and euphausiids (crustaceans), chaetognaths and fish. Lower beak morphology is distinctive in these species: a short rostrum, curved or flat hood, notch absent. The supraoesophageal mass is characterized in these species by a small INFF (with the exception of *Japetella* sp.), small basal lobes and a medium-sized vertical lobe system. The chromatophore and fin lobes are reduced or absent. The optic lobes are large reaching about 70% of the total size of the brain. *Japetella* sp., *Bolitaena* sp., and *T. violaceus* reproduce with an "*Octopus*-type" strategy (*sensu* Nigmatullin and Laptikhovsky, 1994; see also Nesis, 2002) and are simultaneous terminal spawners (*sensu* Rocha et al., 2001), while *G. fabricii* reproduces with a "*Gonatus*-type" strategy (*sensu* Nigmatullin and Laptikhovsky, 1994; see also Nesis, 2002) and is an intermittent terminal spawner (*sensu* Rocha et al., 2001). Recent studies reported that *G. fabricii* exhibit a geographically localized reproduction, relatively uncommon for deep-water squids (Golikov et al., 2019).

Cluster 9 groups seven species of benthic octopuses: *Octopus vulgaris, O. bimaculatus, O. defilippi*[3], *O. macropus*[3], *"Octopus" salutii, Enteroctopus dofleini, and Pteroctopus tetracirrhus.* All of them are "dense," finless, bottom living species inhabiting coastal waters (littoral and continental shelf, with only *O. macropus*, *O. salutii* and *P. tetracirrhus* extending to bathybenthic layers). *Octopus vulgaris*, *O. bimaculatus*, *O. defilippi*, *O. macropus*[3] have a wide geographical distribution; *O. bimaculatus*, *O. salutii, P. tetracirrhus,* and *E. dofleini* occupy a more restricted range (but see also Jereb et al., 2016).

The supraoesophageal mass is characterized by a large "inferior frontal" (INFF) and vertical lobe systems (VERT; although this is quite variable among species) and a well-developed chromatophore lobe (fin lobe absent). The optic lobes are moderately large as they represent about 50% of the entire brain. *Octopus vulgaris* is reported to be a generalist species, while the other taxa in this cluster are described in our dataset with a more restricted diet[4]. The lower beak is described with a short-broad rostrum, narrow curved hood, broad notch, and obtuse jaw angle. All seven species reproduce following an "Octopus-type" strategy (*sensu* Nigmatullin and Laptikhovsky, 1994; see also Nesis, 2002) and are simultaneous terminal spawners (*sensu* Rocha et al., 2001). The eggs, small-intermediate in size, are laid in clusters on the substrate (e.g., females of *O. vulgaris* lay their egg strings in their den).

[3]Current valid species: *Macrotritopus defilippi, Callistoctopus macropus*.
[4]But see: Quetglas et al. (2009), Scheel and Anderson (2012), Villegas et al. (2014).

Cluster 10 groups the remaining octopods: *Eledone moschata*, *E. cirrhosa*, *Bathypolypus sponsalis*, *Benthoctopus piscatorum*[5]. These species (like in *Cluster 9*) are also benthic (dense body tissues) and finless. However, they are known to occupy deeper layers than the species included in *Cluster 9*; particularly an exclusively bathybenthic distribution is known for *B. sponsalis* and *B. piscatorum*. In contrast to *Cluster 9*, these species occupy a relatively restricted geographical distribution (*E. moschata* and *B. sponsalis* being even more restricted).

The supraoesophageal mass is characterized by large INFF, VERT and basal lobes. The chromatophore lobe is moderately large; the fin lobe is absent. The optic lobes are the smallest in our dataset: 26-33% of the total brain size; 45% for *E. cirrhosa*. The diet is reported as specialized[6], with the exception of *E. cirrhosa*. The lower beak is described as short, with broad rostrum, variable curved hood, broad notch, and acute or recessed jaw angle. The reproductive and spawning strategies of the two *Eledone* species are comparable to those of shallow water octopods (see cluster 9). Contrarily, the deep-sea octopuses (*B. sponsalis* and *B. piscatorum*) are reported to be continuous spawners in our source of data. There is no consensus on the spawning pattern of deep-sea octopuses due to lack of data. The eggs of all four species are large (when compared with other octopods), and are in clusters which are laid on substrate.

At first glance, the dendrogram (**Figure 3**) reveals the strong division of coleoid cephalopods into Decapodiformes (clusters 1–7) and Octopodiformes (clusters 8–10), which corresponds to a high dissimilarity index (Squared Euclidean Distance coefficient = 2579.3). The sole exception to this general pattern is represented by the oegopsid squid, *Gonatus fabricii*, which is clustered with pelagic octopuses. As already described (see *Cluster 8*), this association may be explained by the fact that Armhook squids share with free-swimming octopuses the common behavior of females brooding eggs on their arms, instead of releasing them in the water column (as do other oceanic squids) or laying them on the ground (as do neritic squids). However, a more attentive analysis of the figure suggests that the separation of coleoids in two distinct lineages (i.e., decapods vs. octopods) is less clear-cut than expected. The relative affinity or relatedness among species—within and between clusters—seems largely to depend on the life adaptations they share in common (e.g., buoyancy mechanisms, habitats occupied, reproductive strategies) that, in turn, has brought about a similar differentiation in the lobes of the brain, as for our hypothesis.

For example, Bobtail squids (cluster 3) and inshore squids (cluster 4) are grouped together because they share several features in common. They both live in coastal waters (although sepiolids may reach deeper layers of the water column), have "dense" bodies and fins (see **Supplementary Table 3**). Both sepiolids and loliginids are intermittent terminal spawners (*Illex*-type strategy) so that the eggs are laid, in separate clutches, over a relatively long-time frame (Rocha et al., 2001). The eggs (small-intermediate in size) are laid on the substrate in batches (sepiolids) or collective capsules (loliginids). Moreover, both taxa are reported in our data-set with brains characterized by a very small "inferior frontal," medium-sized vertical lobe system and considerable basal lobes. The optic lobes are also well developed in these species, representing roughly 60 and 70% of the total brain size in sepiolids and loliginids, respectively. The fin lobe is rather more conspicuous in loliginids which can be explained by their strictly pelagic life style, in contrast to sepiolids live mostly in contact with the bottom.

Differences Between Cephalopod Cerebrotypes as Identified by Hierarchical Cluster Analysis

To illustrate whether the ten clusters identified after Ward's hierarchical method correspond to a characteristic "cerebrotype," we calculated mean values of the proportions for each of the brain functional sets of species belonging to every cluster identified (**Table 3**). Differences between the proportions for each of the eight brain regions (brain-functional sets) shown for species belonging to the clusters identified, were significant according to Kruskal–Wallis one-way analysis of variance (**Supplementary Table 5**). *Post hoc* pairwise comparisons further confirmed a composite variation of cephalopod brain areas attributed to different clusters (see **Supplementary Table 5.2**). In particular we found significant differences for INFF (clusters 9 and 10 vs. others), VERT in more than 12 pairwise comparisons (26% of the total), BASAL (cluster 6 vs. 1-3, 5, 10; 2 vs. 7, 8; 3 vs. 6-9, to mention some; see **Supplementary Table 5.2**) when considering the supraoesophageal mass. Differences between areas also emerged when suboesophageal mass was considered: e.g., PEDAL - cluster 1 vs. 9, 10; cluster 3 vs. 6, 7; cluster 4 vs. 9, 10; cluster 5 vs. 6, 7; cluster 6 vs. 2, 5, 9, 10, cluster 7 vs. 3, 9, 10 (see **Supplementary Table 5** for details).

