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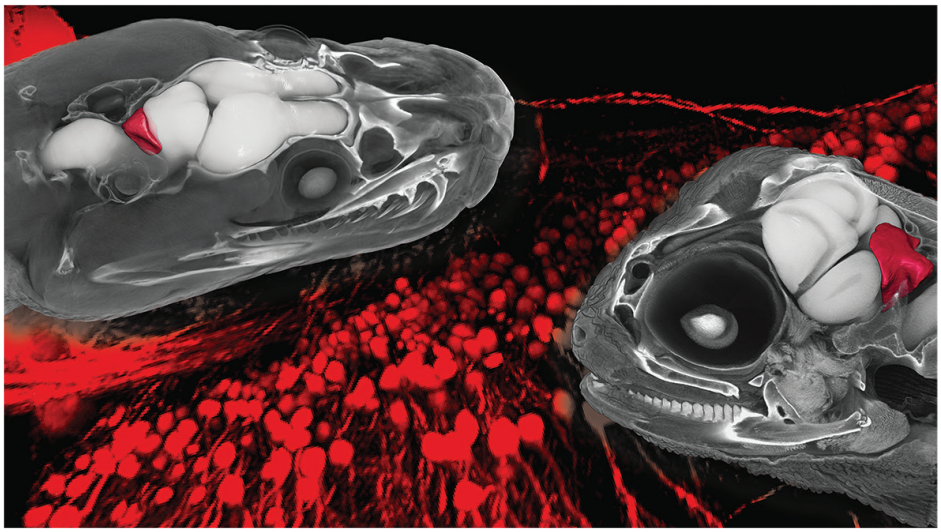
FROM LOCOMOTOR BEHAVIOR TO CEREBELLUM EVOLUTION AND DEVELOPMENT IN SQUAMATE MODELS



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FROM LOCOMOTOR BEHAVIOR TO CEREBELLUM EVOLUTION AND DEVELOPMENT IN SQUAMATE MODELS

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CONTENTS

ABBREVIATIONS

µCT	Micro computed tomography
AGA	Ascending granule cell axon
ATP	adenosine triphosphate
ApoER2	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor
ATOH1	Atonal homolog bHLH transcription factor 1
BC	Basket cell
BG	Bergmann glia
BMP	Bone morphogenetic protein
CALB	Calbindin
CF	Climbing fiber
CNS	Central nervous system
CP	Choroid plexus
CUBIC	Clear, unobstructed brain imaging cocktails and computational analysis
DAB	Disabled homolog
DCN/DCNi	Deep cerebellar nucleus/i
DVR	dorsal ventricular ridge
dph	days post hatching
dpo	days post oviposition
EGL	External granular layer
GABA	gamma-Aminobutyric acid
GC	Granule cell
GCP	Granule cell precursor
GDF	Growth/differentiation factor
GoC	Golgi cell
IGL	Internal granular layer
IHC	Immunohistochemistry
IO	Inferior olive
IRX	Iroquois homeobox
ISH	*In situ* hybridization
LSFM	Light sheet fluorescence microscopy
LHX1	LIM/homeobox protein 1
MEIS	Myeloid ecotropic viral integration site
MF	Mossy fiber

mGluR1	Metabotropic glutamate receptor1
ML	Molecular layer
PAX	Paired box
PC	Purkinje cell
PCA	Principal component analysis
PCC	Purkinje cell cluster
PCL	Purkinje cell layer
PCNA	Proliferating cell nuclear antigen
P-DAB1	Phospho-DAB1
PF	Parallel fiber
PH3	Phospho-histone H3
PS	Pial surface
P-SMAD	Phospho-SMAD
PTF1	Pancreas transcription factor 1
RELN	Reelin
RORA	Retinoic acid receptor-related orphan receptor alpha
RP	Roof plate
SC	Stellate cell
SHH	Sonic hedgehog
SMAD	Mothers against decapentaplegic homolog
SPT11	Spatacsin
STRN	Striatin
TSC1	Hamartin
UBC	Unipolar brush cell
URL	Upper rhombic lip
VLDLR	Very low-density lipoprotein receptor
VS	Ventricular surface
VZ	Ventricular zone
ZIC	Zinc finger of the cerebellum

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. **Simone Macrì**, Yoland Savriama, Imran Khan, and Nicolas Di-Poï. 2019. "Comparative analysis of squamate brains unveils multi-level variation in cerebellar architecture associated with locomotor specialization". Nature Communications. https://doi.org/10.1038/s41467-019-13405-w

S.M. participated in the design of the overall experimental approach and species selection; performed all the micro-CT scans, generated all 3D reconstructions and collected the 3D landmark data; carried out all the 2D and 3D molecular experiments and cell quantification assays; performed part of both the geometric morphometric and transcriptomic analysis; interpreted and analyzed all the results and participated in the preparation of the figures and in the writing of the manuscript.

II. **Simone Macrì** and Nicolas Di-Poï. 2020. "Heterochronic Developmental Shifts Underlying Squamate Cerebellar Diversity Unveil the Key Features of Amniote Cerebellogenesis". Frontiers in Cell and Developmental Biology. https://doi.org/10.3389/fcell.2020.593377

S.M. participated in the design of the overall experimental approach and performed all the experiments; interpreted and analyzed all the results; produced the figures, the first draft and wrote the manuscript.

The publications are referred to in the text by their roman numerals.

ABSTRACT

Locomotor behavior, the entire set of movements an individual utilizes to modify its spatial location in time, is a crucial attribute of an organism's life. Though not responsible for movement initiation or rhythmic locomotor pattern generation, the cerebellum, an ancient and functionally conserved feature of the vertebrate brain, plays a key role in many aspects of motor performance. Variations in its morphology, relative size and cortical organization, likely resulting from divergent developmental programs, have been observed even in closely related vertebrate species, often reflecting a tight linkage between cerebellar organization and functional demands associated with ecologically relevant factors and distinct behavioral traits.

Taking advantage of the extraordinary ecomorphological diversity of squamates (lizards and snakes) and adopting a multidisciplinary approach, this thesis explores the impact of locomotor behavior on squamate brain, particularly on different levels of cerebellar biological organization, and investigates cerebellar morphogenesis in two squamate species to gain insights on the developmental mechanisms potentially responsible for squamate cerebellar divergence.

Along with significant variations in cerebellar morphology and relative size across squamates, this thesis first highlights a wide heterogeneity in Purkinje cell (PC) spatial layout as well as in gene expression pattern, all correlating with specific locomotor behaviors, unveiling unique relationships between a major evolutionary transition and organ specialization in vertebrates. At the developmental level, the thesis indicates that developmental features considered, so far, exclusive hallmarks of avian and mammalian cerebellogenesis characterize squamate cerebellar morphogenesis. Furthermore, the thesis suggests that variations in the spatiotemporal patterning of different cerebellar neurons could be, at least partially, at the base of the large phenotypic diversification of the squamate cerebellum.

Finally, this thesis reveals that squamates provide an important framework to expand our knowledge on organ system-ecology relationships and central nervous system (CNS) development and evolution in vertebrates.

1 INTRODUCTION

The brain is the command-and-control center coordinating the most diverse and fundamental processes of an animal's life. By integrating sensory information originating from both the external world and the individual's internal environment it enables an animal to perform the proper set of actions in response to perceived environmental changes. Thus, the precise control of movement execution, the capability to learn new motor tasks and to quickly react to unexpected discrepancies between the planned and executed movement are among brain functions of crucial ethological importance. Not surprisingly, motor behavior, the entire spectrum of movements an organism can perform, is finely governed by an entangled network of highly interconnected brain areas and nuclei as well as by specialized neuron pools in the spinal cord.

The cerebellum, a major feature of the vertebrate hindbrain, occupies a pivotal position in this network and it is fundamental for fine movement execution, motor error correction and motor learning. Cerebellar functions, cellular organization and both intrinsic and extrinsic connectivity are well-conserved, nonetheless, this brain subdivision exhibits a high degree of variation in morphology, relative size and cortical organization, not only between different vertebrate groups, but also in closely related taxa characterized by divergent ecology and behavioral traits. Such apparent dichotomy between conservation and diversification, together with the relatively simple cytoarchitecture of the cerebellar cortex, have made this brain subdivision an attractive model to understand basic principles of brain evolution and development. Several comparative analyses have, indeed, correlated the relative size or other neuroanatomical features of the cerebellum with specific behavioral traits in all major vertebrate lineages. Moreover, the developmental characterization of this brain subdivision in different vertebrates has highlighted the existence of shared morphogenetic patterns as well as of group-specific developmental strategies, these latter likely playing a key role in determining various aspects of cerebellar phenotypic diversity across vertebrates.

Squamates (lizards and snakes) are a heterogeneous vertebrate group, characterized by an extreme ecomorphological diversification. They also feature a wide repertoire of motor behaviors, ranging from various kinds of limbless locomotion to the highly sophisticated aerial gliding of some snake and lizard species, paralleled by a large diversity in both whole-brain and major brain subdivision organization. In particular, the squamate cerebellum extensively varies in morphology, size, and cortical arrangement. Despite all these characteristics, prior to this thesis work, squamate cerebellar morphogenesis and evolution have been poorly investigated. This thesis now demonstrates that squamates are a key model not only to assess the potential role of ecology and behavior on brain evolution, but also to expand our understanding of the developmental mechanisms responsible for the wide array of vertebrate cerebellar morphologies and cortical arrangements.

2 REVIEW OF THE LITERATURE

2.1 OVERVIEW OF THE VERTEBRATE BRAIN

The brain of vertebrates consists of five major subdivisions: the telencephalon, the diencephalon, the mesencephalon or midbrain, the metencephalon, which dorsally includes the cerebellum and, finally, the medulla oblongata or myelencephalon. Telencephalon and diencephalon collectively constitute the forebrain while the cerebellum with the ventral metencephalic structures and the medulla oblongata are referred to as the hindbrain.

Despite even remarkable anatomical and physiological differences subsist across groups, such basic brain organization is common to all vertebrates (**Fig. 1**; Butler and Hodos, 2005; Striedter, 2005; Sugahara et al., 2016; Sugahara et al., 2017).

2.1.1 THE FOREBRAIN

The forebrain, or prosencephalon, is the rostral-most portion of the brain and includes the extensively interconnected telencephalon and diencephalon. It has a pivotal role in controlling purposeful behaviors and it modulates instinctive actions and reflexes generated in lower CNS centers as a response to environmental or internal stimuli. The basic organization and functions of the forebrain are well conserved across vertebrates, nonetheless, this brain region exhibits a remarkable heterogeneity in terms of relative size, cytoarchitectonic organization and connectivity with other brain districts. The divergent evolution of the prosencephalon has led to an increase in its size and to the elaboration of an entangled network of interconnected centers which paralleled the emergence of complex, highly sophisticated, behaviors in some vertebrate groups.

2.1.1.1 The telencephalon

The vertebrate telencephalon can be subdivided in a dorsal and ventral component, the pallium and the subpallium, respectively (Medina et al., 2005; Northcutt, 1977; Northcutt, 1981; Pombal and Puelles, 1999; Puelles et al., 1999; Wullimann and Rink, 2002). The subpallium is the telencephalic region which shows the highest degree of developmental, structural, physiological and functional conservation across vertebrates (Moreno et al., 2009). Comparative studies highlighted a shared developmental blueprint underlying the origin of the different subpallial derivatives, including the territorial specification of progenitor pools and cell migration. Among the subpallial derivatives, the striatopallidal complex (dorsal and ventral striatum and pallidum) plays a key role in movement control as part of the highly elaborated basal ganglia system which, by interconnecting the striatopallidal complex with both diencephalic (subthalamic nucleus) and mesencephalic (substantia nigra and ventral tegmental area) nuclei, integrates proprioceptive and motivational state information and assists in planning appropriate motor responses.

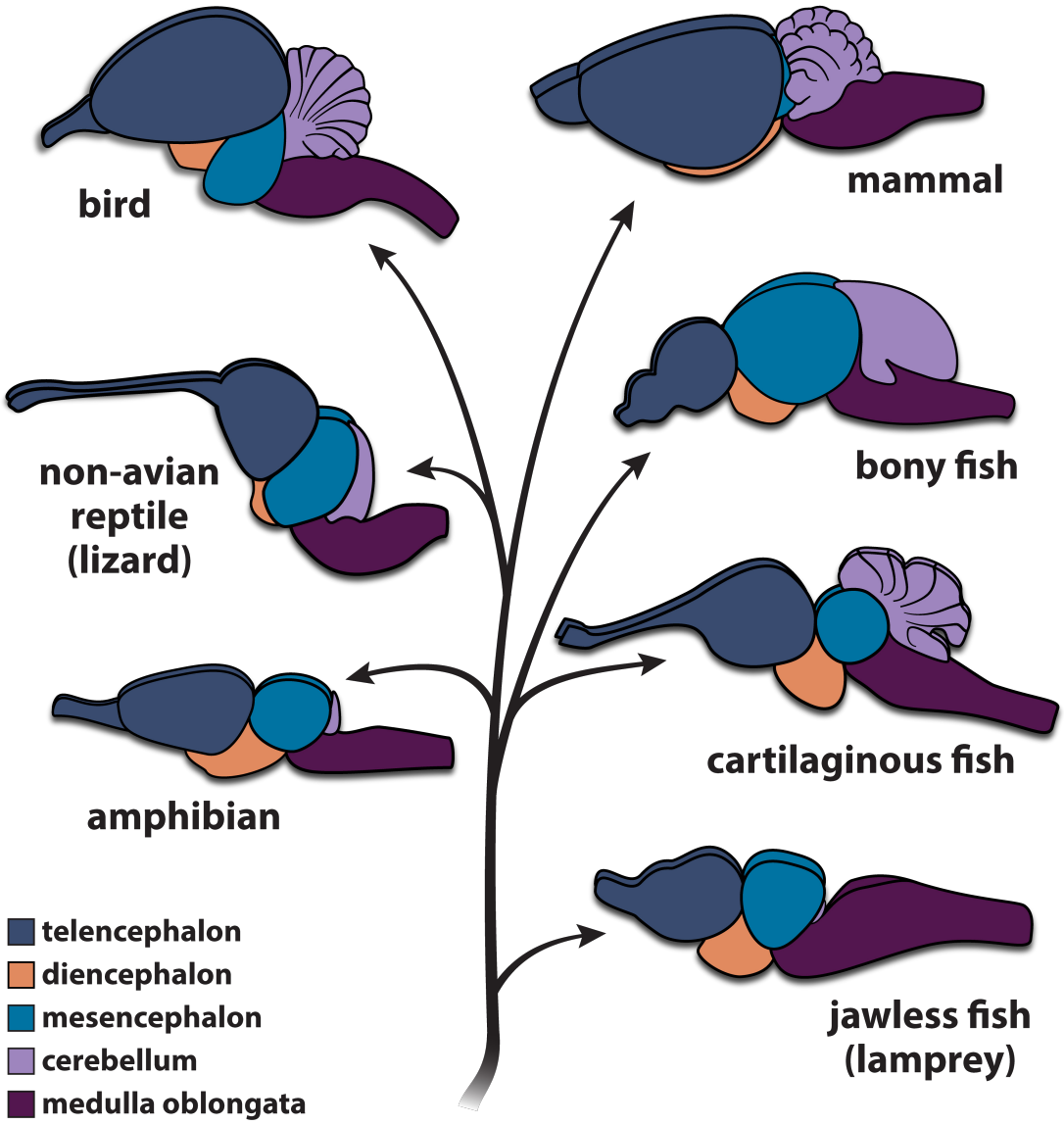

Figure 1 **Gross brain morphology in vertebrates.** Schematic drawings illustrating the morphological heterogeneity of major brain subdivisions in different vertebrate lineages.

While the dorsal component of the striatopallidal complex is present in most vertebrate lineages, the segregation of its ventral component appeared in anurans (Butler and Hodos, 2005; Endepols et al., 2004; Marín et al., 1998; Rink and Wullimann, 2001; Tay et al., 2011). Furthermore, the striatopallidal complex underwent an increase in complexity both in its composition and connectivity at the anamniote-amniote transition, especially with the development of additional pathways to the dorsal thalamus. The evolution of these circuits, involved in both involuntary movement suppression and purposeful movement initiation,

likely paralleled the emergence of new ecological and behavioral challenges imposed by land colonization (Butler and Hodos, 2005).

In contrast to the conserved structural organization of the subpallium, the potential homologies between pallial derivatives across the different vertebrate lineages are only partially resolved. Substantial evidences indicate a well conserved spatial compartmentation of the pallium in four main regions, medial, dorsal, lateral, and ventral (Butler and Hodos, 2005; Ebbesson and Schroeder, 1971; Ganz et al., 2015; Harvey-Girard et al., 2012; Kicliter and Northcutt, 1975; Kokoros and Northcutt, 1977; Mueller et al., 2011; Sugahara et al., 2016; Sugahara et al., 2017; Suryanarayana et al., 2017). However, while a general consensus has been reached on the topographical and functional correspondences of the medial pallium, which in in anamniotes and non-avian reptiles gives rise to structures involved in spatial cognition and map-like memory representations of the allocentric space, similar to the more complex mammalian and avian hippocampus (Broglio et al., 2015; Butler and Hodos, 2005; Holtzman et al., 1999; LaDage et al., 2012; López et al., 2001; Nieuwenhuys et al., 1998; Rodríguez et al., 2002a; Rodríguez et al., 2002b), the evolution and diversification of the other pallial subregions is less clear. In mammals, the dorsal pallium gives rise to the cerebral cortex, the lateral pallium to the olfactory cortex whereas the ventral pallial regions contribute to the amygdalar complex, a set of nuclei relevant for emotional processing and motivation. The cerebral cortex has a high elaborated, six-layered structure clearly distinguished from the simpler three-laminar olfactory cortex. It is site of higher associative areas involved in learning capabilities, memory formation and storage, emotions and social behavior and, in humans, is ultimately responsible for conscious thinking. As a consequence, great efforts have been made to identify putative homologs of the six-layered mammalian cerebral cortex in the other amniote radiations. Non-avian reptile and bird dorsal pallia are characterized by a three-layered structure and by a dorsal pallial thickening, the dorsal cortex and the hyperpallium, respectively. Additionally, they both feature a large non cortical region, the dorsal ventricular ridge (DVR), which extensively protrudes in the lateral ventricle. Despite an apparent and radically different anatomical organization, comparative analyses of sensory afferents to pallial regions, in mammals and birds, evidenced putative homologies between the avian hyperpallium and DVR with the visual and temporal areas of the mammalian cortex, respectively (Butler et al., 2011; Karten, 1969). Moreover, recent studies highlighting the existence of a comparable microcircuitry and input-output configuration in subregions of the avian DVR and areas of the mammalian cortex (Ahumada-Galleguillos et al., 2015; Calabrese and Woolley, 2015; Wang et al., 2010), further suggest a dual origin of the mammalian cerebral cortex. On the other hand, developmental and gene expression analyses (Aboitiz, 1992; Aboitiz et al., 2003; Bruce and Neary, 1995; Montiel and Molnár, 2013; Puelles et al., 2000; Striedter, 1997), clearly indicating a lateral and ventral pallial origin of both the reptilian and avian DVR, rather support homologies between the reptilian and avian DVR with pallial components of the mammalian amygdalar complex, and favor the hypothesis of the expansion of the dorsal pallium as the principal event in mammalian cortex origin.

2.1.1.2 The diencephalon

The diencephalon lies caudal to the telencephalon and it consists of the thalamus and the pretectum. The thalamus can be subdivided along its dorsoventral axis in epithalamus, dorsal thalamus, ventral thalamus and hypothalamus. Furthermore, some anamniotes feature an additional structure, the posterior tuberculum. The complexity of the different diencephalic components varies across vertebrates with anamniotes, actinopterygians (ray-finned fishes) in particular, displaying a complex organization of the posterior tuberculum, pretectum and hypothalamus, whereas amniotes exhibit more elaborated dorsal thalamic regions (Butler and Hodos, 2005).

The nuclei of the pretectum occupy a transitional area over the forebrain and midbrain and are spatially arranged in three different zones which are in continuation with analogous regions in the optic tectum (dorsal midbrain) which, together with retinal projections represents the major pretectal input. Outputs from the pretectum give rise to pathways mediating eye movements in relation to salient visual stimuli (Gamlin, 2006).

The ventral-most division of the diencephalon is the hypothalamus which is functionally interconnected with a rostrally adjacent diencephalic region, the preoptic area. The hypothalamus and the preoptic areas are connected with the pituitary gland by both neuronal projections and hormonal systems, and with some brainstem nuclei and components of the limbic system. Such highly interconnected and entangled system is fundamental in regulating a wide array of relevant functions ranging from circadian rhythm and body temperature regulation to feeding and emotional responses (Saper and Lowell, 2014).

The ventral thalamus, lying in an intermediate position between the hypothalamus and the dorsal thalamus, is part of circuits related to both sensory processing and motor control. Three ventral thalamic nuclei, all receiving retinal projections, are present in anamniotes. Like anamniotes, mammals feature three nuclei, tightly interconnected both with the globus pallidus in the striatopallidal telencephalic complex and the substantia nigra in the midbrain tegmentum, which are primary involved in motor control. These nuclei participate in basal ganglia-related circuitry and, so far, similar connections have not been identified in anamniote ventral thalamic nuclei. They may be an exclusive hallmark of amniotes, related to increased motor control demands associated with the transition to terrestrial environment (Butler and Hodos, 2005). The ventral thalamus includes also the ventral lateral geniculate nucleus which, in mammals, relays inputs from the retina and from superficial and intermediate areas of the dorsal midbrain carrying visual and both motor and multimodal sensory inputs, respectively. Moreover, it receives inputs from the pretectum and from different visual areas of the cerebral cortex. The ventral lateral geniculate nucleus projections do not ascend to the cortex but rather feedback to the intermediate dorsal midbrain and pretectum and target also the cerebellum via the pontine nuclei. Owing to its connectivity the ventral lateral geniculate nucleus is involved in the control of eye movements upon relevant stimuli presentation and in the regulation of coordinated head and eye movements (Butler and Hodos, 2005).

The dorsal thalamus comprises nuclei that transmit different modality sensory information to the telencephalon and, in some vertebrates, nuclei that are highly integrated in circuits with pallial telencephalic areas. Sensory information reaching the dorsal thalamic nuclei are largely relayed through bilateral projection in anamniotes while ipsilateral projections to the

telencephalon are predominant in amniotes. In addition, the majority of anamniotes feature three dorsal thalamic nuclei while, in contrast, amniotes show many distinguished nuclei (Butler, 1994; Butler, 2008). However, the basic structural organization of the dorsal thalamus is largely conserved across vertebrate radiations. In all gnathostomes, indeed, the dorsal thalamic nuclei can be grouped into a lemnothalamic (rostral) and a collothalamic (caudal) division, receiving either direct visual and somatosensory stimuli or indirect sensory information through relays in the midbrain roof, respectively. Within amniotes, a similar configuration of both lemnothalamic and collothalamic nuclei have been identified, nonetheless the putative homology and correspondence of individual nuclei are not fully elucidated (Butler and Hodos, 2005).

2.1.2 THE MIDBRAIN

The vertebrate midbrain, or mesencephalon, consists of a dorsal part or roof, including two laminar structures—the tectum and the tori semicirculari, corresponding to mammalian superior and inferior colliculi, respectively—and a ventral region, known as the tegmentum mesencephali, with a nuclear structure. Both mesencephalic subdivisions play a relevant role in sensorimotor integration as part of an extensively interconnected system which includes sensory and motor nuclei, as well as integrative structures residing in the hindbrain and forebrain. In addition, the transitional region between the mesencephalon and the hindbrain, the isthmus, plays a crucial role during embryonic brain patterning and it is site of nuclei with a widespread neuromodulatory function and of the midbrain locomotor region, a conserved and likely ancestral system that mediates locomotor behavior.

2.1.2.1 The optic tectum and tori semicirculari

The tectum, or superior colliculi in mammals, is the most conserved brain region in both anamniotes and amniotes. Its multi-layered architecture, cell subtypes and connectivity are, indeed, quite similar in all vertebrates (Butler and Hodos, 2005). Owing to the conspicuous inputs received from the retina it is generally called optic tectum and it is remarkably expanded in animals with a particularly developed visual system. However, while retinal connections predominate in the superficial layers of the optic tectum, the most internal laminae are provided with auditory, somatosensory and, in organisms with specialized receptive organs, infrared-sensory and electro-sensory information (Hartline et al., 1978; Ingle, 1973; Schaefer, 1970; Zeymer et al., 2018). These extra-visual stimuli are relayed to the optic tectum by nuclei in the hindbrain or by a paired structure, forming two bilateral bulges in the lower midbrain roof, the tori semicirculari—known as inferior colliculi in mammals. The tori semicirculari are present, except for hagfishes, in all vertebrates and mediate auditory stimuli as well as mechano- and electro-sensory information transmitted by the lateral line system in aquatic anamniotes (Syka and Straschill, 1970; Zeymer et al., 2018).

Both the number and the thickness of the tectal layers vary among taxa, nonetheless, the majority of both incoming and outgoing projections are arranged in a similar topographic fashion in all vertebrates. Such organization, together with the segregation of tectal afferents in different layers, results in the formation of stacked three-dimensional unimodal sensory

maps of the external environment, mutually in register in a way that a specific point in the visual space has a corresponding point in the in the auditory space (Drager and Hubel, 1976; Hartline et al., 1978; Stein et al., 1976). Furthermore, this configuration is paralleled by a similar distribution of the efferent projections descending to brainstem motor nuclei in most vertebrates as well as to the spinal cord in mammals. This congruence of sensory and motor maps allows the optic tectum to play a central role in motor responses following the individuation of a relevant stimulus in the environment, such as reorienting eyes and head towards a prey or escaping from predators (Evans et al., 2018; Schneider, 1967; Schneider, 1969; Sprague and Meikle, 1965). In addition, the sensory spatiotopic information generated in the tectal laminae is also relayed, via efferents to the thalamus, to upper pallial and subpallial areas in the telencephalon, which, in turn, exert a positive or negative selection on tectum-mediated motor behaviors (Everling and Johnston, 2013; Hu et al., 2019).

Both the optic tectum and the tori semicirculari show variations in their relative size, complexity and organization which, in parallel with the degree of development of the sensory systems they are interconnected to, have been correlated to specific ecological and behavioral traits in different vertebrate groups, ranging from fishes to birds and mammals (Barton and Harvey, 2000; Barton et al., 1995; Corfield et al., 2011; Crish et al., 2003; Gutiérrez-Ibáñez et al., 2013; Hoops et al., 2017; Iglesias et al., 2018; Wagner, 2001a; Wagner, 2001b). Such findings highlight the role of ecological factors in shaping morphological and structural features of the vertebrate brain and the tight relationships linking the form and the function of specific brain areas.

2.1.2.2 The midbrain tegmentum

Ventral to the optic tectum, the midbrain tegmentum contains a collection of nuclei completely lying in the midbrain floor, like the red nucleus, the substantia nigra and the nuclei of the ventral tegmental area—all playing a key role in motor behaviors, the former involved in limb movement coordination and the others in mediating the initiation and control of voluntary movements. Other tegmental nuclei, instead, are integral part of diffuse motor-related networks that extend to the hindbrain, such as the reticular formation and the nucleus of the III cranial nerve (oculomotor). Moreover, the tegmentum mesencephali is extensively traversed by both ascending and descending fiber systems which transmit sensory information to forebrain areas and motor commands to brainstem nuclei and spinal cord, respectively. Data regarding the structure and composition of the tegmentum in different vertebrate groups are fragmented, nonetheless, despite anamniotes (except for sharks, rays and skates) seem to lack both the ventral tegmental area and substantia nigra, most of the tegmental structures and fiber systems appear to be well-conserved, at least among gnathostomes (Butler and Hodos, 2005).

2.1.3 THE HINDBRAIN

The hindbrain is composed by the medulla oblongata, the floor of the metencephalon and the cerebellum (for a detailed description of the cerebellum, please, see chapter 2.2). While in most vertebrates the ventral hindbrain floor constitutes an homogeneous structure with no

obvious structural and morphological transition between the metencephalic and myelencephalic subdivision, in mammals, the ventral metencephalon presents a highly developed and distinctive formation, the pons, which hosts several cranial nerve nuclei and it is massively crossed by projections from the cerebral cortex that either terminate on site (corticopontine tract) or descend to the spinal cord (corticospinal tract). The corticopontine tract is part of a bi-synaptic pathway connecting cortical areas in the telencephalon with the contralateral cerebellum through a relay in pontine nuclei. Pontine nuclei, though much smaller than the mammalian counterpart, have been identified in birds while their presence in reptiles is debated.

The hindbrain contains most of the nuclei of the cranial nerves (III-X, XII) and has a pivotal role in in key biological functions like the control of breathing, alertness, sleep and motor coordination. Large part of these functions is mediated by a complex, extensively ramified coordinating system, the reticular formation which, made up of loosely packed neuronal clusters and fibers interspersed between the sensory and motor nuclei of the cranial nerves, spans the entire hindbrain and extends to forebrain areas. The coordinating activity of the reticular formation is elicited through its extensive and capillary interconnection with sensory, and both somatomotor and visceromotor systems. The reticulospinal tract, conveying information from upper motor systems to spinal cord motor neurons largely involved in axial and proximal limb musculature activity and tone (Davidson and Buford, 2004; Davidson et al., 2007; Drew et al., 2004; Lawrence and Kuypers, 1968; Mewes and Cheney, 1991; Prentice and Drew, 2001), is a crucial route in motor control and execution in all vertebrates. Embedded in the entangled mesh of the reticular formation, specialized groups of neurons characterized by the production of distinctive neuropeptides form, together with other brainstem and hypothalamic nuclei, a neuromodulatory system that regulates key physiological processes, including sleep and wakefulness, heart rate and blood pressure, with its pervasive ascending and descending pathways.

In addition to the reticular formation, the motor control-related functions of the hindbrain are also mediated by a nuclear complex localized in the medulla oblongata and strongly interconnected with the cerebellum, the inferior olive (IO). The IO receives motor and sensory, especially proprioceptive, information from multiple CNS regions including cortical motor areas, brainstem nuclei, spinal cord (Berkley and Worden, 1978; Swenson and Castro, 1983; Swenson et al., 1989), and sends efferents to the cerebellar cortex and to the deep cerebellar nuclei (DCNi). The cortico-olivary projections carry information related to ongoing motor activity and intention while spino-olivary fibers transmit information about limb position and muscle tone. IO axons, known as climbing fibers, branch extensively and ascend to the molecular layer of the cerebellum where they wrap around proximal Purkinje cell (PC) dendrites, providing the cerebellum with information crucial for the motor control and motor learning functions exerted by this brain subdivision.

Hindbrain structures have been quite conservative during vertebrate evolution. All jawed vertebrates, indeed, display a well recognizable reticular formation and its principal neuronal groups as well as a similarly organized IO and neuromodulatory system. The acquisition of some additional hindbrain structures in tetrapods, particularly in birds and mammals, paralleled the transition to land and the evolution of a sophisticated use of limbs and digits (Butler and Hodos, 2005).

2.1.4 THEORIES OF BRAIN EVOLUTION

Despite sharing a basic configuration, vertebrate brains display a wide array of variations in their overall- and single subdivision size and morphology, cellular organization and composition, and functional capabilities, but the evolutionary mechanisms underlying such phenotypic diversification are poorly understood.

Two opposing models have been proposed to explain the observed changes in vertebrate brain architecture, the concerted and the mosaic model of brain evolution.