Phylogenetic PCA

The phylogenetic Principal Component Analysis (Revell, 2009) was carried out on a reduced number of species ($n = 38$; 48.7% of the total) because of missing correspondence between organisms selected by Lindgren et al. (2012) and our dataset.

The resulting first two components accounted for 77% of variance (Eigenvalues, %Variance: PC1 = 5.20, 65.0; PC2 = 0.97, 12%); a third component (eigenvalue = 0.82) was also considered accounting for a total of 88% of cumulative variance (for extracted scores see **Supplementary Table 7**). Because of pruning and lack of overlap between our dataset and the species considered by Lindgren et al. (2012) a further reduction in the list of species was required for the following cluster analysis ($n = 24$ out 52; 46% of the species included in **Figure 3**)[7].

[5]The species is currently "unaccepted" with accepted name *Bathypolypus bairdii* (see also **Table 1**).

[6]The diet of these species is defined as 'specialized', but not restricted, in our original data source. Recent studies expand knowledge available of habits of deep-sea species (e.g., Quetglas et al., 2001; Valls et al., 2017).

[7]Species retained (in alphabetical order): *Abralia veranyi, Architeuthis dux, Chiroteuthis veranyi veranyi, Cranchia scabra, Eledone cirrhosa, Enteroctopus dofleini, Gonatus fabricii, Grimalditeuthis bonplandi, Illex coindetii, Japetella sp, Loligo vulgaris, Lolliguncula brevis, Octopus vulgaris, Onychoteuthis banksii, Rossia macrosoma, Sepia elegans, Sepia officinalis, Sepiola affinis, Sepiola robusta, Spirula spirula, Taonius pavo, Teuthowenia megalops, Todaropsis eblanae, Tremoctopus violaceus violaceus.*

The resulting dendrogram (**Supplementary Figure 2**) shows limited similarities with the clustering of **Figure 3**.

In brief, the grouping corresponding to clusters 3 and 4 was almost retained, despite some loss in species (i.e., *Sepiola affinis*, *S. robusta*, *Rossia macrosoma*, *Loligo vulgaris*, *Lolliguncula brevis*; **Supplementary Figure 2**), and is nested with *Abralia veranyi* and *Spirula spirula*, originally belonging to cluster 1, but within the same branching. Species originally included in cluster 5 appear in a different branch mixing with some other members of cluster 1 (see **Supplementary Figure 2**). Furthermore, octopuses and cuttlefishes mixed together (with the exception of *O. vulgaris*), and with different branching when compared with the maximum-likelihood topology as shown by Lindgren et al. (2012).

DISCUSSION

Our study indicates that the cephalopod brain is largely differentiated among species (**Figure 1** and **Table 3**; see also: Maddock and Young, 1987; Nixon and Young, 2003) and evolved specific cerebrotypes in disparate taxa (**Table 3** and **Supplementary Table 5**), similar to what has been reported in vertebrates (e.g., Burish et al., 2004; Iwaniuk et al., 2004; Iwaniuk and Hurd, 2005; Yopak, 2012; Kotrschal et al., 2017).

Many comparative studies on brain evolution in vertebrates utilized multivariate statistics (e.g., van Dongen, 1998; Burish et al., 2004; Iwaniuk et al., 2004; Iwaniuk and Hurd, 2005; Lisney and Collin, 2006; Gonzalez-Voyer et al., 2009a; Yopak, 2012; Steinhausen et al., 2016; Kotrschal et al., 2017; Mai and Liao, 2019). The approach is useful to investigate brain evolution for two main reasons: (i) there are contingencies among brain regions that result in correlated evolution among some areas; (ii) a multitude of selection pressures and constraints determine the composition and evolution of the brain.

Our data reflect the view of Maddock and Young (1987) that there are significant quantitative differences between the brains of different cephalopod species, and that - despite individual variations due to growth or other factors (e.g., seasonal), these differences should reflect the habitat the cephalopod occupies (as also suggested in Nixon and Young, 2003). Most of the clusters identify cerebrotypes that map on to ecological and/or behavioral similarities among cephalopod species. By using a hierarchical cluster analysis approach, we recognize 10 groups of species that reveal differences and analogies among the 52 cephalopod species included in our final data set. The topology of the relationship among species we observed (see dendrogram in **Figure 3**) strongly supports J.Z. Young's view (Young, 1977a) and our working hypothesis that analysis combining relative brain size and life strategies may provide the basis for assumptions on the pressures and adaptations that drove cephalopods to evolve.

Evolutionary speculations are beyond our data and approach. However, to control for phylogenetic dependence/independence of traits here considered—i.e., cephalopod brain diversity—and possibly ruling out bias in detecting relationships we ran phylogenetic principal component analysis (Revell, 2009) thus to explore association between brain proportions of different cephalopods taking into account the phylogenetic relationship between species. Our pPCA benefits from the data of Lindgren et al. (2012). Unfortunately, the currently available phylogenetic data and limited correspondence with detailed "brain data" did not allow us to achieve enough resolution in the phylogeny to be utilized as additional information in this work. The data we present have to be considered a preliminary outcome and the basis of future work.

Octopods are characterized by large brachial and inferior frontal lobes and smaller optic lobes as opposed to decapods (see **Figures 1**, **2**; see also Maddock and Young, 1987). It appears evident that differences in relative proportions of the inferior frontal lobe system and brachial lobes of octopuses, as compared with decapods, are largely linked with the large use of arms associated with the benthic habitat (Young, 1977a; see also Hanlon and Messenger, 2018).

Figure 1 highlights a large variability in the proportions of the lobes within decapods (see also **Table 3** for differences between cerebrotypes). This was already noticed by Maddock and Young (1987) and is not surprising considering the numerous families included within the taxon. However, the most striking differences among cephalopod species emerged when considering the vertical lobe system (e.g., about four times differences between species included in cluster 6 vs. cluster 2; **Table 3**). The five lobules of the vertical lobe in *O. vulgaris*—for example—allow a packing-effect and a volume reduction of the structure by increasing the surface area and the corresponding number of cells counting the lobe (Young, 1963). The small cells (amacrine) also minimize the length of connections, increase connectivity and computational abilities (Young, 1991, 1995; see also Shigeno et al., 2018), and reduce neuropilar space, as occurs in higher vertebrates (e.g., Hofman, 1985; Sherman and Duda, 1999; Hof et al., 2005; Toro and Burnod, 2005; Molnar et al., 2006; Geschwind and Rakic, 2013; Van Essen et al., 2018; Amiez et al., 2019). In *Loligo* (and *Sepia*) we find the opposite: there is no folding of the surface of the vertical lobe, an estimated reduced number of cells, a correspondingly huge neuropil (Young, 1979). In the words of J.Z. Young: "The octopod condition seems to favor a large number of small cells, the decapod a large number of large cells. The large cells are numerous even within the neuropil. These differences are very striking and call for further knowledge of fine structure and experiments on function" (Young, 1979, p. 352; see also for example Shomrat et al., 2011).