The concerted model (Finlay and Darlington, 1995) considers the brain as an integrated whole and suggests that brain parts evolve in a coordinated fashion. Strict developmental interdependencies between brain regions, according to this hypothesis, would limit the range of potential changes involving individual brain subdivisions without affecting the rest of the brain, thus, leading to a uniform scaling of brain structures, even in the case of selective pressure acting on specific brain regions. Such model emphasizes shared features between individuals and attributes to developmental pattern modifications, such as temporal variations in neurogenetic events, the key process driving brain evolution (Anderson and Finlay, 2014; Cahalane et al., 2014; Finlay et al., 1998; Finlay et al., 2001; Reep et al., 2007; Workman et al., 2013).

The mosaic model of brain evolution (Barton and Harvey, 2000), on the other hand, views the brain as a collection of independently evolving units. It suggests that brain evolution occurs through alterations in individual functional brain modules in response to specific selective pressures, thus, correlating the phenotypic diversity observed in brain architecture across vertebrates to their different ecological and behavioral traits. According to this model, brain diversity would result from the summation of isolated changes in individual brain areas, derived from modifications in selected genes influencing the structure of a brain module functionally involved in a specific behavior (Hager et al., 2012).

Experimental evidences in support of both theories have emerged in comparative studies of different vertebrate group brain gross anatomy, in the last decades. Indeed, while several volumetric analyses on mammalian and fish brains highlighted a significant proportionality in brain region volume changes (Finlay and Darlington, 1995; Yopak et al., 2010), other studies outlined mosaic changes and significant correlations between different brain module development and specific ecological or behavioral traits, in different vertebrate groups (Barton and Harvey, 2000; Boire and Baron, 1994; Dobson and Sherwood, 2011; Gonzalez-Voyer and Kolm, 2010; Gonzalez-Voyer et al., 2009; Iwaniuk et al., 2004; Kotrschal et al., 1998; Yao et al., 2021). Furthermore, quantitative genetic studies, indicating the existence of low levels of phenotypic and genetic correlations among different brain regions in sticklebacks (Noreikiene et al., 2015) and that volumetric variation of brain subdivisions is regulated by distinct loci independent of each other and of whole-brain size in chicken and mice, (Hager et al., 2012; Höglund et al., 2020) have provided a genetic base for the mosaic model of brain evolution.

A possible reconciliation of these two contrasting views emerged from quantitative analyses on the brain composition of different mammalian taxa (insectivores, tree shrews and primates), evidencing a tendency for each brain subdivision to occupy a nearly-fixed fraction of the whole-brain volume in a given taxon, whereas significant deviations in brain subdivision proportions were found between taxa (Clark et al., 2001). These observations suggested the

existence of evolutionary shifts in the balance between conservation and variability—this latter driven by ecological or behavioral requirements—in developmental processes regulating brain regions growth within and across taxa, and led to the elaboration of the concept of cerebrotypes (Clark et al., 2001), specific patterns of brain composition that diverge among taxa and ecological niches. Cerebrotypes have been recently demonstrated in amphibians, birds as well as in invertebrates (Doré et al., 2002; Iwaniuk and Hurd, 2005; Ponte et al., 2021). Moreover, several studies conducted before the formulation of the cerebrotype concept had already revealed the existence of specific brain subdivision patterns in a broad range of mammals (De Winter and Oxnard, 2001; Lapointe et al., 1999; Legendre et al., 1994) and fish (Huber et al., 1997; Wagner, 2001a; Wagner, 2001b), often associated to specific ecological niches or behavioral traits.

Importantly, large part of the comparative studies on brain evolution has focused on volumetric measurements and the possibility that evolutionary principles similar to those proposed to act in defining brain composition and size could be extended to other, volume-independent, aspects of brain organization, like brain subdivision morphological features or cytoarchitecture, has been poorly investigated.

2.2 THE CEREBELLUM

The cerebellum or “little brain”, is the main feature of the vertebrate hindbrain. It is highly involved in sensorimotor integrative processes underlying the execution of coordinated movements, motor learning, and motor error correction. However, along with this well-recognized role in motor dynamics, evidences accumulating from physiological, neuroanatomical and behavioral studies are highlighting a pivotal role of this brain subdivision also in higher cognitive functions, ranging from working memory to language and emotions (Balsters et al., 2013; Baumann et al., 2015; Buckner, 2013; Koziol et al., 2014; Schmahmann, 2019; Strick et al., 2009; Vandervert, 2016).

A rudimental and poorly recognizable plate-like cerebellum is present in jawless vertebrates such as lampreys, where PCs and granule cells (GCs), the main cerebellar cell types have been observed despite the absence of cortical lamination (Dow, 1942; Johnston, 1902; Pearson, 1936; Sugahara et al., 2017). All gnathostomes possess a morphologically distinct cerebellum featuring a peculiar laminar organization. Cerebellar neurons and accessory interneurons are, indeed, orderly segregated in three distinct layers, the internal GC layer (IGL), the PC layer (PCL) and the molecular layer (ML).

The sensorimotor integrative function exerted by the cerebellum relies on an extensive connectivity bridging this brain subdivision with vestibular, somatosensory, auditory, visual and motor systems (D’Angelo, 2011). Sensory signals from multiple nuclei both in the spinal cord and brain stem (Gould, 1980; Matsushita et al., 1979; Sotelo, 2004) as well as inputs carrying motor information from the cerebral cortex and relayed to nuclei in the pons (Apps and Watson, 2013) are conveyed by mossy fibers (MFs) to GCs while climbing fibers (CFs), extending from the IO, transfer signals coming from the spinal cord (Armstrong, 1974), and higher brain areas to PCs (Crill, 1970; Dias-Ferreira et al., 2010; Lang et al., 2006; Onodera, 1984; Watson et al., 2009). Such a remarkably diffuse pattern of connectivity characterizes

also cerebellar outputs. Signals generated by the cerebellar processing activity are transmitted from PC axons to DCNi neurons which, in turn, sort them towards multiple destinations, including the thalamus in the forebrain, the red nucleus and reticular formation nuclei in the brain stem (Batton et al., 1977; Buisseret-Delmas et al., 1998; D'Angelo, 2018; Homma et al., 1995; Nowak et al., 2007; Teune et al., 2000).

The cerebellum shows a wide range of variation in its overall morphology and relative size, both within and between vertebrate groups (**Fig. 2**).

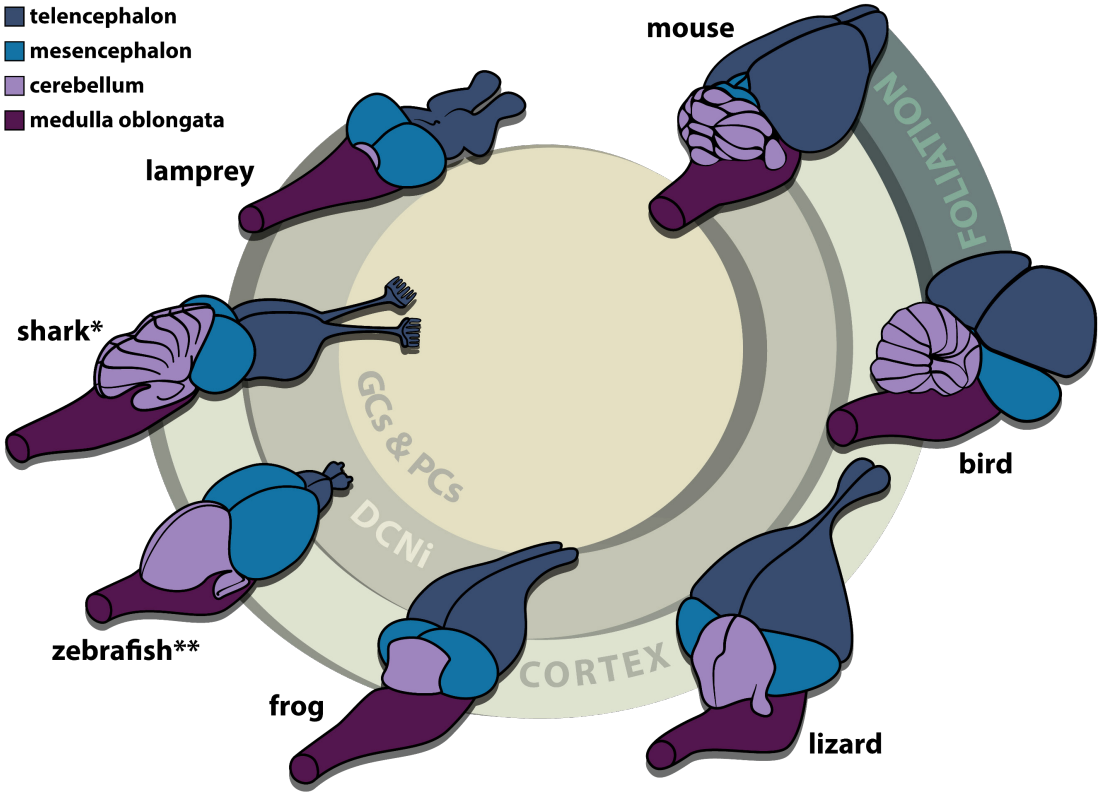

Figure 2 **Phenotypic diversity and structural features of the cerebellum across vertebrates.** GCs, granule cells; PCs, Purkinje cells; DCNi, deep cerebellar nuclei; (*) The cerebellar foliation displayed by some shark species is achieved through mechanisms different than those of birds and mammals. (**) In ray-finned fishes, the cerebellar output is relayed to target regions by the eurydendroid cells, which lack a nuclear organization.

Several modifications, indeed, occurred during the gnathostome radiation involving both the basic cerebellar architecture and connectivity which paralleled an increase in cerebellar complexity. The appearance of DCNi in amphibians and their progressive multiplication in amniotes, together with an increase in the number of accessory interneuron types likely contributed to a better resolution and fine tuning of incoming and outgoing signals. Moreover, the extreme cortical surface expansion in birds and mammals and the establishment of new routes connecting the cerebellar hemispheres with different areas of cerebral cortex in mammals, enhanced the power of cerebellar computations expanding its functional repertoire

(Apps and Watson, 2013). Nonetheless, the main cellular, structural, functional and developmental features of the cerebellum are remarkably conserved among all vertebrates (Butler and Hodos, 2005; Dow, 1942; Larsell, 1923; Larsell, 1926; Larsell, 1932; Nieuwenhuys, 1967; Voogd and Glickstein, 1998) making this brain subdivision an excellent model to study basic brain pattern evolution and development.

2.2.1 CORTICAL STRUCTURE AND INTRINSIC CIRCUITRY

Except for agnathans, the cerebellar cortex displays a tripartite organization (**Fig. 3A**), which, with only few exceptions, is highly stereotyped across vertebrate groups. The IGL is densely populated by the abundant, glutamatergic, small GCs and by excitatory interneurons. The PCL contains the large and pear-shaped somata of the GABAergic PCs, which in most vertebrates are distributed in a well-ordered monolayer along the outer contour of the IGL. The ML is largely dominated by the entangled network formed by GC axons and PC dendrites, and hosts two types of inhibitory interneurons, the basket cells (BCs) and stellate cells (SCs), which occupy distinct subregions of the ML (**Fig. 3A**). The diverse types of accessory interneurons of the cerebellar cortex establish specific connections with PCs, GCs as well as with afferent fiber terminals. This internal connectivity, built upon the broader main incoming and outgoing circuitry of the cerebellum, exerts a modulatory effect that aids in sculpting cerebellar response.

Together with neurons, cerebellum hosts a particular type of astroglial cells, known as the Bergmann glia (BG). The BG plays a crucial role in cerebellar cortex patterning. During development, in fact, it provides a radial scaffold both PCs and GCs use as guidance during the migration from their place of origin towards their final destination (Consalez et al., 2021; Yuasa et al., 1991; Yuasa et al., 1993). In the adult cerebellum, BG is essential for cerebellar physiology and signal processing optimization by actively participating to a vast gamut of functions (De Zeeuw and Hoogland, 2015).

The output cells of the cerebellum are the DCNi neurons. They lie in the deep regions of the cerebellum and are contacted both by incoming afferents (MFs and CFs) and PC axons. The product of the integrative activity occurred in the cerebellar cortex is relayed by bundles of fibers (the cerebellar peduncles), originating in the DCNi, to different locations both in upper and lower brain areas (**Fig. 3A**; Batton et al., 1977; Buisseret-Delmas et al., 1998; D'Angelo, 2018; Homma et al., 1995; Teune et al., 2000; Voogd et al., 2013). Among vertebrates, ray-finned fish cerebella are devoid of DCNi. In these organisms the cerebellar output is transmitted from PCs to specific cells, the eurydendroid cells, which lack a defined nuclear organization (Ikenaga et al., 2006). DCNi number shows a progressive increase during vertebrate evolution (Butler and Hodos, 2005; Nieuwenhuys, 1967), with lampreys, sharks and amphibians displaying a single nucleus, non-avian reptiles having two and birds and mammals featuring three DCNi (Butler and Hodos, 2005; Larsell, 1923; Larsell, 1926; Paul and Roberts, 1984; Pose-Méndez et al., 2016).

2.2.1.1 The internal granular layer

The IGL is the internal-most layer of the cerebellar cortex and it consists of a tightly packed array of GCs, the most abundant cells of the entire brain (Sawtell and Abbott, 2015), but it also includes local excitatory interneurons. Large Golgi cells (GoCs), absent in amphibians (Llinás and Hillman, 1969), are intermingled between GCs, exerting their inhibitory action at the level of MF-GC synapses. GoCs display an elaborated branching with basal and apical dendrites differing in their spatial extension. The basal dendrites are confined in the IGL, while the apical ones penetrate into the ML where they establish contacts with PFs (**Fig. 3A**).

The mammalian cerebellum features additional interneurons, either widely distributed, like the inhibitory Lugaro cells, or restricted to areas involved in vestibular information processing (the vestibulocerebellum), like the unipolar brush cells (UBCs), which stand out as the only glutamatergic interneurons of the cerebellum (Butler and Hodos, 2005; Mugnaini et al., 2011; Schilling et al., 2008).

GCs are characterized by their small dimension and by the peculiar morphology of their axons. GC neurite ascends towards the upper layers of the cortex as a single process and, once reached the molecular layer, it bifurcates towards opposite directions conferring GCs a typical T-shaped appearance. The geometry of these parallel fibers (PFs) is such that the direction of each parallel fiber is orthogonal to the plane of PC dendrites, thus allowing each GC to contact hundreds of PCs (**Fig. 3A,B**; Eccles et al., 1967; Ito, 2006). GC neurite bifurcation has not been described in weakly electric fishes where a divergent arrangement of cortical layers causes the ascending GC processes to lie already in a plane orthogonal to PC dendrites. The contacts between GCs and PCs, however, are not exclusively restricted to PFs. *En passant* synapses between the ascending GC axon (AGA) and PCs are, indeed, recurrent (**Fig. 3B**; Bower, 2002; Bower and Woolston, 1983; Gundappa-Sulur et al., 1999; Huang et al., 2006; Sims and Hartell, 2005). In contrast with the weak and delayed stimulation provided by PFs innervating the distal portion of PC arborization, these *en passant* synapses, located in proximal regions of PC arborization, are capable to deliver a powerful and instantaneous excitatory input to PCs (Bower, 2002; Huang et al., 2006). These divergent transmission modalities likely play distinct role in cerebellar physiology. Synapses between AGA and proximal PC dendrites are, indeed, thought to work as coincidence detectors whereas PF-PC synapses, owing to their high plasticity, are likely to act as modulators of cerebellar signal processing (Sims and Hartell, 2005). Excitatory inputs from different brain nuclei and spinal cord reach the IGL through MFs. MFs, GCs and GoCs terminals form a specialized synaptic complex, known as *glomerulus*. In the *glomerulus* GoCs receive excitatory input from the MFs and exert an inhibitory action on neighboring GCs causing waves of lateral inhibition that propagate beyond the afferent synaptic field. These effects have been correlated with specific physiological and morphological properties of GoCs and are considered fundamental in fine tuning long-term synaptic plasticity at the MF-GC interface (D'Angelo et al., 2011; Galliano et al., 2010).