Figure 3 presents the outcome of the hierarchical cluster analysis and highlights several common features, for example, between cuttlefish (cluster 2) and shallow-water octopuses (cluster 9), which probably reflect similar adaptations to the environment and/or common mechanisms evolved to counteract predation. Both are reported to have a wide distribution and to have colonized mainly coastal waters of both temperate and tropical regions, although cuttlefish are completely absent from the Americas (but fossil records provide evidence of their existence in those areas). As bottom living organisms, both have had to adapt to various types of substrate (e.g., rubble, rocky reefs, open sand plains) and prey (generalists). The

"ecological" demands, in terms of relative habitat complexity and predation pressure, have brought to the evolution of cephalopods in both taxa, development of rich behavioral repertoires, complex cognitive capabilities (for review see for example: Marini et al., 2017; Mather and Dickel, 2017; Hanlon and Messenger, 2018) and reproductive strategies (i.e., intermittent and simultaneous terminal spawning; review in Rocha et al., 2001) capable of dealing with unstable environments. Again as an example, it is not surprising that *O. vulgaris* has the most conspicuous chromatophore lobe (5%) and that *S. officinalis* is characterized by the largest VERT complex (9.7% of total brain size; see **Supplementary Information** and **Figure 1**) among the cephalopods included in this study. Following Maddock and Young (1987) "cirrates are sharply distinguished from other octopods by their relatively large brachial lobes and small vertical lobes. [...]" and "among the octopods other than cirrates, the benthic species are distinct from the pelagic, largely on a basis of greater brachial and inferior frontal systems. [...] epipelagic and bathypelagic [octopods are] broadly separate [... with] the inferior frontal systems [...] reduced and the optic lobes [...] large. It would not be legitimate to separate the epipelagic from the bathypelagic octopods on the brains alone [...] but there are other features that clearly separate the groups such as the arms and web, [...] the whole body form and habitat" (Maddock and Young, 1987, p. 765).

The application of a phylogenetic PCA provided an additional interesting approach. We had access to a large dataset (Lindgren et al., 2012), but the convergence in terms of species included was very limited (38 out 188 species corresponding to 20% of species; only 52% of the species of the dataset of this study were retained for pPCA), limiting the potential of this approach.

Our analyses suggest that the phylogenetic signal alone is not a justification for the grouping of species we found (see **Figure 3** and **Supplementary Figure 2**). Due to the limited set of data available to us, we can only hypothesize that brains evolved in cephalopods on the basis of different factors including phylogeny, development and the third factor (life-style adaptations).

Future research will be required extending the dataset by including all different categories of variables here considered and a strong set of phylogenetic signals as recently applied to decapodiforms by Anderson and Lindgren (2021).

In her original study, Borrelli (2007) attempted to correlate cephalopod' relative brain size with species richness (e.g., Lynch, 1990; Owens et al., 1999; Nicolakakis et al., 2003; Sol et al., 2005; Sayol et al., 2019). Species richness, and possibly subspecies (Sol et al., 2005) appears to be affected by behavioral flexibility, so that taxa appearing more flexible to environmental changes (i.e., opportunistic species) are also those that are represented by a higher number of species as opposed to those characterized by specialist species, and which are less speciose. In a preliminary analysis, Borrelli calculated the total number of species per family in the entire class Cephalopoda noting that species counts were differently distributed among the class with some families more speciose. Borrelli was able to obtain mantle length and brain size of 32 species (a single species per family/subfamily was chosen as representative of the taxon) belonging to 28 families (data deduced from Maddock and Young, 1987). Standardized residuals of brain size[8] were regressed against the log transformed values of the number of species per family/subfamily providing a significant relationship between the two (Linear regression: $\beta = 0.31 \pm 0.10$, $F_{1,31} = 8.97$, $P = 0.005$, $R^2 = 0.23$; see Figure 2.1 in Borrelli, 2007) that—according to the original study—supports the idea of the behavioral drive hypothesis in cephalopods, *sensu* Wilson (1985): the most speciose cephalopod families were also those having larger brains, as opposed to families with less species. Unfortunately, such an approach was not possible in this work.

During the analysis of the data set and in agreement to what was reported by Borrelli (2007), we faced a problem in attempting to correlate the relative size (volume) of brain areas with body size. A similar issue was also encountered by Maddock and Young (1987) when comparing their data with "previous measurements"; in the words of the Authors "for the optic lobes (which are relatively easy to measure) our figures are about equal to hers [Wirz, 1959, *NdA*] in eight genera, rather more in four genera and less in nine genera. The only serious discrepancies are for *Eledone* and *Bathypolypus* where she records values equivalent to 154 and 94 compared with our 35 and 44. We have checked our figures and, finding no reason to doubt their accuracy, conclude that the differences may be due to differences in the sizes of animals. We intend to undertake a study brain/body sizes in *Eledone* and other cephalopods, which should help to clear up this point" (Maddock and Young, 1987, p. 762-763). The Authors also extend this remark by comparing with data from Frösch (1971) pointing out that the "vertical lobes were considerably smaller in all the newly-hatched forms than in the adults, but the superior frontals and subverticals were larger [...]. The subfrontals, like the vertical lobes, were much smaller in the younger animals. Presubably, they develop with learning experience" (Maddock and Young, 1987, p. 764).

The main reason is that "brain scaling" has to be accurately assessed in cephalopod species given the marked variation in the volume of the brain, and of the single lobes within it, with the size and age of the individual (e.g., Packard and Albergoni, 1970; Frösch, 1971; Dickel et al., 1997, 2006; Shigeno et al., 2001a). As a consequence, it is not possible to identify a "reference" or "type" body size at maturity for cephalopods, as it is in many vertebrate species including fish (e.g., Huber et al., 1997), birds (e.g., Portmann, 1947) and mammals (e.g., Stephan and Pirlot, 1970; Marino, 1998). Therefore, we utilized only a-dimensional measurements (i.e., percentages) of the different sections of the brain from our data sources.

It could be extremely interesting and informative in the future to focus attention on a qualitative assessment of the gross brain morphology in cephalopods. This may help in assessing the degree of inter-specific variability in gross brain structures and in finding potential similarities among morphotypes, other than those that result from comparing the typical decapod vs. octopod brain (i.e., *Loligo* or *Octopus*, respectively).

In spite of the wealth of data available from the literature on the organization of the nervous system of cephalopods (e.g.,

[8]Factoring out body size. Mantle length and total brain volume were log transformed to normalize variances and consequently regressed (Borrelli, 2007).

Young, 1971, 1974, 1976, 1977b, 1979; Messenger, 1979; Shigeno et al., 2001a), complete atlases and accurate 3D rendering of the brain morphology are not available (but see Chung et al., 2020). Therefore, a quantitative assessment of gross brain morphology based on the degree of "encephalization" (*sensu lato*), as recently carried out for example in fish (Lisney and Collin, 2006) is still not possible in cephalopods.