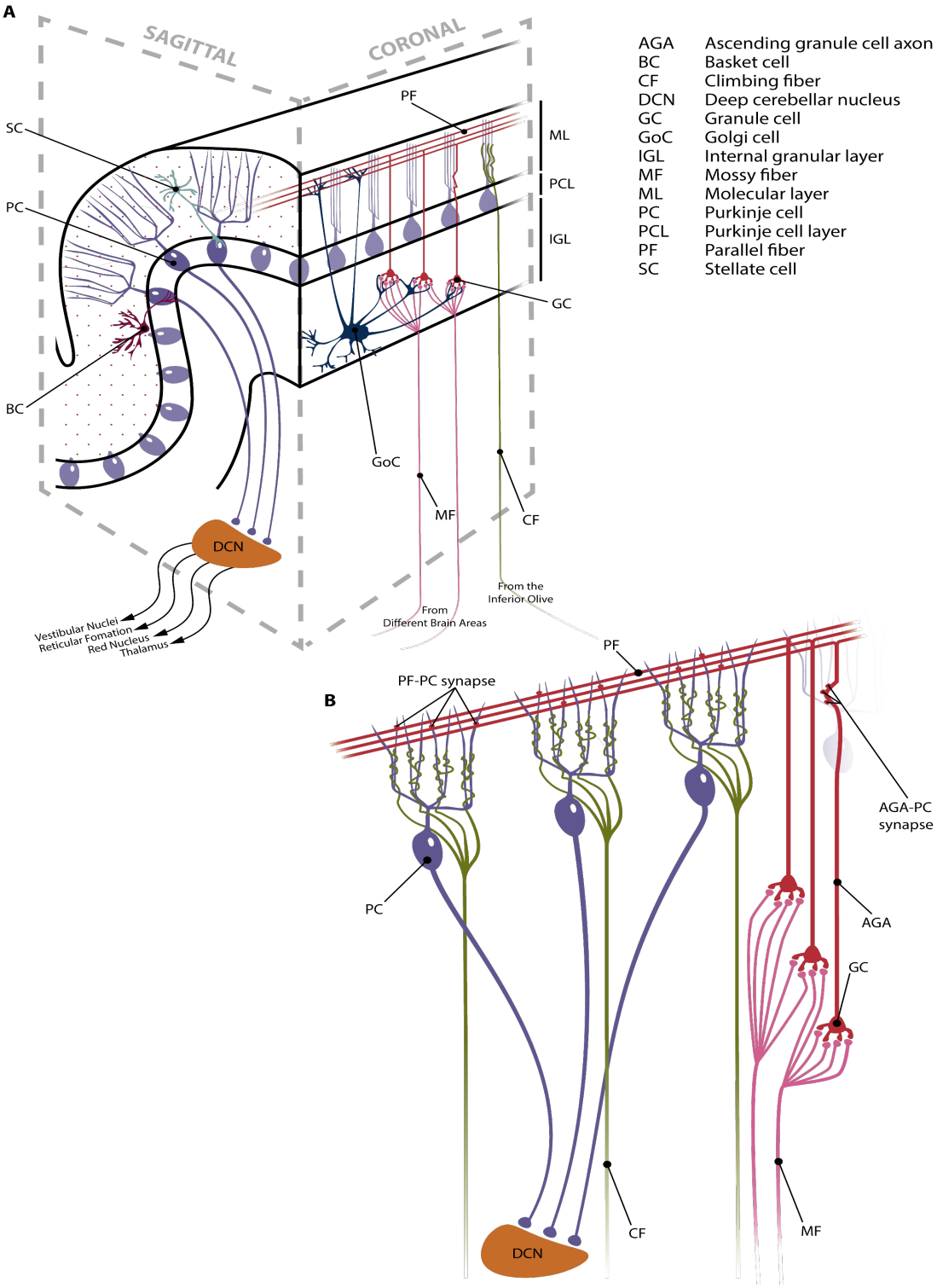

Figure 3 **Structure of the cerebellar cortex. (A)** Cerebellar cytoarchitecture and circuitry. The presence and type of cerebellar interneurons varies across vertebrates. **(B)** Synapse distribution and spatial relationships between main cerebellar cell types and their afferents. CF and MF synapses with DCN are not represented.

In the mammalian cerebellum and in weakly electric fishes, an additional and particular type of interneuron participates in GC mossy fiber synapses, the UBCs. UBCs are small, excitatory interneurons that possess a single and stubby dendrite which ends in a brush-like structure and contacts a single mossy fiber terminal carrying a vestibular input. UBC axons locally arborize in the granular layer to contact proximal GCs and MFs forming rosette-like structures. The main function of UBCs is to generate an intracortical circuitry responsible for a sustained feed-forward amplification of single mossy fiber input (Schilling et al., 2008). In contrast with the other accessory interneurons, uniformly distributed in all cerebellar areas, UBCs are almost exclusively located in cerebellar region targeted by vestibulocochlear stimuli, suggesting a pivotal role of these cells in balance and posture (Mugnaini et al., 2011).

2.2.1.2 The Purkinje cell layer

Purkinje cells are among the largest cells of the entire brain (Dusart and Flamant, 2012) and display peculiar morphological features. They show a pear-shaped cell body and an elaborated dendritic arborization which ramifies along a single plane. PCs somata lie along the outer contour of the IGL and are distributed in a well-ordered monolayer in most vertebrate groups (**Fig. 3A,B),** exception being some cartilaginous fishes, lungfishes and snakes which display, instead, various nuances of PC scattering. The reciprocal organization of PC dendrites is also highly stereotyped. PC dendritic trees, in fact, extend along parallel planes, orthogonal to PF direction. This overall PC spatial layout allows single GCs to propagate their output to several thousand PCs via their PFs. PCs are inhibitory GABAergic cells capable of a vast repertoire of electrophysiological responses, mediated by complex sets of ion channels, and are the sole output of the cerebellum. Their myelinated axons contact the underlying DCNi that will eventually relay the outcome of cerebellar computation to multiple brain nuclei in the brain stem and forebrain (**Fig. 3A,B)**.

PCs are the direct target of signals originating in various districts of both brain and spinal cord (Armstrong, 1974; Crill, 1970; Dias-Ferreira et al., 2010; Lang et al., 2006; Onodera, 1984; Watson et al., 2009), relayed by IO CFs (Desclin, 1974; Sotelo et al., 1975) which tightly wrap around PC dendrite initial segment and proximal portion (**Fig. 3A,B**). PC innervation by CFs represents a unicum in the entire CNS due to an almost exact numerical matching between PCs and CFs. CF ramifications establish extensive contacts with PC dendrites but each PC is contacted by only one CF (Armstrong and Schild, 1970; Cesa and Strata, 2009; Eccles et al., 1966; Hashimoto and Kano, 2013; Kano et al., 2018). Such a precise pattern of innervation is grossly set already during development and refined postnatally (Cesa and Strata, 2009).

In addition to PC somata, the PCL also hosts the cell bodies of a highly specialized type of astrocytes, the BG. BG physiological properties and morphology are finely adapted to the cerebellar network where BG tasks are thought not be restricted to simple housekeeping processes but rather strongly connected to cerebellar computational dynamics. BG have been shown, indeed, to both regulate the extracellular ionic milieu and have a neuroprotective function (Jakoby et al., 2014; Wang et al., 2012), but also to extensively contribute both to synaptic stability and plasticity (Balakrishnan and Bellamy, 2009; Balakrishnan et al., 2014; Iino et al., 2001; Saab et al., 2012).

2.2.1.3 The molecular layer

The ML is outermost layer of the cerebellar cortex and it is dominated by PC dendritic arborizations and GC parallel fibers which give rise to an ordered, though extremely entangled, network spanning the entire ML thickness. Due to the peculiar structure and orientation of GC axons, PC somata and dendrites, each parallel fiber synapses with a multitude of PCs along its way. The synapses between PFs and PCs are highly plastic with adaptive rearrangements in their strength and location reflecting modifications of both environmental and internal parameters induced by a wide gamut of events, including physical exercise, social interaction and neurotransmitter modulation (Floeter and Greenough, 1979; Ito and Schuman, 2008; Pysh and Weiss, 1979). Therefore, the ML is a dynamic environment where crucial events underlying cerebellar processing capabilities occur.

The cellular fraction of the ML is constituted by two types of inhibitory interneurons, the BCs and SCs. BC and SC position in the internal cerebellar circuitry is quite similar as they both receive inputs almost exclusively from the PF system and direct their output to PC. They are distinguished by the branching pattern of their axons and dendrites, by the topography of their synapse on PCs, and by the different regions their perikarya occupy in the ML (Chan-Palay and Palay, 1970; Chan-Palay and Palay, 1972; Lemkey-Johnston and Larramendi, 1968; Schilling et al., 2008). Moreover, while SCs have been thoroughly documented in all jawed vertebrates, BCs are an exclusive feature of the avian and mammalian cerebellum (Butler and Hodos, 2005; Chan-Palay and Palay, 1972; Eccles et al., 1970; Kaslin and Brand, 2013; Llinás and Hillman, 1969; Midtgaard, 1992; Rushmer and Woodward, 1971).

SCs populate the upper part of the ML and their axons run parallel to PFs before descending along an almost orthogonal plane to contact multiple PC dendrites with their branches. SC axons rarely reach PC somata and they generally terminate abruptly (Chan-Palay and Palay, 1972). BCs, instead, are found in the lower third of the ML and directly inhibit PCs by forming remarkably elaborated pericellular cages (baskets) with their axons, giving rise to numerous perisomatic synapses with PCs. Additionally, BC axons form specialized terminals, characterized by a brush-like appearance and known as *pinceaux*, which contact the initial segment of PC axons (Chan-Palay and Palay, 1970; Zhou et al., 2020). The divergent topography of SC and BC synapses on PC strongly influences the intensity of the inhibition these interneurons exert on target PCs, likely reflecting a differential contribution to PC response modulation. The contacts between SCs and PCs, occurring in distal regions of the PC dendrites are, indeed, only capable of weakly altering PC membrane potential, whereas, the perisomatic stimulation from BCs exerts a much powerful effect. Altogether, ML inhibition is thought to give a crucial contribution to cerebellar computational processes aiding in dynamically sculpting PC output, thanks to the diversified structural and physiological properties of SCs and BCs, via complex combinations of lateral, and both feedforward and feedback inhibition mechanisms (Ito, 2014; Prestori et al., 2019).

2.2.2 CONNECTIVITY OF THE CEREBELLUM

On par with the internal architecture and circuitry of the cerebellar cortex, the organization of both cerebellar inputs and outputs is highly stereotyped and well conserved among vertebrates

(Butler and Hodos, 2005). Virtually, all important districts of the whole CNS are in connection, either directly or indirectly, with the cerebellum, including brain stem, spinal cord, basal ganglia, limbic system, thalamus and telencephalic motor areas (Bangma and ten Donkelaar, 1982; Bangma et al., 1984; Bostan et al., 2010; Bostan et al., 2013; Gonzalez et al., 1984; Gould, 1980; Matsushita et al., 1979; Sotelo, 2004). Moreover, multiple non motor, associative regions of the mammalian cerebral cortex show an extensive bi-directional connectivity with the cerebellum (Glickstein et al., 1985; Middleton and Strick, 1994; Middleton and Strick, 2001; Sasaki et al., 1979; Schmahmann, 1996), forming a closed loop circuit influencing cognitive functions such as attention, executive control, language, working memory, and learning (Strick et al., 2009). Such extensive and capillary connectivity provides the cerebellum with the anatomical foundation to perform its diffuse and heterogeneous tasks.

2.2.2.1 Climbing fibers and inferior olive

CFs originate solely from the IO, a large and composite nucleus in the ventrolateral surface of brainstem (Szentágothai and Rajkovits, 1959). IO neurons send axons that travel to the contralateral ML where they contact discrete groups of PCs, distributed along a parasagittal stripe of the cerebellar cortex. Once in the cortex, IO neuron axons extensively branch giving rise to an average of seven CFs which wrap around proximal regions of PC dendrites establishing a conspicuous number of large synapses (**Fig. 3B)**. A remarkable feature of CF innervation, revealed by studies in rat, is the nearly perfect match between IO neurons and PCs. In fact, although each IO cell targets an average of seven PCs, each PC is innervated by one IO neuron only (Armstrong and Schild, 1970; Cesa and Strata, 2009; Eccles et al., 1966; Hashimoto and Kano, 2013; Kano et al., 2018). This precise innervation pattern is refined from the originally overconnected network generated during embryogenesis through a thorough process of synapse elimination taking place during the first postnatal weeks in rodents (Crepel et al., 1976; Hashimoto and Kano, 2013; Kano et al., 2018; Mariani and Changeux, 1981). CF input to PC stands as one of the strongest synaptic connections in the whole-brain and it has been proposed to act as an error signal, modulating associative plasticity at the level of PF-PC synapses (Albus, 1971; Ito, 2001; Marr, 1969). CFs form wide synapses capable to induce strong depolarizations in PCs and trigger distinctive complex spike responses, which are remarkably different from the graded potentials induced by PF stimulation. The information relayed by CFs originates from many brain districts connected to the IO, each targeting specific IO subnuclei. IO is provided with somatosensory, motor, visual, optokinetic and vestibular information by afferents from the spinal cord, vestibular nuclei, midbrain superior colliculi and from nuclei at the mesodiencephalic junction relaying inputs from the cerebral cortex (De Zeeuw and Ruigrok, 1994; Onodera, 1984; Onodera and Hicks, 1995; Swenson and Castro, 1983). Furthermore, IO neurons constitute an entangled network of communicating cells thanks to gap junctions interconnecting them (Condorelli et al., 1998). Such configuration allows the electrotonic coupling of IO cells, facilitating the generation of synchronous, subthreshold, oscillations among them. Such properties of IO neurons, influencing both the rate and the timing of PC complex spikes are thought to be fundamental for learning-dependent timing in motor control (Devor and Yarom, 2002; Lampl and Yarom, 1993; Van Der Giessen et al., 2008).

2.2.2.2 Mossy fibers

Mossy fibers are the second major input to the cerebellar cortex. Like CFs, MFs arise from multiple sources in the brain and spinal cord, and compose a heterogeneous population of cerebellar afferents, conveying an highly diversified range of information (Gould, 1980; Matsushita et al., 1979). However, both the trajectory and the destination of mossy fibers substantially differ from those of CFs. In fact, MF projections are bilateral, innervating multiple groups of GCs along the medio-lateral extent of the cerebellum. Moreover, MFs do not extend to upper cortical areas but terminate in the IGL (**Fig. 3B**) where they participate in the formation of remarkable synaptic structures, described for the first time by Ramón y Cajal (1888), and known as *glomeruli*. *Glomeruli* are tripartite anatomic elements composed by the enlarged loops of MF terminals, the clawed edges of GC dendrites and GoC axons and basal dendrites. They allow stimuli conveyed by MFs, in form of either prolonged discharges or as bursts of very high instantaneous firing frequencies (Arenz et al., 2008; Chadderton et al., 2004; Kase et al., 1980; Rancz et al., 2007), to activate both GCs and GoCs. Each mossy fiber can contact up to fifty GCs and form several tens of *glomeruli*. The combined effect of the peculiar arrangement of glomerular synapses and of the asymmetrical morphology of GoC dendrites, is that GoCs can exert their inhibitory activity on GCs by means of both feedforward and feedback loops (Cesana et al., 2013; D'Angelo, 2009; D'Angelo et al., 2013; Kanichay and Silver, 2008). The feedforward inhibition is driven by MF stimulation of the glomerular basal dendrites of GoCs which, in turn, inhibit groups of neighboring GCs in the range of its axonal field. The inhibitory feedback loop is, instead, triggered by AGAs and PFs and mediated by GoC apical arborizations.