An experimental and data-analysis strategy similar to what has been carried out in vertebrates (e.g., Barton, 1996; Clark et al., 2001; Iwaniuk and Hurd, 2005; Kalisinska, 2005; Ratcliffe et al., 2006; Macrì et al., 2019) may reveal important and significant scientific outcomes for cephalopod biology.

CLOSING REMARKS

Despite the intrinsic limitations of our dataset, the results provide support for a close relationship between "cerebrotypes" and life styles in cephalopods. This, again, supports our working hypothesis that this taxon evolved different sensory (and computational) strategies to cope with the demands of life in the ocean (Amodio et al., 2019a,b). These resemble similar adaptations achieved by fish (e.g., Lisney and Collin, 2006) and other vertebrates. By sharing the same environments and ecological niches, octopuses, squids and their allies were forced to compete with fish (their primary predators), which drove cephalopods to colonize and radiate across the world's oceans (Packard, 1972; O'Dor and Webber, 1986; see also, e.g., Aronson, 1991). Our results strongly support Young's view (1977a) of the evolution of the cephalopod brain.

The evolution of cephalopod cerebrotypes could be related to a number of factors. As mentioned in the introduction, phylogenetic (e.g., closely related species should have a similar brain composition) and developmental (e.g., paralarvae or miniature adults at hatching; see for example: Frösch, 1971; Young and Harman, 1988; Sweeney et al., 1992; Shigeno et al., 2001b) constraints could largely dictate brain composition. In addition, behavior and ecology—as a third factor—appeared to influence cephalopod "cerebrotypes": species occupying similar niches appear to possess similar brain organization/composition.

Here we attempted to relate the cephalopod cerebrotypes to their "adaptive features" and niches that species occupy. We selected only a number of possible variables to consider, based on the data available for the largest number of species included in this study and our aim: a first attempt to provide a revisited glance to the outcome of observational approach originally driven by Young (1977a). We are fully aware that the other two constraints (phylogenetic and developmental) play an important role in the "evolution" of cerebrotypes in this taxon.

A comparison between the dendrograms (**Figure 3** and **Supplementary Figure 2**) and a phylogenetic tree of the species (see Figure 1 in Lindgren et al., 2012), clearly show that the overall clustering pattern is not congruent with phylogeny to some extent, and suggest that many of the clusters reflect similarities in brain and their relation with behavior and ecology. We found relationships between clustering pattern and behavior and ecology analagous to those found in fish (e.g., Huber et al., 1997), birds (e.g., Iwaniuk and Hurd, 2005) and mammals (e.g., de Winter and Oxnard, 2001).

A future effort should focus on testing the interplay between the above-mentioned factors, with a focus on evolution and phylogeny, thus to test whether the aforementioned constraints are independent or interlinked in the overall evolution of cephalopod brains.

Cephalopod molluscs represent a promising group among invertebrates for studies concerning the organizing principles that underlie the architecture and ontogeny of complex brains. In a similar fashion to multivariate analyses of brain composition in other taxa, our study indicates that the cephalopod brain evolved specific cerebrotypes that have evolved in disparate taxa.

DATA AVAILABILITY STATEMENT

All the data presented in this study are included in the paper and in the **Supplementary Material**. Further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the study is based on historical data obtained from the literature.

AUTHOR CONTRIBUTIONS

LB built the original dataset. GP and MT drafted the manuscript. AT helped with the phylogenetic PCA analysis. All authors contributed to this study and finalized the manuscript.

FUNDING

This work has been supported by the Stazione Zoologica Anton Dohrn.

ACKNOWLEDGMENTS

Part of this work was included in the Ph.D. Thesis of LB (Stazione Zoologica Anton Dohrn, Italy and Open University, UK). We thank Dr. Annie Lindgren for providing access to the dataset included in Lindgren et al. (2012). We are also grateful to Dr. M. V. Modica for suggestions and Ms. Marisa Barjami for assistance. MT is supported by an Irish Research Council Government of Ireland Postgraduate Scholarship and the Dr. Tony Ryan Research Fund. The study has been supported by the Stazione Zoologica Anton Dohrn (Napoli, Italy) to GP and GF.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnana.2020.565109/full#supplementary-material

REFERENCES

Abbott, N. J., and Pichon, Y. (1987). The glial blood-brain barrier of crustacea and cephalopods: a review. *J. Physiol. Paris* 82, 304–313.

Adams, D. C., and Collyer, M. L. (2017). Multivariate phylogenetic comparative methods: evaluations, comparisons, and recommendations. *Syst. Biol.* 67, 14–31. doi: 10.1093/sysbio/syx055

Albertin, C. B., and Simakov, O. (2020). Cephalopod biology: at the intersection between genomic and organismal novelties. *Ann. Rev. Anim. Biosci.* 8, 71–90. doi: 10.1146/annurev-animal-021419-083609

Allcock, A. L., Lindgren, A., and Strugnell, J. (2015). The contribution of molecular data to our understanding of cephalopod evolution and systematics: a review. *J. Nat. Hist.* 49, 1373–1421. doi: 10.1080/00222933.2013.825342

Allcock, A. L., Strugnell, J. M., Ruggiero, H., and Collins, M. A. (2006). Redescription of the deep-sea octopod *Benthoctopus normani* (Massy 1907) and a description of a new species from the Northeast Atlantic. *Mar. Biol. Res.* 2, 372–387. doi: 10.1080/17451000600973315

Amiez, C., Sallet, J., Hopkins, W. D., Meguerditchian, A., Hadj-Bouziane, F., Ben Hamed, S., et al. (2019). Sulcal organization in the medial frontal cortex provides insights into primate brain evolution. *Nat. Commun.* 10:3437. doi: 10.1038/s41467-019-11347-x

Amodio, P., Boeckle, M., Schnell, A. K., Ostojic, L., Fiorito, G., and Clayton, N. S. (2019a). Shell loss in cephalopods: trigger for, or by-product of, the evolution of intelligence? A Reply to Mollo et al. *Trends in Ecology & Evolution* 34, 690–692. doi: 10.1016/j.tree.2019.05.005

Amodio, P., Boeckle, M., Schnell, A. K., Ostojíc, L., Fiorito, G., and Clayton, N. S. (2019b). Grow smart and die young: why did cephalopods evolve intelligence? *Trends Ecol. Evol.* 34, 45–56. doi: 10.1016/j.tree.2018.10.010

Anderson, F. E., and Lindgren, A. R. (2021). Phylogenomic analyses recover a clade of large-bodied decapodiform cephalopods. *Mol. Phylo. Evol.* 156:107038. doi: 10.1016/j.ympev.2020.107038

Aronson, R. B. (1991). Ecology, paleobiology and evolutionary constraint in the octopus. *Bull. Mar. Sci.* 49, 245–255.

Barton, R. A. (1996). Neocortex size and behavioural ecology in primates. *Proc. R. Soc. Lond. B. Biol. Sci.* 263, 173–177.

Borrelli, L. (2007). *Testing the Contribution of Relative Brain Size and Learning Capabilities on the Evolution of Octopus vulgaris and Other Cephalopods*. Ph.D. Thesis, Stazione Zoologica Anton Dohrn, Italy & Open University, Napoli, United Kingdom.