Among MF sources in the brainstem are nuclei that also provide important descending motor tracts (rubrospinal, reticulospinal, vestibulospinal), like the red nucleus, the reticular formation and the vestibular nuclei. MFs originating in these nuclei provide the cerebellum with relevant information related to the ongoing motor performance and body segment spatial arrangement. Such connection pattern is highly conserved in and it reflects the common sensorimotor integrative function the cerebellum exerts in vertebrates (Bangma and ten Donkelaar, 1982; Butler and Hodos, 2005; Künzle, 1983b; Pose-Méndez et al., 2016). Differences exist, however, in some groups, and are linked both to the presence of additional cerebellar structures or to the remarkable development of dorsal telencephalic structures which established a tight functional link with the cerebellum. In ray-finned fishes, for instance, the *valvula cerebelli*, an accessory and specialized part of the cerebellum associated with mechano- photo- and electro-sensory functions, is target of a conspicuous number of mossy fibers arising from a special mesencephalic nucleus—the nucleus lateralis valvulae—which has no homologue in other vertebrates (Meek et al., 1986; Meek et al., 2008). In mammals, instead, the most important source of mossy fibers originates in the pons, which mostly relays inputs generated in the cerebral cortex from both sensory and motor areas (Glickstein, 2013; Glickstein et al., 1985). The pons is a prominent structure of the mammalian brain extending from the posterior end of the mesencephalon to the anterior edge of the medulla and is absent in other vertebrate groups. In fact, despite two pontine nuclei (lateral and ventral) have been described in birds, they relay only sparse afferents to the cerebellum, and a distinctive pons is absent in the avian brain. Remarkably, the pons shows an increase in its relative size which parallels the expansion of cerebral and cerebellar hemispheres (the

destination of mossy fibers from pontine nuclei) occurred in mammal brain evolution, outlining a tight functional link between the cerebellum and the cerebral cortex, and highlighting the importance of distributed neural systems in the evolution of complex behaviors (Balsters et al., 2010; Barton and Venditti, 2014; Brodal and Bjaalie, 1992; Whiting and Barton, 2003).

2.2.2.3 Deep cerebellar nuclei

The DCNi distribute the ultimate result of cerebellar computation to the rest of the brain (**Fig. 3A)**. Cerebellar outgoing projections are directed to the upper encephalic areas and to a wide set of brainstem nuclei. With the exclusion of teleosts, which feature specialized cerebellar efferent cells (the eurydendroid cells), lacking a nuclear organization, all vertebrates possess one or more DCNi. In particular, anamniotes have one, non-avian reptiles two (a medial and a lateral), while both birds and mammals show three nuclei (a medial, an interposed and a lateral nucleus). The higher number of DCNi in birds and mammals is likely linked to the enlargement of their cerebellum compared to other vertebrates and to a more refined system for pallial control of medullary and spinal pathways. DCN projections can be grouped in two main categories. The first one, comprising efferents that ultimately influence motor neurons in the spinal cord, and the second one, composed by projections ascending to nuclei in the thalamus relaying, in turn, the signal to telencephalic motor areas. Subsets of fibers arising from the DCN are bundled together and constitute the so-called cerebellar peduncles. In gnathostomes the most relevant cerebellar efferent pathway is via the *brachium conjunctivum* (the superior cerebellar peduncle of mammals) which massively targets neurons in the red nucleus. Neurons in the red nucleus are the origin of the rubrospinal tract, which descends in the contralateral spinal cord and, by influencing the activity of motor neurons, exerts a fundamental role in limb movements. A second destination of *brachium conjunctivum* fibers are the nuclei of the reticular formation. Such diffuse brainstem nuclei give rise to the reticulospinal tract, another primary descending spinal pathway bilaterally influencing motor circuits, involved in axial and proximal limb movements. Furthermore, *brachium conjunctivum* efferents also ascend to diencephalic districts, in particular to the dorsal thalamic division, which provides motor-related areas in the telencephalon with important feedback information from the periphery. In addition to the *brachium conjunctivum*, a minor efferent pathway, displaying a hooked trajectory and known as the *fasciculus uncinatus* in mammals, contributes projections to the vestibular nuclei, to the cervical region of spinal cord, as well as to several motor nuclei of the hindbrain, including the reticular formation. Surprisingly, regardless of the number of individual DCN featured, though a certain heterogeneity may exist in the relative innervation of the different targets, such efferent pattern appears well conserved among gnathostomes (Arends and Zeigler, 1991; Bangma, 1983; Bangma et al., 1984; Ebbesson and Campbell, 1973; Faull, 1978; Finger, 1978; Hindenach, 1931; Künzle, 1985a; Larsell, 1923; Montgomery, 1988; New et al., 1998; Wullimann and Northcutt, 1988).

DCN, however, are not simply a relay station distributing cerebellar outputs but are highly integrated in the cerebellar circuitry. The signal transmitted by the DCN is, in fact, not only influenced by the inhibitory stimuli from PC axons. Both CFs and MFs, before ascending to

their destinations in the cerebellar cortex, send axon collaterals conveying excitatory inputs to DCN (De Zeeuw et al., 1997; Gerrits and Voogd, 1987; Shinoda et al., 1992).

2.2.3 FUNCTIONAL REGIONALIZATION OF THE CEREBELLUM

One of the most striking differences between the cerebellar and the cerebral cortex is the extreme cytoarchitectural homogeneity the former exhibits. While the neocortex microstructure shows a marked divergence in terms of single layer thickness, cellular density and intracortical connectivity across its surface (Brodmann, 1909; Charvet et al., 2015; Defelipe et al., 1999; Scala et al., 2019; Watson and Puelles, 2017), revealing the presence of a functional diversification between cortical areas (Bayer and Altman, 1991; Elston, 2003; Hof and Nimchinsky, 1992), the array of layers and cells as well as the internal circuitry of the cerebellum displays a uniform pattern which is replicated, uninterrupted, along its entire extent.

However, in sharp contrast with such regular and homogeneous internal organization and circuitry, both cerebellar afferents and corticonuclear projections, show a compartmentalized distribution pattern, suggesting that a functional regionalization may exist also in this brain subdivision, though imparted by its connectivity rather than its cortical cytoarchitecture.

At the beginning of the twentieth century, comparative analyses, electrophysiological measurements and lesion studies, aiming to characterize cerebellar functional compartmentation, evidenced the existence of a somatotopic organization of the cerebellum, where representations of different body parts were topographically mapped in specific cerebellar domains (Adrian, 1943; Bolk, 1906; Dow, 1938; Ferraro and Davidoff, 1931; Larsell, 1934; Snider and Stowell, 1944). Since then, technical progress allowed to improve the resolution and refinement of the cerebellar somatotopic map, offering a much more complex picture than previously thought (Manni and Petrosini, 2004). Experimental evidences indicate that body segments are not represented as a continuum over a wide area of the cerebellar cortex but are fragmented into discontinuous patches. In addition, the same body parts are mapped in multiple locations. Such topographic representation is defined as fractured somatotopy (Grodd et al., 2001; Shambes et al., 1978).

Furthermore, investigations on the fine structure and circuitry of cerebellum in the last decades highlighted an exquisite parasagittal modular arrangement of cerebellar inputs and outputs, based on the topography of corticonuclear PC projections and both olivocortical and olivonuclear innervation by climbing fibers and IO cell axon collaterals, respectively. In such pattern, spatially defined subsets of PCs project to specific DCN microdomains which, in turn, convey the information to a circumscribed IO area which supply innervation to both same PCs and DCN subregions (Trott and Armstrong, 1987a; Trott and Armstrong, 1987b; Voogd and Glickstein, 1998). These discrete olivo-cortico-nuclear domains, known as microzones (Oscarsson, 1979) are, thus, believed to constitute the cerebellar functional units for information processing (Apps and Garwicz, 2005; Sugihara et al., 2001; Voogd and Glickstein, 1998).

Further evidence corroborating the modular organization of the cerebellum derives from molecular and physiological analyses outlining the existence of an alternated striped pattern

of PCs and DCN neurons sharing same physiological properties and protein expression profile, highly congruent with the modular distribution of incoming and outgoing cerebellar projections. Among such differentially expressed markers, Aldolase C (ZebrinII) shows a characteristic parasagittal pattern, made of positive cell stripes interspersed with bands of immunonegative neurons (Apps and Hawkes, 2009; Hawkes and Leclerc, 1987; Sugihara and Shinoda, 2004; Sugihara et al., 2009; Voogd, 2014; Voogd and Ruigrok, 2004; Zhou et al., 2014)..

Moreover, recent tracing experiments (Pijpers et al., 2006), revealed an exact spatial correlation between MFs and CFs in the cerebellar cortex of rats, where the convergence of MF and CF potentials onto a single somatotopic map, evoked by stimulating either peripheral nerves or somatosensory areas of the cerebral cortex, had already been documented (Brown and Bower, 2001; Provini et al., 1968). In particular, despite an extensive and bilateral branching (Odeh et al., 2005; Scheibel, 1977; Wu et al., 1999), single MF terminals have been shown to exclusively contact GC clusters subjacent to PC microzones sharing the same ZebrinII expression profile and physiological characteristics (Pijpers et al., 2006).

Though most of the investigations leading to the identification of the somatotopic organization of the cerebellum has been conducted in mammals, a spatial segregation in specific cerebellar regions of both main cerebellar inputs and cortico-nuclear projections has been described in many vertebrates studied so far, independently of cerebellar complexity (Bangma, 1983; Bangma and ten Donkelaar, 1984; Bangma et al., 1984; Iwaniuk et al., 2007; Matsui et al., 2014; Pose-Méndez et al., 2014). In this respect, a distinctive zonal pattern of cerebellar afferents and cortico-nuclear projections has been described in reptiles, including squamates (Bangma and ten Donkelaar, 1982; Bangma and ten Donkelaar, 1984; Bangma et al., 1983; Künzle, 1983a; Künzle, 1985b), suggesting the involvement of different zones in mediating motor functions related to particular body districts (Larsell, 1926).

2.2.4 CEREBELLUM AND MOTOR CONTROL

Motor behavior, the full repertoire of movements an organism can perform, has a crucial ethological relevance as it represents the ultimate form an animal interacts with the surrounding environment. The contribution of the cerebellum is fundamental for motor behavior control both within the context of single body part movements as well as of whole-body locomotion. Moreover, the cerebellum is also central in the acquisition and refinement of new motor tasks.

Initial indications about the potential involvement of the cerebellum in motor processes derived in large part from clinical observations of patients with cerebellar damage and from ablation studies in laboratory animals (Botterell and Fulton, 1938a; Botterell and Fulton, 1938b; Dow, 1938; Holmes, 1917). Such studies, confirmed by more recent investigations, showed the recurrence of motor dysfunctions both in patients and treated animals, and highlighted the correlation between cerebellar damage localization and specific motor impairments, thus corroborating the hypothesis of cerebellar functional compartmentation (Holmes, 1917; Ye et al., 2010). Together with severe balance, posture and oculomotor deficits, cerebellar lesions have been shown to cause a wide range of motor symptoms including the fragmentation of complex motor sequences in series of small steps (decomposition of

movements), a tendency to under/overshoot targets (dysmetria), the incapability to perform alternating movements (dysdiadochokinesis) and the oscillation of a limb preceding or following a purposeful movement (intention tremor), collectively referred to as cerebellar ataxia (Trouillas et al., 1997).

However, despite a considerable expansion of studies on cerebellar functions in these last decades, the mechanisms through which the cerebellum exerts its role in movement control, refinement, and learning are only partially understood. Considering its homogeneous, crystal-like cytoarchitecture, the extensive connectivity with upper and lower sensorimotor centers, and PC firing patterns upon motor task execution, the cerebellum has been suggested to be crucial in the acquisition and storage of movement-related sensorimotor representations, required to estimate the consequences of motor acts (Blakemore et al., 2001; Ebner and Pasalar, 2008; Gilbert and Thach, 1977; Ito, 2006; Ramnani, 2006; Wolpert et al., 1998). In such context, the cerebellum is thought to work as a comparator, capable to adequately remodulate the descending motor system outputs in response to detected mismatches between the ongoing performance and the intended movement (Brooks and Thach, 2011; Glickstein and Doron, 2008). Moreover, the capability to induce long-term modifications of central motor commands in response to deviations from planned movement execution, contributes in narrowing the range of prediction error in subsequent motor acts and is considered to be at the base of the role of the cerebellum in motor learning (Kawato, 1999; Miall et al., 1993).

2.3 DEVELOPMENT OF THE CEREBELLUM

The cerebellum is among the latest CNS structure to develop and shows an extended morphogenesis. In mammals, the vertebrate group displaying the highest level of cerebellar complexity, cerebellogenesis protracts over a long time, from early embryonic stages until early postnatal life (Larsell, 1948; Phemister and Young, 1968; ten Donkelaar et al., 2003). Owing to its conserved architecture and connectivity and to the relative simplicity of its laminar organization, the cerebellum has been an attractive and powerful model to investigate the cellular and molecular processes underlying vertebrate brain development and evolution.

The cerebellum is derived from two paired structures (alar plates) in the dorsal part of the rhombomere 1, the anterior-most portion of the hindbrain (Zervas et al., 2005) but, as recently shown in birds and mammals it receives contributions also from rhombomere 1 basal plate and isthmic subregions (Martinez et al., 2013; Watson et al., 2017). Thus, in the early phases of its morphogenesis the cerebellum develops as a bilateral structure. As cerebellogenesis proceeds, the alar plates converge and fuse along the midsagittal plane, giving rise to the unpaired cerebellar primordium laying above the 4th ventricle. The cerebellar primordium is a thin, plate-like sheet of neural tissue connected anteriorly to the dorsal midbrain and laterally to the hindbrain. Its posterior margin, known as the upper rhombic lip (URL; Miale and Sidman, 1961), is, instead, in continuation with a non-neural structure, the thin roof plate (RP) which covers the 4th ventricle.

After the territorial specification and the formation of the primordium, cerebellar histogenesis strongly relies on the crucial activity of two germinative regions, the ventricular

zone (VZ) and the URL. These two highly proliferative districts, in fact, will give rise to all the cell types residing in the mature cerebellum (Englund, 2006; Fink, 2006; Hoshino, 2006; Hoshino et al., 2005; MacHold and Fishell, 2005; Wang et al., 2005). In addition, cerebellar integrity also depends on the interaction between the cells derived from these two germinative epithelia as well as on signals originating in adjacent non-neural tissues (Carletti et al., 2008; Chizhikov et al., 2006; Corrales et al., 2006; Krizhanovsky and Ben-Arie, 2006; Miyata et al., 1997; Tong et al., 2015; Wechsler-Reya and Scott, 1999). Cerebellar morphogenesis and cortical lamination are, thus, the result of an intense and fine-tuned communication between different developing structures and cells, mediated by secreted factors.

As with cortical organization, internal and external connectivity, and functions, also the morphogenetic and molecular processes at the base of cerebellar development have been quite conservative during vertebrate evolution. Nonetheless, along with minor variations in germinative compartments extension and precursor migration in some anamniotes, significant changes have occurred in birds and mammals at the level of GC generation (Butts et al., 2014a; Iulianella et al., 2019). Such a fine combination of conservation and variation in cerebellar developmental processes across groups is likely the key to interpret the apparent dualism between the remarkable morphological heterogeneity the cerebellum exhibits and its shared cytoarchitecture, connectivity and functions in vertebrates (**Fig. 4**).

2.3.1 THE VENTRICULAR ZONE

PCs, DCN GABAergic neurons and cortical inhibitory interneurons are produced in the VZ of the cerebellum. In mammals, the timing of generation of VZ-derived cells has been thoroughly characterized by lineage tracing analyses (Miale and Sidman, 1961; Morales and Hatten, 2006; Sotelo, 2004). The first cell types to emerge from the VZ are the GABAergic DCN nucleo-olivary projection neurons and nuclear interneurons followed by PCs and, finally, by cortical inhibitory interneurons (Carletti and Rossi, 2008; Leto et al., 2006). A fundamental role in inducing a GABAergic fate to VZ-derived cells is played the bHLH pancreas transcription factor 1 (*Ptf1*; Hoshino, 2006). In absence of *Ptf1*, mouse cerebellar GABAergic precursor acquire a granule cell-like phenotype (Pascual et al., 2007) and deletions causing *PTF1* truncation have been linked to pancreatic and cerebellar agenesis in humans (Sellick et al., 2004). In contrast to the well-established role of *Ptf1* in committing VZ progenitors towards an inhibitory fate, the molecular mechanisms underlying the sequential differentiation of the various GABAergic cell subtypes are only partially resolved. However, evidences exist about the presence in the VZ of different progenitor subsets, each expressing a particular combination of transcription factors, and responsible for the generation of particular neuron phenotypes (Maricich and Herrup, 1999; Morales and Hatten, 2006). In particular, studies in mouse transgenic lines, demonstrated that the GABAergic component of DCNi derives from progenitors expressing the transcription factors iroquois homeobox (IRX3), myeloid ecotropic viral integration site 1 and 2 (*Meis1/2*) and the LIM homeodomain proteins LHX2/9, while GoCs, BCs and SCs emerge from a common progenitor pool characterized by the expression of the paired homeobox 2 (*Pax2*) gene (Maricich and Herrup, 1999; Weisheit et al., 2006). In a similar fashion, PC progenitors feature high expression levels of other members of the LIM homeobox protein family, LHX1/5 (Morales and Hatten, 2006). Moreover, PCs also feature

the expression of the retinoic acid receptor-related orphan receptor alpha (*Rora*), a thyroid hormone-regulated transcription factor necessary for PC maturation, survival, and lifelong morpho-functional integrity (Chen et al., 2013; Dussault et al., 1998; Gold et al., 2003; Gold et al., 2007; Hamilton et al., 1996; Sidman et al., 1962; Takeo et al., 2015).

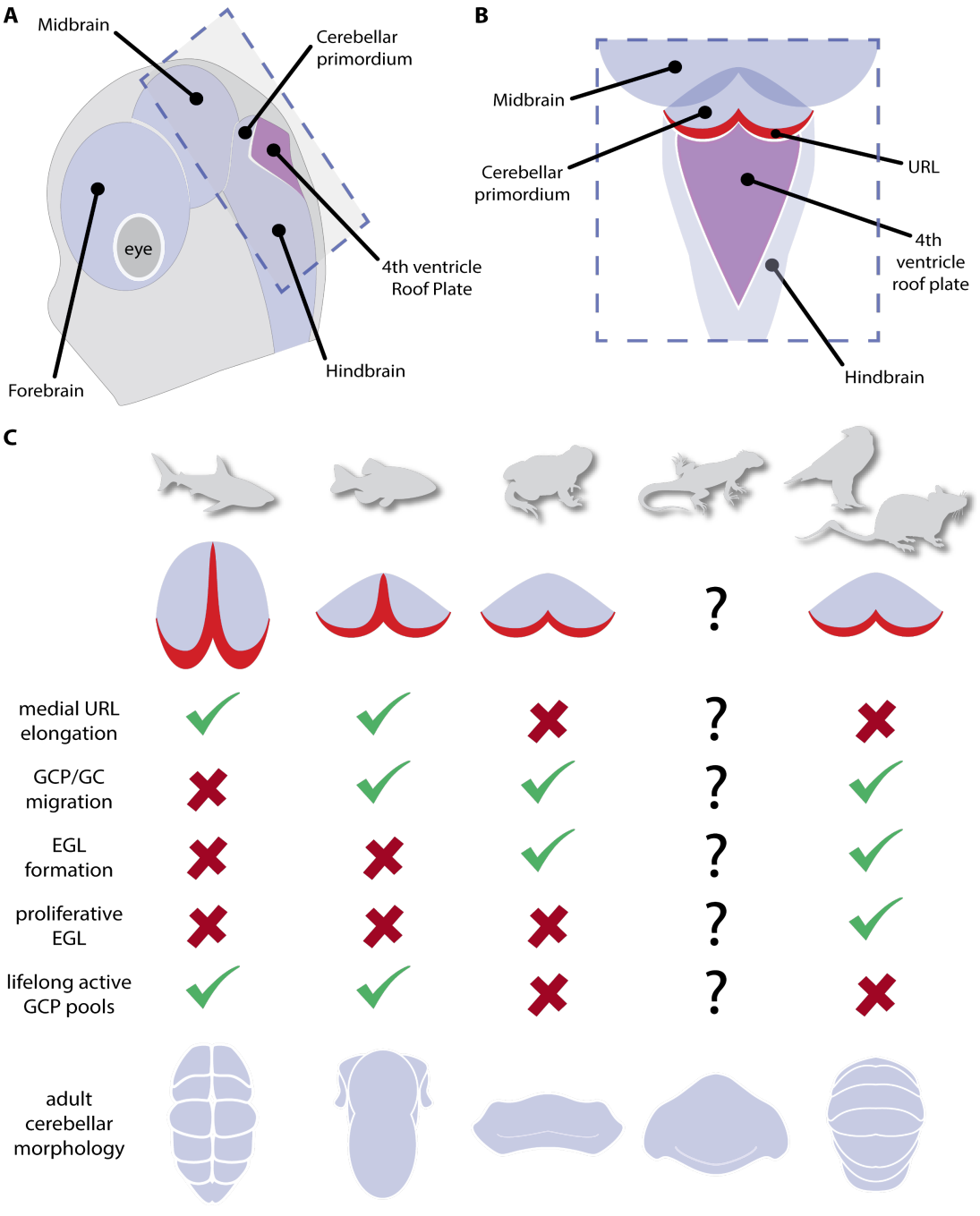

Figure 4 **Different strategies of granule cell generation and morphological diversity of the adult cerebellum across vertebrates. (A)** Schematic representation of a generalized vertebrate embryo head and brain in lateral view. **(B)** Dorsal view of the embryonic brain region shown in the blue dashed rectangle in **(A)**. **(C)** State of the knowledge prior to this thesis work regarding the mechanisms underlying cerebellar morphological diversification across vertebrates. GC, granule cell; GCP, granule cell precursor; EGL, external granular layer; URL, upper rhombic lip.

The pivotal role of this gene in the morphogenetic program of the cerebellum clearly emerged from knockout mouse phenotypic characterization which highlighted severe derangements involving multiple aspects of cerebellar structure and organization (Dussault et al., 1998; Sidman et al., 1962).

In addition to their temporal and molecular segregation, the different precursor subtypes generated in the VZ epithelium undertake divergent migratory routes to reach their destination in the mature cerebellum. DCN neuron precursors leave the VZ and form a compact cluster located below the pial surface (PS) in the anterior region of the developing cerebellum, populating the so called nuclear transitory zone, whereas GABAergic interneuron precursors disperse diffusely throughout the cerebellar parenchyma. PC precursors, on the other hand, follow a radial migration towards the pial surface and aggregate in multilayered clusters, the PC clusters (PCCs), spanning the entire rostro-caudal cerebellar extent, before acquiring their peculiar monolayer arrangement along the outer edge of the IGL.

Though reports in non-mammalian vertebrates give only a fractured picture on the evolution of VZ dynamics, evidence coming from lineage tracing studies and mutant line analyses in zebrafish as well as marker expression profiling in chondrichthyans and amphibians, suggest that the molecular, spatial and temporal processes occurring in this germinative epithelium are well-conserved between anamniote and amniote cerebella despite their structural differences (Bae et al., 2009; Gona, 1972; Uray et al., 1998; Kani et al., 2010; Kaslin et al., 2013; Nimura et al., 2019; Rodríguez-Moldes et al., 2008).

2.3.2 THE UPPER RHOMBIC LIP AND GC GENERATION

The URL is the proliferative epithelium which gives rise to the glutamatergic cell population of both the cerebellar cortex and DCNs (Alder et al., 1996; Fink, 2006; MacHold and Fishell, 2005; Wang et al., 2005). It is located at the interface between the posterior rim of the metencephalic alar plates and the non-neural complex formed by the 4th ventricle choroid plexus (CP) and RP. Like in the VZ, the generation of the different cell types produced by the URL follows a well-defined temporal, spatial and molecular pattern with precursors of DCN projection neurons, glutamatergic interneurons and GCs emerging in sequential order (Carletti and Rossi, 2008; Englund, 2006; Fink, 2006; Sekerková et al., 2004).

The master regulator of the proliferative activity of the URL is the bHLH transcription factor *Atoh1*, related to the product of the atonal gene of *Drosophila melanogaster* (Akazawa et al., 1995; Ben-Arie et al., 1997; MacHold and Fishell, 2005). *Atoh1* is, indeed, essential in promoting and supporting the germinative activity of the URL and its deletion in mouse mutants causes severe impairments during cerebellar development, leading to the formation of an agranular, unfoliated cerebellum, displaying also defects in PC spatial arrangement (Ben-Arie et al., 1997). The *Atoh1* molecular signature of the URL connotes as a constant in vertebrates as even in lampreys, which only possess a rudimentary, unlayered cerebellum, *Atoh1* expression marks dorsal metencephalic regions homologous to the gnathostome URL (Butts et al., 2014b; Chaplin et al., 2010; Green et al., 2014; Kani et al., 2010; Sugahara et al., 2016; Sugahara et al., 2017).

URL cell dynamics, including *Atoh1* transcription initiation, cell proliferation, GC differentiation and survival, are regulated by instructive signals originating from neighboring non-neural signaling centers, the 4th ventricle CP and RP, mediated by secreted morphogens (Broom et al., 2012; Chizhikov et al., 2006; Krizhanovsky and Ben-Arie, 2006; Rook et al., 2020; Yamamoto et al., 1996). Among the diffusible factors produced by the 4th ventricle CP and RP are members of the bone morphogenetic protein (BMP) family, which are crucial regulators in CNS patterning and development. Tissue and cell culture assays and *in vivo* experiments (Alder et al., 1996; Alder et al., 1999; Krizhanovsky and Ben-Arie, 2006; Lee et al., 1998; Rook et al., 2020; Salero and Hatten, 2007; Su et al., 2006) indicate BMP7, BMP4, BMP6 and the growth/differentiation factor 7 (GDF7) as fundamental factors in modulating URL morphogenetic program. Moreover, canonical BMP signaling through mothers against decapentaplegic homolog (SMAD) proteins has been documented both in the URL and in tangentially migrating GCPs (Fernandes et al., 2012; Owa et al., 2018; Rook et al., 2020), and the phenotypic analysis of *Smad1/5*-deficient embryos and adult mouse mutants highlighted anomalies in URL development as well as a reduction in GCP production, accompanied by PC spatial disorganization (Tong and Kwan, 2013).

Several developmental comparative studies have outlined GC generation and migration dynamics as key processes in determining cerebellar morphological traits and complexity (**Fig. 4**; Butts et al., 2014a; Butts et al., 2014b; Chaplin et al., 2010; Hibi et al., 2017; Rodríguez-Moldes et al., 2008). In fact, although both the territorial domain and early steps in GC developmental program are conserved at least within gnathostomes, heterogeneity exists in GCP/GC cellular dynamics and migration (Hibi et al., 2017). In particular, mammal, bird and metamorphic frog URL-generated GCPs undertake a tangential, subpial migration and colonize the entire cerebellar PS giving rise to a transitory, multilayered domain, the external granular layer (EGL), before switching, upon differentiation to GC, to a radial, glia-guided, migration towards the ventricular surface to form the IGL (Altman, 1972; Gona, 1972; Husmann et al., 1992; Lin et al., 2001). In addition, the avian and mammalian EGL features a unique transit amplification phase, leading to the exponential increase of the GCP population, a crucial step for the formation of the foliated cerebellar architectures these vertebrates exhibit (Ben-Arie et al., 1997; Lewis et al., 2004; Lorenz et al., 2011). On the other hand, fishes (both chondrichthyans and teleosts) display a persistent proliferative activity of the midline URL, a domain spanning the entire rostro-caudal extent of the cerebellar primordium which, in birds and mammals, instead, disappears in concomitance to alar plate fusion (Louvi, 2003; Sgaier et al., 2005). The cerebellar midline, which in teleosts comprises in its anterior-most portion the presumptive *valvulus* domain, is a significant source of GCPs in these animals. Its axial elongation during early cerebellogenesis, together with its maintenance till adulthood as a GCP stem cell niche in zebrafish, and likely in sharks, is thought to play a key role in the ultimate morphology and, in some cases, extreme development of the cerebellum in some fishes (Kaslin et al., 2009; Kaslin et al., 2013). Moreover, the lack of migration of GCPs away from the midline URL in sharks causes their peculiar arrangement of GCs which, rather than forming a continuous layer, are clustered in two paramedian columns, known as *eminentiae granularis*, extending along the antero-posterior cerebellar axis (Chaplin et al., 2010).

These comparative developmental analyses suggest that the additional proliferative step occurring in the EGL—a transient, *Atoh1*-positive progenitor domain spanning the entire

cerebellar pial surface—is an exclusive feature of bird and mammal cerebellogenesis, likely aiming to maximize GC production in a short developmental time window (Butts et al., 2014b; Chaplin et al., 2010; Iulianella et al., 2019; Pose-Méndez et al., 2016; Rodríguez-Moldes et al., 2008). In contrast, the presence of stem progenitor niches in teleosts and probably in chondrichthyans, allows some members of these groups to attain extremely complex cerebellar morphologies through a lifelong process of GC production (Butts et al., 2014a; Candal et al., 2005; Chaplin et al., 2010; Kaslin et al., 2013; Rodríguez-Moldes et al., 2008; Zupanc et al., 2005).

Surprisingly, no reports exist on cerebellar development in non-avian reptiles such as squamates (lizards and snakes) which occupy a major branch of the amniote tree and show a remarkable diversification in both cerebellar shape and organization (Aspden et al., 2015; Larsell, 1926), thus representing a key model to elucidate the evolutionary origin and development of the amniote cerebellum.

2.3.3 CEREBELLAR CELL INTERACTION AND CORTICAL LAMINATION

The generation of cerebellar cell types and their migration are only the initial steps of cerebellar morphogenesis. Rather than just being the product of cell proliferation and migration, the final morphology and crystal-like internal structure of the cerebellum is, indeed, the culmination of a temporally coordinated series of dynamic interactions between different cell types, mediated by diffusible signals. In particular, an active molecular crosstalk between GCPs in the EGL, maturing PCs and radially migrating GCs is fundamental for the cerebellum to achieve a correct size, degree of foliation and cortical layering (**Fig. 5;** Corrales et al., 2006; Dahmane and Ruiz, 1999; Miyata et al., 1997; Wechsler-Reya and Scott, 1999).

Among signaling molecules, a pivotal role in maintaining EGL GCPs in an undifferentiated state and promoting their exponential amplification is played by the mitogenic factor Sonic hedgehog (SHH), secreted by PCs (Corrales et al., 2006; Dahmane and Ruiz, 1999; Wechsler-Reya and Scott, 1999). The relevance of *Shh* pathway activation in GCPs has been thoroughly assessed and elegantly documented in a series of experiments involving conditional mouse mutants and highlighting a positive correlation between *Shh* signaling intensity and the degree and complexity of cerebellar foliation pattern (Corrales et al., 2004; Corrales et al., 2006). Results from such analyses showed that *Shh* deletion causes the formation of a thinner and less persistent EGL, associated with a small and unfoliated cerebellum in adult mutant mice while its upregulation results in the enlargement of cerebellar folia and in a thicker IGL (Corrales et al., 2004; Corrales et al., 2006). Consistent with the absence of a proliferative EGL in anamniotes, SHH has not been detected in zebrafish and sharks PCs, further connoting the appearance of this transient, secondary germinative domain as an evolutionary hallmark of avian and mammalian cerebellogenesis (Butts et al., 2014c; Butts et al., 2014a; Chaplin et al., 2010; Iulianella et al., 2019).