Borrelli, L., Gherardi, F., and Fiorito, G. (2006). *A Catalogue of Body Patterning in Cephalopoda.* Stazione Zoologica A. Dohrn, Firenze University Press, Napoli, United Kingdom.

Brown, E. R., and Piscopo, S. (2013). Synaptic plasticity in cephalopods; more than just learning and memory? *Invert. Neurosci.* 13, 35–44. doi: 10.1007/s10158-013-0150-4

Budelmann, B. (1995). "The cephalopod nervous system: what evolution has made of the molluscan design," in *The Nervous Systems of Invertebrates: An Evolutionary and Comparative Approach*, eds O. Breidbach and W. Kutsc (Basel: Springer), 115–138.

Budelmann, B. U. (1995). "The cephalopod nervous system: what evolution has made of the molluscan design," in *The Nervous Systems of Invertebrates: An Evolutionary and Comparative Approach,* eds. O. Breidbach and W. Kutsch. (Basel: Birkhäuser Verlag), 115–138.

Budelmann, B. U., Schipp, R., and Boletzky, S. V. (1997). "Cephalopoda," in *Microscopic Anatomy of Invertebrates,* eds. F. W. Harrison and A. J. Kohn. (New York, NY: Wiley-Liss, Inc.), 119–414.

Bullock, T. H. (1965). "Mollusca: Cephalopoda," in *Structure and Function in the Nervous Systems of Invertebrates*, eds. T. H. Bullock and G. A. Horridge. (San Francisco, CA and London: W.H. Freeman and Company), 1433–1515.

Bullock, T. H., and Budelmann, B. U. (1991). Sensory evoked potentials in unanesthetized unrestrained cuttlefish: a new preparation for brain physiology in cephalopods. *J. Comp. Physiol. A* 168, 141–150.

Burish, M. J., Kueh, H. Y., and Wang, S. S. H. (2004). Brain architecture and social complexity in modern and ancient birds. *Brain Behav. Evol.* 63, 107–124. doi: 10.1159/000075674

Charvet, C. J., Sandoval, A. L., and Striedter, G. F. (2010). Phylogenetic origins of early alterations in brain region proportions. *Brain Behav. Evol.* 75, 104–110. doi: 10.1159/000300573

Chung, W.-S., Kurniawan, N. D., and Marshall, N. J. (2020). Toward an MRI-based mesoscale connectome of the squid brain. *iScience* 23, 100816. doi: 10.1016/j.isci.2019.100816

Cianchetti, M., Follador, M., Mazzolai, B., Dario, P., and Laschi, C. (2012). "Design and development of a soft robotic octopus arm exploiting embodied intelligence," in: *Robotics and Automation (ICRA), 2012 IEEE International Conference on IEEE* (Saint Paul, MN), 5271–5276. doi: 10.1109/ICRA.2012.6224696

Clark, D. A., Mitra, P. P., and Wang, S. S.-H. (2001). Scalable architecture in mammalian brains. *Nature* 411, 189–193. doi: 10.1038/35075564

De Marianis, B., Olmo, E., and Giuditta, A. (1979). Excess DNA in the nuclei of the subseophagel region of octopus brain. *J. Comp. Neurol.* 186, 293–300. doi: 10.1002/cne.901860211

de Winter, W., and Oxnard, C. E. (2001). Evolutionary radiations and convergences in the structural organization of mammalian brains. *Nature* 409, 710–714. doi: 10.1038/35055547

Dickel, L., Chichery, M. P., and Chichery, R. (1997). Postembryonic maturation of the vertical lobe complex and early development of predatory behavior in the cuttlefish (*Sepia officinalis*). *Neurobiol. Learn. Mem.* 67, 150–160. doi: 10.1006/nlme.1996.3754

Dickel, L., Darmaillacq, A. S., Poirier, R., Agin, V., Bellanger, C., and Chichery, R. (2006). Behavioural and neural maturation in the cuttlefish *Sepia officinalis*. *Vie et Milieu* 56, 89–95.

Dunn, O. J. (1964). Multiple comparisons using rank sums. *Technometrics* 6, 241–252. doi: 10.1080/00401706.1964.10490181

Edelman, D. B., and Seth, A. K. (2009). Animal consciousness: a synthetic approach. *Trends Neurosci.* 32, 476–484. doi: 10.1016/j.tins.2009.05.008

Everitt, B., Landau, S., and Leese, M. (2001). *Cluster Analysis.* London: Edward Arnold Publishers Ltd.

Everitt, B. S. (1993). *Cluster Analysis.* London: Edward Arnold Publishers Ltd.

Frösch, D. (1971). Quantitative untersuchungen am zentralnervensystem der schlüpfstadien von zehn mediterranen cephalopodenarten. *Rev. Suisse Zool* 78, 1069–1122.

Geschwind, Daniel, H., and Rakic, P. (2013). Cortical evolution: judge the brain by its cover. *Neuron* 80, 633–647. doi: 10.1016/j.neuron.2013.10.045

Golikov, A. V., Blicher, M. E., Jørgensen, L. L., Walkusz, W., Zakharov, D. V., Zimina, O. L., et al. (2019). Reproductive biology and ecology of the boreoatlantic armhook squid *Gonatus fabricii* (Cephalopoda: Gonatidae). *J. Mollusc. Stud.* 85, 341–353. doi: 10.1093/mollus/eyz023

Gonzalez-Voyer, A., Winberg, S., and Kolm, N. (2009a). Brain structure evolution in a basal vertebrate clade: evidence from phylogenetic comparative analysis of cichlid fishes. *BMC Evol. Biol.* 9:238. doi: 10.1186/1471-2148-9-238

Gonzalez-Voyer, A., Winberg, S., and Kolm, N. (2009b). Social fishes and single mothers: brain evolution in African cichlids. *Proc. R. Soci.* 276, 161–167. doi: 10.1098/rspb.2008.0979

Gorsuch, R. L. (1983). *Factor Analysis.* Hillsdale, NJ: Lawrence Erlbaum Associates.

Hamodeh, S., Bozkurt, A., Mao, H., and Sultan, F. (2017). Uncovering specific changes in network wiring underlying the primate cerebrotype. *Brain Struct. Funct.* 222, 3255–3266. doi: 10.1007/s00429-017-1402-6

Hanlon, R. T., and Messenger, J. B. (2018). *Cephalopod Behaviour.* Cambridge: Cambridge University Press.