An active interaction between GCPs, migrating GCs and PCs is also required for a proper lamination of the cerebellar cortex. In this case, the release of a large glycoprotein, Reelin (RELN), in the extracellular matrix by both GCPs in the EGL and by radially migrating GCs,

triggers a series of cytoskeletal rearrangements in PCs, leading to the dispersion of the PCCs and culminating with the formation of a monolayered and uniform PCL.

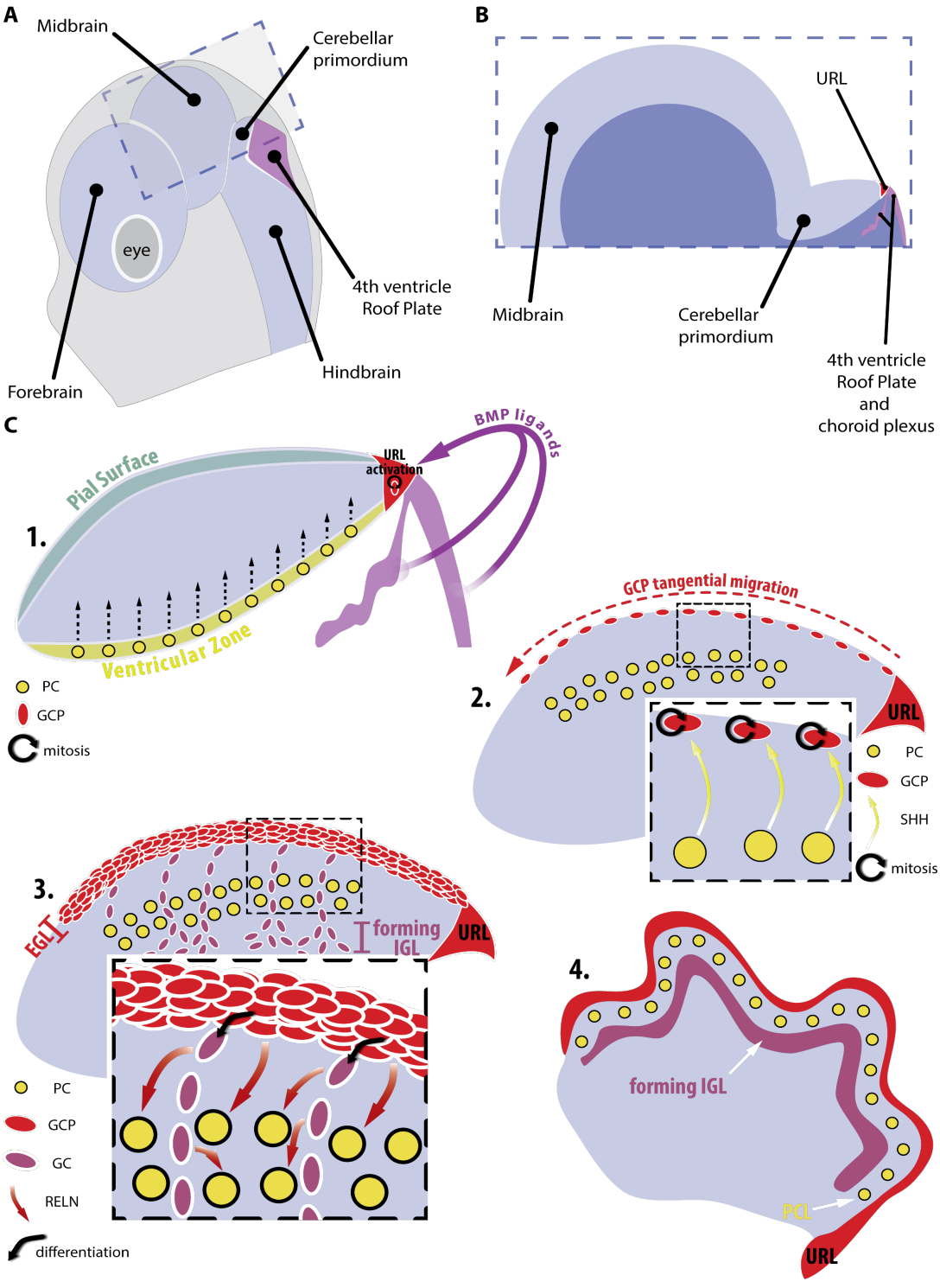

Figure 5 **Cellular and molecular events during cerebellogenesis in birds and mammals.** **(A)** Schematic representation of a generalized vertebrate embryo head and brain in lateral view. **(B)** Schematic mid-sagittal section of the brain region shown in the blue dashed rectangle in **(A)**. **(C)** Generation, migratory routes and molecular interactions of developing cell types define cerebellar foliation extent and cortical lamination. EGL, external granular layer; GC, granule cell; GCP, granule cell precursor; IGL, internal granular layer; PC, Purkinje cell; PCL, Purkinje cell layer; URL, upper rhombic lip.

The *Reln* pathway integrity is crucial for the development of several brain districts, and its activation is fundamental for a correct layering of both cerebellar and cerebral cortex, and hippocampus (Förster et al., 2006; Jossin, 2020).

Moreover, RELN functions extend beyond embryogenesis and early postnatal life as recent studies pointed out the important contribution of this glycoprotein in facilitating synaptic plasticity and in modulating memory, learning and neurotransmitter secretion in adult cerebral cortex and hippocampus (Beffert et al., 2005; Hellwig et al., 2011; Herz and Chen, 2006; Weeber et al., 2002). Dysfunction at any level of the *Reln* pathway causes comparable cortical and structural alterations in both telencephalon and cerebellum, and severe locomotor deficits in mammals and zebrafish (D'Arcangelo et al., 1995; Gallagher et al., 1998; Nimura et al., 2019; Niu et al., 2004; Sheldon et al., 1997; Trommsdorff et al., 1999; Ware et al., 1997). The phenotype of *Reln* knockout mice, known as *reeler*, is indeed characterized by an hypoplastic and agranular cerebellum, featuring a conspicuous cortical disorganization and heterotopic PCs. In particular, PCs are found amassed in large clusters in the white matter or within the IGL (Goffinet et al., 1984), likely due to an altered migration. As a consequence of such abnormalities mice, as well as humans, affected by mutations in the *Reln* gene display typical symptoms of cerebellar dysfunction like gait ataxia and hypotonia (Chang et al., 2007; Falconer, 1951; Hong et al., 2000).

Despite a thorough investigation of the molecular cascade triggered by this glycoprotein has led, in the last decades, to the identification of both RELN receptors and *Reln* pathway effector molecules, the mechanisms underlying its effect on neuronal migration, and specifically on PC spatial rearrangements, are only partially resolved (D'Arcangelo, 2014; Jossin, 2020; Lee and D'Arcangelo, 2016). RELN binds to the extracellular domain of two transmembrane receptors, the very-low density lipoprotein receptor (VLDLR) and the low density lipoprotein receptor-related protein 8, apolipoprotein e receptor (APOER2), localized on target-cell membrane, and promotes the phosphorylation of the disabled-1 protein (DAB1) by Src family tyrosine kinases, Src and Fyn (Arnaud et al., 2003; Bock and Herz, 2003; D'Arcangelo et al., 1999; Hiesberger et al., 1999; Howell et al., 1999). The phosphorylated form of DAB1 (P-DAB1) is capable to physically interact with several partners and form molecular complexes which, either directly or through the activation of distinct downstream effectors, mediate several aspects of RELN-dependent neuronal migration in various brain regions (Bock et al., 2003; Chai et al., 2009; Franco et al., 2011; Hashimoto-Torii et al., 2008; Jakob et al., 2017; Jossin and Cooper, 2011).

Different models, mostly deriving from analyses on developing neocortex, have been proposed to explain the effects of RELN on neuronal migration. It has been suggested that RELN might act as an attractant, inducing responsive cells to move following a gradient of increasing *RELN* concentration (Gilmore and Herrup, 2000), or that it might impart an arrest signal promoting the detachment of migrating neurons from radial glia processes ("detach and stop signal"; Jossin, 2004; Pinto-Lord et al., 1982; Sanada et al., 2004). Furthermore, the recent identification of a modality of locomotion alternative to the glia-guided one—the somal translocation mode, characterizing the late phase of neocortical neuron migration—has led to the formulation of the so called "detach and go signal" model. In this model, in parallel with triggering the detachment from the glia scaffold, RELN would provide target cells with an instructive signal to switch from the glia-guided locomotion to the somal translocation mode

(Borrell et al., 2006; Cooper, 2008; Franco et al., 2011; Nadarajah et al., 2001; Olson et al., 2006; Sekine et al., 2011).

Consistent with both glia-detachment signal models, neocortical neurons with impaired *Reln* pathway fail to disconnect from radial glia fibers while wild-type cells disengage from them during late migration phase (Dulabon et al., 2000; Pinto-Lord et al., 1982; Sanada et al., 2004). Importantly, these findings corroborate previous observations conducted in the developing *reeler* cerebellum (Yuasa et al., 1991; Yuasa et al., 1993; Yuasa et al., 1996) outlining the presence of tight attachments between radial glia fibers and PCs amassed in heterotopic clusters, and suggest that RELN signaling may regulate through similar mechanisms both cerebral and cerebellar cortex lamination.

2.4 THE SQUAMATE BRAIN AND CEREBELLUM

Squamates occupy a key phylogenetic position among vertebrates and, with more than 10.000 recognized species widely distributed across the world, represent a major segment of the amniote tree. They populate a broad spectrum of habitats and display an extraordinary level of morphological diversification, associated with unique ecological and behavioral features, including a vast repertoire of locomotor strategies (Da Silva et al., 2018; Gagliano et al., 2020; Irschick and Garland T., 2001; ten Donkelaar and Bangma, 1992).

This heterogeneity, encompassing multiple aspects of squamate ecobiology, provides an exceptionally fertile ground to investigate key biological and evolutionary processes. In order to have a broad view on such a wide and varied diversity, this thesis analyzes data derived from the brains of 40 squamate species adult individuals. Furthermore, embryonic series from a lizard (*Pogona vitticeps*) and a snake (*Boaedon fuliginosus*), two species with comparable gestation periods and well-established breeding conditions, have been used to characterize squamate cerebellar morphogenesis.

2.4.1 OVERVIEW OF THE SQUAMATE BRAIN

The large ecomorphological heterogeneity of squamates has a deep impact on squamate outer appearance—the most evident the limb loss or reduction and body elongation in snakes and some lizard species—but it also involves substantial changes in brain areas devoted to environmental stimuli processing and sensorimotor integration (Allemand et al., 2017; Eymann et al., 2019; G. Senn, 1969; Hoops et al., 2017; LaDage et al., 2009; Larsell, 1926; Senn and Northcutt, 1973). Compared to amphibians, reptiles—including squamates—display larger brains relative to body weight (Platel, 1979). Such an increase is mostly linked to the relevant expansion of 3 brain regions, the telencephalon, the optic tectum and the cerebellum (Nieuwenhuys et al., 1998; for a detailed description of the squamate cerebellum, please, see next chapter). Within this general trend, nonetheless, substantial differences exist among squamates in terms of the degree of development of specific brain subdivisions, particularly those involved in sensory processing, reflecting the multifaceted ecological and behavioral repertoire characterizing this group of reptiles. The tectum mesencephali, which receives the majority of optic nerve fibers, is particularly developed in diurnal lizards while burrowing

species, generally featuring reduced eyes, show poorly expanded tectal hemispheres. In a similar fashion, both the main and accessory olfactory bulbs and tracts of squamate species especially relying on odorants or chemical cues to perform essential biological activities, like prey catching and mating, are markedly enlarged (Bellairs, 1969; Gabe and St. Girons, 1976; Halpern, 1992; Martinez-Garcia et al., 1991).

A feature of the squamate brain, shared with other reptiles (including birds), is a discrete telencephalic structure, the DVR, which protrudes, almost obliterating it, in the lateral ventricle (Northcutt, 1981). The DVR can be divided into an anterior and a posterior part. The anterior DVR is formed by 3 longitudinal regions, each targeted by specific ascending sensory projections (somatosensory, acoustic, visual and gustatory; Guirado et al., 2000; Manger et al., 2002; Nieuwenhuys et al., 1998; Pritz et al., 1992), while the posterior DVR connections show similarities with those of the amygdalar complex of mammals (Nieuwenhuys et al., 1998). In squamates, the posterior DVR is characterized by a distinctive formation, particularly developed in snakes, known as the *nucleus sphericus*, extensively targeted by accessory olfactory bulb efferents (Halpern, 1976; Lanuza and Halpern, 1997; Lohman and Smeets, 1993; Martinez-Garcia et al., 1991). The DVR, however, is not the sole pallial derivative present in squamate telencephalon. Together with mammals, in fact, non-avian reptiles are the only vertebrate group to feature a multilaminar cortex. The squamate cerebral cortex is formed by 3 layers that can be distinguished by variations in neuronal density. The outermost and innermost layers are relatively cell-sparse while the intermediate lamina contains an almost uninterrupted and densely packed layer of neurons. Such a tripartite pattern spans the entire extent of the dorsal telencephalon and characterizes the different cortical subdivisions that follow one another along the medio-lateral plane: medial, dorsal, and lateral. Most of the research on the reptilian cortex has largely focused on the identification of putative structural and functional homologies with the mammalian counterpart, and some studies (Baird Day et al., 1999; Calisi et al., 2017; LaDage et al., 2009; Roth et al., 2006) have correlated variations in the relative size of the medial cortex, which is involved in navigational tasks and suggested to be a putative homolog of the avian and mammalian hippocampus (Bruce and Butler, 1984; Butler and Hodos, 2005; Striedter, 2015), with habitat complexity and prey capture strategy, further highlighting, thus, the tight relationships between ecology, behavior and squamate brain structure.

2.4.2 THE SQUAMATE CEREBELLUM

Among the major brain subdivisions showing relevant modifications in squamates, the cerebellum exhibits a high degree of morphological and cytoarchitectural variation (Aspden et al., 2015; Hoops et al., 2017; Larsell, 1926; ten Donkelaar and Bangma, 1992).

Most of the anatomical data obtained on the cerebellum of lizards derives from studies on quadrupedal species and describes it as a single, leaf-shaped, folium of neural tissue lying above the 4th ventricle, curved towards the anterior part of the brain and overarching the tectal hemispheres (**Fig. 6A bottom**; Larsell, 1926; Nieuwenhuys, 1967; Nieuwenhuys et al., 1998). The cerebellar cortex of quadrupedal lizards shows the trilaminar organization typical of most vertebrates with a PCL constituted by PCs orderly distributed, though often in multiple rows rather than in a monolayer, along the outer contour of the IGL (**Fig. 6C**). Compared to

the relatively canonical morphology and cytoarchitecture of the lizard cerebellum, the snake counterpart shows important peculiarities. It exhibits an opposite tilting respect to lizards and lies almost completely embedded in the 4th ventricle. It is relatively small, poorly developed along the medio-lateral axis and displays a trapezoidal shape tapering towards the caudal end of the brain (**Fig. 6A top**). Moreover, it is characterized by a completely scattered PC topological distribution (**Fig. 6B**; Aspden et al., 2015; Larsell, 1926).