Hochner, B., Shomrat, T., and Fiorito, G. (2006). The octopus: a model for a comparative analysis of the evolution of learning and memory mechanisms. *Biol. Bull.* 210, 308–317. doi: 10.2307/4134567

Hof, P. R., Chanis, R., and Marino, L. (2005). Cortical complexity in cetacean brains. *Anat. Rec.* 287A, 1142–1152. doi: 10.1002/ar.a.20258

Hofman, M. A. (1985). Size and shape of the cerebral cortex in mammals. I. The cortical surface. *Brain Behav. Evol.* 27, 28–40. doi: 10.1159/000116529

Huber, R., van Staaden, M. J., Kaufman, L. S., and Liem, K. F. (1997). Microhabitat use, trophic patterns, and the evolution of brain structure in African cichlids. *Brain Behav. Evol.* 50, 167–182. doi: 10.1159/000113330

Huffard, C. L. (2013). Cephalopod neurobiology: an introduction for biologists working in other model systems. *Invert. Neurosci.* 13, 11–18. doi: 10.1007/s10158-013-0147-z

Iwaniuk, A. N., Dean, K. M., and Nelson, J. E. (2004). A mosaic pattern characterizes the evolution of the avian brain. *Proc. R. Soc. Lond. Ser.* 271, S148–S151. doi: 10.1098/rsbl.2003.0127

Iwaniuk, A. N., and Hurd, P. L. (2005). The evolution of cerebrotypes in birds. *Brain Behav. Evol.* 65, 215–230. doi: 10.1159/000084313

Jereb, P., Roper, C., Norman, M., and Finn, J. (2016). "Cephalopods of the World," *An Annotated and Illustrated Catalogue of Species Known to Date. Octopods and Vampire Squids*. Roma: FAO, Food and Agriculture Organization of the United Nations.

Kalisinska, E. (2005). Anseriform brain and its parts versus taxonomic and ecological categories. *Brain Behav. Evol.* 65, 244–261. doi: 10.1159/000084315

Kamhi, J. F., Ilie,ş, I., and Traniello, J. F. A. (2019). Social complexity and brain evolution: comparative analysis of modularity and integration in ant brain organization. *Brain Behav. Evol.* 93, 4–18. doi: 10.1159/000497267

Keynes, R. D. (1989). The role of giant axons in studies of the nerve impulse. *BioEssays* 10, 90–93. doi: 10.1002/bies.950100213

Klug, C., Landman, N. H., Fuchs, D., Mapes, R. H., Pohle, A., Guériau, P., et al. (2019). Anatomy and evolution of the first Coleoidea in the Carboniferous. *Commun. Biol.* 2:280. doi: 10.1038/s42003-019-0523-2

Kotrschal, A., Zeng, H.-L., van der Bijl, W., Öhman-Mägi, C., Kotrschal, K., Pelckmans, K., et al. (2017). Evolution of brain region volumes during artificial selection for relative brain size. *Evolution* 71, 2942–2951. doi: 10.1111/evo.13373

Kröger, B., Vinther, J., and Fuchs, D. (2011). Cephalopod origin and evolution: a congruent picture emerging from fossils, development and molecules. *BioEssays* 33, 602–613. doi: 10.1002/bies.201100001

Lee, P. N., Callaerts, P., de Couet, H. G., and Martindale, M. Q. (2003). Cephalopod Hox genes and the origin of morphological novelties. *Nature* 424, 1061–1065. doi: 10.1038/nature01872

Lewitus, E., Kelava, I., Kalinka, A. T., Tomancak, P., and Huttner, W. B. (2014). An adaptive threshold in mammalian neocortical evolution. *PLoS Biol.* 12:e1002000. doi: 10.1371/journal.pbio.1002000

Lindgren, A. R., Pankey, M. S., Hochberg, F. G., and Oakley, T. H. (2012). A multi-gene phylogeny of Cephalopoda supports convergent morphological evolution in association with multiple habitat shifts in the marine environment. *BMC Evol. Biol.* 12:129. doi: 10.1186/1471-2148-12-129

Liscovitch-Brauer, N., Alon, S., Porath, H. T., Elstein, B., Unger, R., Ziv, T., et al. (2017). Trade-off between transcriptome plasticity and genome evolution in cephalopods. *Cell* 169, 191–202.e111. doi: 10.1016/j.cell.2017.03.025

Lisney, T., and Collin, S. (2006). Brain morphology in large pelagic fishes: a comparison between sharks and teleosts. *J. Fish Biol.* 68, 532–554. doi: 10.1111/j.0022-1112.2006.00940.x

Lundmark, C. (2001). Evolution of mammalian brains. *BioScience* 51, 992–992. doi: 10.1641/0006-3568(2001)051[0992:Eomb]2.0.Co;2

Lynch, M. (1990). The rate of morphological evolution in mammals from the standpoint of the neural expectation. *Am. Nat* 136, 727–741.

Macrì, S., Savriama, Y., Khan, I., and Di-Poï, N. (2019). Comparative analysis of squamate brains unveils multi-level variation in cerebellar architecture associated with locomotor specialization. *Nat. Commun.* 10:5560. doi: 10.1038/s41467-019-13405-w

Maddock, L., and Young, J. Z. (1987). Quantitative differences among the brains of cephalopods. *J. Zool.* 212, 739–767.

Mai, C. L., and Liao, W. B. (2019). Brain size evolution in anurans: a review. *J. Evol. Biol.* 69:265. doi: 10.1163/15707563-00001074

Marini, G., De Sio, F., Ponte, G., and Fiorito, G. (2017). "Behavioral analysis of learning and memory in cephalopods," in *Learning and Memory: A Comprehensive Reference (Second Edition),* ed. J. H. Byrne. (Amsterdam: Academic Press, Elsevier), 441–462.

Marino, L. (1998). A comparison of encephalization between odontocete cetaceans and anthropoid primates. *Brain Behav. Evol.* 51, 230–238. doi: 10.1159/000006540

Mather, J. A., and Dickel, L. (2017). Cephalopod complex cognition. *Curr. Opin. Behav. Sci.* 16, 131–137. doi: 10.1016/j.cobeha.2017.06.008

McClain, C. R. (2019). Likes, comments, and shares of marine organism imagery on Facebook. *Peer J.* 7:e6795. doi: 10.7717/peerj.6795

Messenger, J. (1979). The nervous system of Loligo: IV. The peduncle and olfactory lobes. *Philos. Trans. R. Soc. Lond.* 285, 275–309.

Messenger, J. B. (1996). Neurotransmitters of cephalopods. *Invertebr. Neurosci.* 2, 95–114. doi: 10.1007/BF02214113

Messenger, J. B. (2001). Cephalopod chromatophores: neurobiology and natural history. *Biol. Rev.* 76, 473–528. doi: 10.1017/S1464793101005772

Molnar, Z., Metin, C., Stoykova, A., Tarabykin, V., Price, D. J., Francis, F., et al. (2006). Comparative aspects of cerebral cortical development. *Eur. J. Neurosci.* 23, 921–934. doi: 10.1111/j.1460-9568.2006.04611.x

Mouritsen, O. G., and Styrbæk, K. (2018). Cephalopod gastronomy—a promise for the future. *Front. Commun.* 3:38. doi: 10.3389/fcomm.2018.00038

Naef, A. (1928). *Die Cephalopoden (Embryologie). Fauna und Flora des Golfes von Neapel.* Roma; Berlin: Bardi; R. Friedländer & Sohn.

Nakajima, R. (2018). "Can I Talk to a Squid? The origin of visual communication through the behavioral ecology of cephalopod," in *Human Interface and the Management of Information. Interaction, Visualization, and Analytics,* eds. S. Yamamoto and H. Mori. (Cham: Springer International Publishing), 594–606.

Nakajima, R., Shigeno, S., Zullo, L., De Sio, F., and Schmidt, M. R. (2018). Cephalopods between science, art, and engineering: a contemporary synthesis. *Front. Commun.* 3:20. doi: 10.3389/fcomm.2018.00020

Nesis, K. N. (2002). Life style strategies of recent cephalopods: a review. *Bull. Mar. Sci.* 71, 561–579.

Nicolakakis, N., Sol, D., and Lefebvre, L. (2003). Behavioural flexibility predicts species richness in birds, but not extinction risk. *Anim. Behav.* 65, 445–452. doi: 10.1006/anbe.2003.2085

Nigmatullin, C. M., and Laptikhovsky, V. V. (1994). Reproductive strategies in the squids of the family Ommastrephidae (preliminary report). *Ruthenica* 4, 79–82.

Nixon, M., and Young, J. Z. (2003). *The Brains and Lives of Cephalopods*. New York, NY: Oxford University.

O'Dor, R. K., and Webber, D. M. (1986). The constraints on cephalopods: why squid aren't fish. *Can. J. Zool.* 64, 1591–1605.

Owen, R. (1832). *Memoir on the Pearly Nautilus (Nautilus Pompilius, Linn.)*. London: Richard Taylor.

Owens, I. P. F., Bennett, P. M., and Harvey, P. H. (1999). Species richness among birds: body size, life history, sexual selection or ecology? *Proceed. R. Soc. Lond. Biol. Sci.* 266, 933–939.

Packard, A. (1972). Cephalopods and fish: the limits of convergence. *Biol. Rev.* 47, 241–307.

Packard, A. (1988). "The skin of cephalopods (coleoids): general and special adaptations," in *The Mollusca*, eds E. R. Trueman and M. R. Clarke (San Diego, CA: Academic Press), 37–67.

Packard, A., and Albergoni, V. (1970). Relative growth, nucleic acid content and cell numbers of the brain in *Octopus vulgaris* (Lamarck). *J. Exp. Biol.* 52, 539–552.

Packard, A., and Hochberg, F. G. (1977). Skin patterning in *Octopus* and other genera. *Symposia of the Zoological Society of London* 38, 191–231.

Plän, T. (1987). *Funktionelle Neuroanatomie sensorisch/motorischer loben im gehirn von Octopus vulgaris.* Doktorgrades der Naturwissehschaften (Dr. Rer. Nat.), Universität Regensburg.

Portmann, A. (1947). Études sur la cérébralisation chez les oiseaux. II. Les indices intracérébraux. *Alauda* 15, 1–15.

Pozzo-Miller, L. D., Moreira, J. E., and Llinás, R. R. (1998). The first-order giant neurons of the giant fiber system in the squid: electrophysiological and ultrastructural observations. *J. Neurocytol.* 27, 419–429.

Quetglas, A., González, M., Carbonell, A., and Sánchez, P. (2001). Biology of the deep-sea octopus *Bathypolypus sponsalis* (Cephalopoda: Octopodidae) from the western Mediterranean Sea. *Marine Biol.* 138, 785–792. doi: 10.1007/s002270000495

Quetglas, A., Ordines, F., González, M., and Franco, I. (2009). Life history of the bathyal octopus *Pteroctopus tetracirrhus* (Mollusca, Cephalopoda) in the Mediterranean Sea. *Deep Sea Res.* 56, 1379–1390. doi: 10.1016/j.dsr.2009.02.007

Ratcliffe, J. M., Brock Fenton, M., and Shettleworth, S. J. (2006). Behavioral flexibility positively correlated with relative brain volume in predatory bats. *Brain Behav. Evol.* 67, 165–176. doi: 10.1159/000090980

Revell, L. J. (2009). Size-correction and principal components for interspecific comparative studies. *Evolution* 63, 3258–3268. doi: 10.1111/j.1558-5646.2009.00804.x

Rezende, E. L., and Diniz-Filho, J. A. F. (2012). Phylogenetic analyses: comparing species to infer adaptations and physiological mechanisms. *Compreh. Physiol.* 2, 639–674. doi: 10.1002/cphy.c100079

Rocha, F., Guerra, A., and Gonzalez, A. F. (2001). A review of reproductive strategies in cephalopods. *Biol. Rev.* 76, 291–304. doi: 10.1017/S1464793101005681

Sayol, F., Lapiedra, O., Ducatez, S., and Sol, D. (2019). Larger brains spur species diversification in birds. *Evolution* 73, 2085–2093. doi: 10.1111/evo.13811

Scheel, D., and Anderson, R. (2012). Variability in the Diet Specialization of *Enteroctopus dofleini* (Cephalopoda: Octopodidae) in the Eastern Pacific examined from midden contents. *Am. Malacol. Bull.* 30, 267–279. doi: 10.4003/006.030.0206

Schuppli, C., Graber, S. M., Isler, K., and van Schaik, C. P. (2016). Life history, cognition and the evolution of complex foraging niches. *J. Hum. Evol.* 92, 91–100. doi: 10.1016/j.jhevol.2015.11.007

Sherman, K., and Duda, A. M. (1999). An ecosystem approach to global assessment and management of coastal waters. *Mar. Ecol. Prog. Ser.* 190, 271–287.

Shigeno, S., Andrews, P. L. R., Ponte, G., and Fiorito, G. (2018). Cephalopod Brains: An overview of current knowledge to facilitate comparison with vertebrates. *Front. Physiol.* 9:952. doi: 10.3389/fphys.2018.00952

Shigeno, S., Kidokoro, H., Tsuchiya, K., Segawa, S., and Yamamoto, M. (2001a). Development of the brain in the oegopsid squid, *Todarodes pacificus*: an atlas from hatchling to juvenile. *Zool. Sci.* 18, 1081–1096. doi: 10.2108/zsj.18.1081

Shigeno, S., Tsuchiya, K., and Segawa, S. (2001b). Embryonic and paralarval development of the central nervous system of the loliginid squid *Sepioteuthis lessoniana*. *J. Comp. Neurol.* 437, 449–475. doi: 10.1002/cne.1295

Shomrat, T., Graindorge, N., Bellanger, C., Fiorito, G., Loewenstein, Y., and Hochner, B. (2011). Alternative sites of synaptic plasticity in two homologous "Fan-out Fan-in" learning and memory networks. *Curr. Biol.* 21, 1773–1782. doi: 10.1016/j.cub.2011.09.011

Smith, M. R., and Caron, J.-B. (2010). Primitive soft-bodied cephalopods from the Cambrian. *Nature* 465, 469–472. doi: 10.1038/nature09068

Sol, D., Gray Stirling, D., and Lefebvre, L. (2005). Behavioral drive or behavioral inhibition in evolution: subspecific diversification in holarctic passerines. *Evolution* 59, 2669–2677. doi: 10.1111/j.0014-3820.2005.tb00978.x

Sörensen, P. M., and Mouritsen, O. G. (2019). Science education and public understanding of science via food, cooking, and flavour. *Int. J. Gastron. Food Sci.* 15, 36–47. doi: 10.1016/j.ijgfs.2018.11.006

Steinhausen, C., Zehl, L., Haas-Rioth, M., Morcinek, K., Walkowiak, W., and Huggenberger, S. (2016). Multivariate meta-analysis of brain-mass correlations in eutherian mammals. *Front. Neuroanat.* 10:91. doi: 10.3389/fnana.2016.00091

Stephan, H., and Pirlot, P. (1970). Volumetric comparisons of brain structures in bats. *Zeitschrift für Zool. Systematik Evolutionsforschung* 8, 200–236.