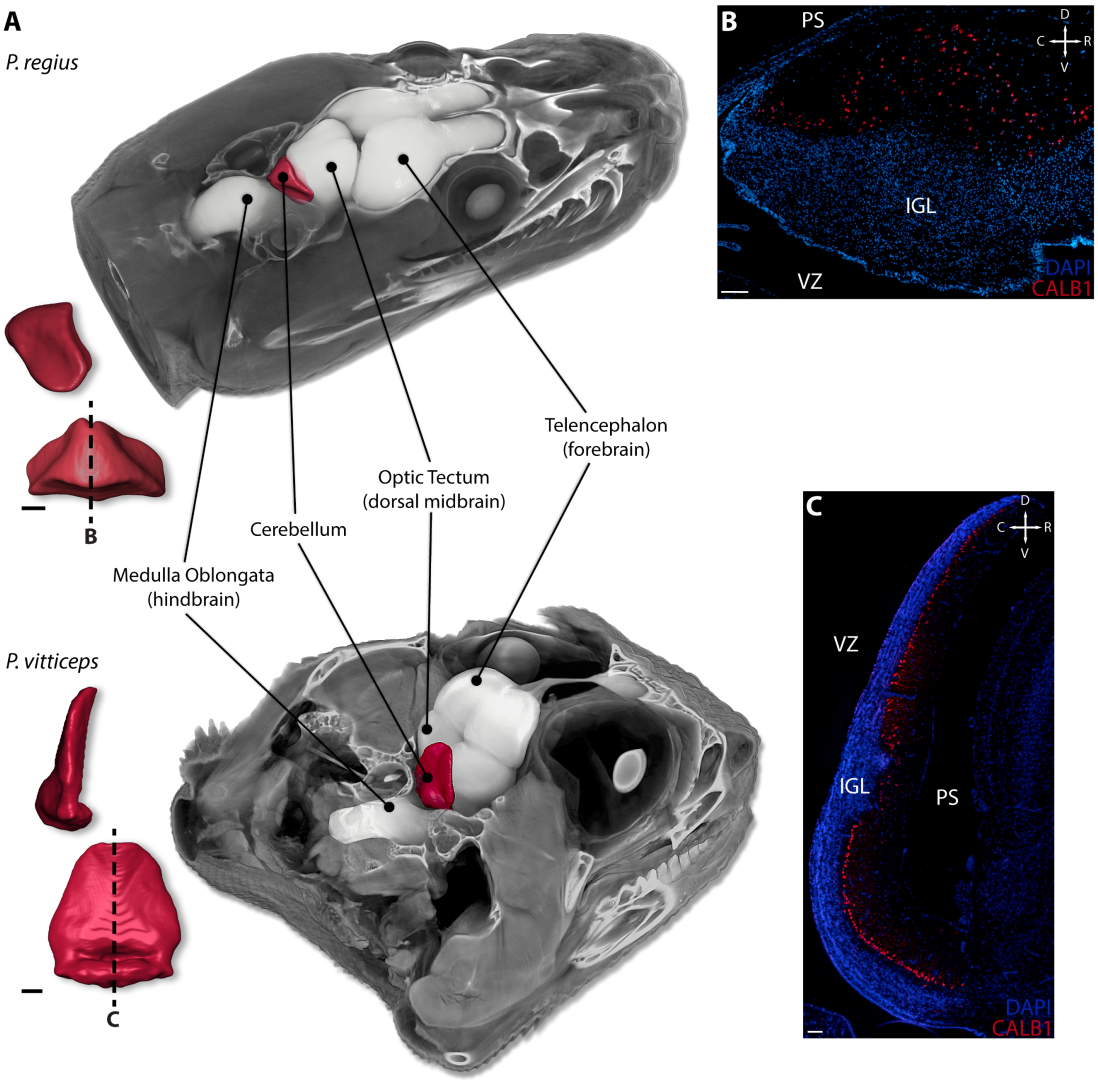

Figure 6 **Morphological and cortical diversity of the squamate cerebellum. (A)** volume rendering and whole-brain segmentation of iodine-stained adult heads of *P. regius* **(top)** and *P.vitticeps* **(bottom)**, highlighting the cerebellum structure (red color). High magnifications of 3D-rendered cerebella **(left column)** are shown in lateral **(top row)** and pial **(bottom row)**. Dashed lines and letters mark the sectioning planes relative to the immunostaining experiments in panels **(B,C)**. **(B,C)** Immunodetection of Purkinje cells with CALB1 marker (red staining), on sagittal sections of *P. regius* **(B)** and *P. vitticeps* **(C)** juvenile cerebellum. Cell nuclei are counterstained with DAPI (blue staining). Crossed white arrows point toward rostral (R), caudal (C), dorsal (D), and ventral (V) directions. IGL, internal granular layer; PS, pial surface; VS, ventricular surface. Scale bars: 500 µm **(A)**, 100 µm **(B,C)**.

However, due to taxon-specific or limited sampling, the few studies on squamate cerebellum, likely reveal only a small fraction of the entire gamut of both cerebellar morphologies and cortical layouts in this order of reptiles. Indeed, the only anatomical study featuring the analysis of a legless and a limb-reduced lizard species (Larsell, 1926) highlighted the existence of additional morphological variants besides the highly divergent cerebellar shapes of quadrupedal lizards and snakes. The morphological comparison between the quadrupedal lizard and the limbless or limb-reduced species suggests that the squamate cerebellum could be somatotopically organized, with the lateral parts involved in the control of limbs and the medial cerebellar region involved, instead, in the control of the axial musculature. In such landscape, the reduction of the lateral extent of the snake cerebellum, but also of the legless and limb-reduced lizards, could reflect the absence or the pronounced reduction of the limbs in these species and, by extension, differences in their locomotor modalities (Larsell, 1926). Moreover, tracing studies on the distribution of the corticonuclear projections in a lizard (*Varanus exanthematicus*) and a snake (*Python regius*) indicate that squamate PCs are organized in three distinct zones—medial, intermediate and lateral—each with its own specific projection site (Bangma, 1983; Bangma and ten Donkelaar, 1984). In particular, PCs in the lateral part of the medial zone and most of those in the lateral zone project to different regions of the vestibular nucleus whereas PCs located in the medial part of the medial zone and those in the dorsolateral intermediate zone target the medial and lateral deep cerebellar nucleus, respectively (Bangma, 1983; Bangma and ten Donkelaar, 1984). Furthermore, analyses of the course and distribution of fibers from the DCNi in *V. exanthematicus* and *P.regius* point out that a large fraction of the lateral DCN efferents in the lizard, but only sparse fibers in the snake, extensively terminates in the red nucleus while part of the medial DCN projections targets the brainstem reticular formation in both species (Bangma, 1983; Bangma et al., 1984). These two nuclei govern, via their spinal projection, the rubro- and reticulospinal tracts, the distal muscles employed for limb and finger movements and the axial and proximal musculature, respectively (Davidson and Buford, 2004; Davidson et al., 2007; Drew et al., 2004; Lawrence and Kuypers, 1968; Mewes and Cheney, 1991; Prentice and Drew, 2001). These observations, in parallel with the absence of a rubrospinal tracts in boid snakes (ten Donkelaar, 1976a; ten Donkelaar, 1976b; ten Donkelaar and Bangma, 1983; ten Donkelaar et al., 1983), support the hypothesis of the subdivision of the squamate cerebellum in two main functional areas, nonetheless, the restricted number of species tested and the exclusively qualitative nature of the studies conducted do not provide sufficient evidence for a generalization of such findings and hypothesis.

In addition to morphological features, variability exists also in squamate cerebellar cytoarchitecture. In fact, while lizards comply to the canonical, ordered PC spatial distribution observed in most vertebrates, several reports highlighted a scattered PC organization in snakes (Aspden et al., 2015; Larsell, 1926; ten Donkelaar and Bangma, 1992). However, due to the limited number of studies, whether such alternative PC layouts are a distinctive trait of snakes and lizards, is yet to be clarified.

Moreover, the lack of any developmental data on the cerebellum of squamates has hindered the possibility to assess if the different spatial layout observed in snakes and lizards are developmentally generated through different morphogenetic programs and to elucidate the

evolutionary origin of key developmental innovations, so far observed only in birds and mammals, like the formation of a proliferative EGL and the secretion of SHH by PCs.

3 AIMS OF THE STUDY

Owing to their phylogenetic position, to their vast repertoire of morphological and behavioral traits, and to the relative simplicity of their brain architecture, squamates represent a suitable model to expand the current knowledge on the basic developmental features of the amniote brain as well as on the contribution of ecological and behavioral specializations on vertebrate brain evolution. Nonetheless, the potential offered by squamates in such contexts has been largely unexplored. Moreover, the influence of environmental and behavioral features on the vertebrate brain have traditionally focused on variations in whole-brain or major brain subdivision morphology or size, while the possibility of potential changes in multiple and deeper aspects of brain biological organization has remained largely untested. In this thesis work I took advantage of squamate diversity to investigate the effects of a distinctive behavioral trait, locomotor behavior, on the evolution of the brain and in particular of the cerebellum, a brain subdivision playing a relevant role in motor control. I adopted a multidisciplinary integrative approach, including the developmental characterization of the cerebellum in 2 squamate species, to explore the following aspects:

- Is locomotor behavior associated with multiple brain or cerebellar structure variations in squamates?
- What are the developmental mechanisms potentially responsible for cerebellar phenotypic diversification across squamates?
- Does squamate cerebellar morphogenesis feature developmental hallmarks displayed by other amniotes?

4 MATERIALS AND METHODS

The lizard and snake species and a summary of the methodologies used in this thesis work are listed in Tables 1 and 2, respectively. Sources of detailed descriptions of methods can be found in the original publications included at the end of this thesis. The primary antibodies employed in IHC experiments and the ISH probes are listed in Table 3 and 4, respectively.

All experiments performed for this thesis work involving animals were conducted in full compliance of the Finnish national guidelines and approved by the Finnish National Board of Animal Experimentation.

Table1. *Species used in this thesis work*

Species	Families	Locomotor modes	Study
Ablepharus kitaibelii	Scincidae	Limbless or limb-reduced facultative burrower	I
Acontias meleagris	Scincidae	Limbless burrower	I
Agama agama	Agamidae	Quadrupedal terrestrial	I
Amphisbaena scutigerum	Amphisbaenidae	Limbless burrower	I
Anguis fragilis	Anguidae	Limbless or limb-reduced facultative burrower	I
Anolis carolinensis	Dactyloidae	Quadrupedal arboreal	I
Bachia flavescens	Gymnophthalmidae	Limbless or limb-reduced facultative burrower	I
Basiliscus vittatus	Corytophanidae	Quadrupedal facultative bipedal/aerial	I
Blanus cinereus	Blanidae	Limbless burrower	I
Bradypodion pumilum	Chamaeleonidae	Quadrupedal arboreal	I
Chalcides chalcides	Scincidae	Limbless or limb-reduced facultative burrower	I
Chalcides sepsoides	Scincidae	Limbless or limb-reduced facultative burrower	I
Trioceros jacksonii	Chamaeleonidae	Quadrupedal arboreal	I
Dasia olivacea	Scincidae	Quadrupedal arboreal	I
Draco volans	Agamidae	Quadrupedal facultative bipedal/aerial	I
Eublepharis macularius	Eublepharidae	Quadrupedal terrestrial	I
Gekko gecko	Gekkonidae	Quadrupedal arboreal	I
Hemiergis quadrilineata	Scincidae	Limbless or limb-reduced facultative burrower	I
Lepidothyris fernandi	Scincidae	Quadrupedal terrestrial	I
Lygodactylus picturatus	Gekkonidae	Quadrupedal arboreal	I
Melanoseps loveridgei	Scincidae	Limbless burrower	I
Ophiodes fragilis	Anguidae	Limbless or limb-reduced facultative burrower	I
Phelsuma grandis	Gekkonidae	Quadrupedal arboreal	I
Plestiodon marginatus	Scincidae	Quadrupedal terrestrial	I
Pogona vitticeps	Agamidae	Quadrupedal terrestrial	I, II
Pseudopus apodus	Anguidae	Limbless multi-habitat lateral undulation	I
Rieppeleon brevicaudatus	Chamaeleonidae	Quadrupedal arboreal	I

Takydromus sexlineatus	Lacertidae	Quadrupedal arboreal	I
Teratoscincus scincus	Gekkonidae	Quadrupedal terrestrial	I
Tropidurus torquatus	Tropiduridae	Quadrupedal facultative bipedal/aerial	I
Boaedon fuliginosus	Lamprophiidae	Limbless multi-habitat lateral undulation	I, II
Cerastes cerastes	Viperidae	Limbless multi-habitat other movements	I
Chrysopelea ornata	Colubridae	Limbless multi-habitat lateral undulation	I
Dasypeltis gansi	Colubridae	Limbless multi-habitat other movements	I
Dendrelaphis pictus	Colubridae	Limbless multi-habitat other movements	I
Epicrates cenchria	Boidae	Limbless multi-habitat other movements	I
Eryx colubrinus	Boidae	Limbless or limb-reduced facultative burrower	I
Eryx jaculus	Boidae	Limbless or limb-reduced facultative burrower	I
Hydrophis platurus	Elapidae	Limbless multi-habitat lateral undulation	I
Pantherophis guttatus	Colubridae	Limbless multi-habitat lateral undulation	I
Python regius	Pythonidae	Limbless multi-habitat other movements	I
Xerotyphlops vermicularis	Typhlopidae	Limbless burrower	I

Table2. *Methods used in this thesis work*

Method	Study
µCT-scan of iodine-stained samples	I, II
Manual segmentation and 3D brain model reconstruction	I, II
Volumetric measurements of 3D-reconstructed brain models	I, II
Landmarking	I
3D Geometric morphometrics	I
Brain tissue clearing using CUBIC protocol	I
Whole-mount IHC	I
Lightsheet fluorescence microscopy and imaging	I
Nissl staining	II
IHC on paraffin sections	I, II
ISH on paraffin sections	I, II
Microscopy	I, II

Table3. *Primary antibodies used in this thesis work*

Antibody	RRID or catalog number	Study
CALB1	AB_10000340	I, II
GFAP	LS-C357895	II
LHX1	AB_2135639	II
PCNA	AB_314691	II
PH3	AB_304763	II
P-DAB1	orb156526	II
P-SMAD 1/5/9	AB_2493181	II

SHH	AB_2285962	II
ZIC 1/2/3	LS-C118695	II

Table4. *riboprobes used in this thesis work*

Probe	Species	Sequence length (bp)	Study
Bmp4	*P. vitticeps*	670	II
Bmp7	*P. vitticeps*	584	II
Atoh1	*P. vitticeps*	966	II
Rora	*P. vitticeps*	942	II
Vldlr	*P. vitticeps*	830	II
Reln	*P. vitticeps*	1237	II
Dab1	*P. vitticeps*	848	II
Bmp4	*B. fulignosus*	785	II
Bmp7	*B. fulignosus*	784	II
Atoh1	*B. fulignosus*	623	II
Rora	*B. fulignosus*	907	II
Reln	*B. fulignosus*	927	II
Dab1	*B. fulignosus*	612	II

5 RESULTS AND DISCUSSION

5.1 LOCOMOTOR MODE IMPACTS MULTIPLE LEVELS OF CEREBELLAR BIOLOGICAL ORGANIZATION IN SQUAMATES (I-II)

5.1.1 LOCOMOTOR MODE DEFINES SQUAMATE CEREBELLAR SHAPE AND SIZE (I)

Squamates display extremely diversified ecological and behavioral characteristics which are well reflected by their body plan and locomotor adaptations. The full range of squamate locomotor specializations, however, goes beyond the obvious physical constraints that either limblessness or different degrees of limb reduction dictate in snakes or in some lizard families. The wide gamut of squamate locomotor behavior is, indeed, influenced by multiple habitat features such as environmental settings, temperature, incline and substrate composition and is associated with specific limb and body kinematics, distinct skin-muscle relationships and with the coordinated activity of particular muscle groups which, in some cases, are exclusively present in species adopting similar locomotion (Carothers, 1986; Gans, 1962; Gans, 1973; Gray, 1946; Irschick and Jayne, 1998; Jayne, 2020; Jayne et al., 