Sweeney, M. J., Roper, C. F., Mangold, K. M., Clark, M., and Boletzky, S.,v. (1992). "Larval" and juvenile cephalopods: a manual for their identification. *Smithson. Contrib. Zool.* 513, 1–282. doi: 10.5479/si.00810282.513

Sylvester, J. B., Rich, C. A., Loh, Y.-H. E., van Staaden, M. J., Fraser, G. J., and Streelman, J. T. (2010). Brain diversity evolves via differences in patterning. *Proc. Natl. Acad. Sci.* 107, 9718–9723. doi: 10.1073/pnas.1000395107

Tanner, A. R., Fuchs, D., Winkelmann, I. E., Gilbert, M. T. P., Pankey, M. S., Ribeiro, Â. M., et al. (2017). Molecular clocks indicate turnover and diversification of modern coleoid cephalopods during the Mesozoic Marine Revolution. *Proc. R. Soc.* 284:20162818. doi: 10.1098/rspb.2016.2818

Toro, R., and Burnod, Y. (2005). A morphogenetic model for the development of cortical convolutions. *Cereb. Cortex* 15, 1900–1913. doi: 10.1093/cercor/bhi068

Valls, M., Rueda, L., and Quetglas, A. (2017). Feeding strategies and resource partitioning among elasmobranchs and cephalopods in Mediterranean deep-sea ecosystems. *Deep Sea Res. Part I* 128, 28–41. doi: 10.1016/j.dsr.2017.09.002

van Dongen, P. A. M. (1998). "Brain Size in Vertebrates," in *The Central Nervous System of Vertebrates*. (Berlin, Heidelberg: Springer Berlin Heidelberg), 2099–2134. doi: 10.1007/978-3-642-18262-4_23

Van Essen, D. C., Donahue, C. J., and Glasser, M. F. (2018). Development and evolution of cerebral and cerebellar cortex. *Brain Behav. Evol.* 91 158–169. doi: 10.1159/000489943

Villegas, E. J. A., Ceballos-Vázquez, B. P., Markaida, U., Abitia-Cárdenas, A., Medina-López, M. A., and Arellano-Martínez, M. (2014). Diet of *Octopus bimaculatus* Verril, 1883 (Cephalopoda: Octopodidae) in Bahía De Los Ángeles, Gulf of California. *J. Shellfish Res.* 33, 305–314. doi: 10.2983/035.033.0129

Ward, J. H. j. (1963). Hierarchical grouping to optimize and objective function. *J. Am. Stat. Ass.* 58, 244.

Willemet, R. (2012). Understanding the evolution of mammalian brain structures; the need for a (new) cerebrotype approach. *Brain Sciences* 2, 203–224. doi: 10.3390/brainsci2020203

Williamson, R., and Chrachri, A. (2004). Cephalopod neural networks. *NeuroSignals* 13, 87–98. doi: 10.1159/000076160

Wilson, A. C. (1985). The molecular basis of evolution. *Sci. Am.* 253, 148–157.

Wirz, K. (1959). Étude biométrique du système nerveux des Céphalopodes. *Bull. Biol. Fr. Belg* 93, 78–117.

Wishart, D. (1987). *Clustan, Cluster Analysis Software.* Edinburgh: Clustan Ltd.

WoRMS Editorial Board, M. A. (2020). *World Register of Marine Species [Online]*. Available online at: http://marinespecies.org (accessed March 29, 2020).

Xie, Z., Domel, A. G., An, N., Green, C., Gong, Z., Wang, T., et al. (2020). Octopus arm-inspired tapered soft actuators with suckers for improved grasping. *Soft Robo.* 7, 639–648. doi: 10.1089/soro.2019.0082

Yopak, K. E. (2012). Neuroecology of cartilaginous fishes: the functional implications of brain scaling. *J. Fish Biol.* 80, 1968–2023. doi: 10.1111/j.1095-8649.2012.03254.x

Young, J. Z. (1963). The number and sizes of nerve cells in *Octopus*. *Proc. Zool. Soc. Lond.* 140, 229–254.

Young, J. Z. (1964). *A Model of the Brain.* Oxford: Clarendon Press.

Young, J. Z. (1965). The central nervous system of Nautilus. *Philos. Trans. R. Soc. Lond.* 249, 1–25.

Young, J. Z. (1967). "Some comparisons between the nervous systems of cephalopods and mammals," in *Invertebrate Nervous Systems, their Significance for Mammalian Neurophysiology*, ed. C. A. G. Wiersma. (Chicago: University of Chicago Press), 353–362.

Young, J. Z. (1971). *The Anatomy of the Nervous System of Octopus vulgaris.* London: Oxford University Press.

Young, J. Z. (1974). The central nervous system of *Loligo* I. The optic lobe. *Philos. Trans. R. Soc. Lond. B* 267, 263–302.

Young, J. Z. (1976). The nervous system of Loligo. II. Suboesophageal centres. *Philos. Trans. R. Soc.* 274, 101–167.

Young, J. Z. (1977a). Brain, behaviour and evolution of cephalopods. *Symp. Zool. Soc. Lond.* 38, 377–434.

Young, J. Z. (1977b). The nervous system of *Loligo* III. Higher motor centres: the basal supraoesophageal lobes. *Philos. Trans. R. Soc. Lond.* 276, 351–398.

Young, J. Z. (1979). The nervous system of Loligo: V. The vertical lobe complex. *Philos. Trans. R. Soc. Lond.* 285, 311–354.

Young, J. Z. (1991). Computation in the learning system of cephalopods. *Biol. Bull.* 180, 200–208.

Young, J. Z. (1995). "Multiple matrices in the memory system of Octopus," in *Cephalopod Neurobiology*, eds. J.N. Abbott, R. Williamson and L. Maddock. (Oxford: Oxford University Press), 431–443.

Young, R. E., and Harman, R. F. (1988). "Larva", "paralarva" and "subadult" in cephalopod terminology. *Malacologia* 29, 201–207.

Zar, J. H. (1999). *Biostatistical Analysis.* Upper Saddle River, NJ: Prentice Hall.

Zarrella, I., Herten, K., Maes, G. E., Tai, S., Yang, M., Seuntjens, E., et al. (2019). The survey and reference assisted assembly of the *Octopus vulgaris* genome. *Sci. Data* 6:13. doi: 10.1038/s41597-019-0017-6

Zullo, L. (2004). *Functional Organisation of the Sensory-Motor Areas in the SNC of Octopus vulgaris.* Ph.D. Università degli Studi di Napoli Federico II. Facoltà di Scienze Matematiche, Fisiche e Naturali.

Zullo, L., Sumbre, G., Agnisola, C., Flash, T., and Hochner, B. (2009). Nonsomatotopic organization of the higher motor centers in octopus. *Curr. Biol.* 19, 1632–1636. doi: 10.1016/j.cub.2009.07.067

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership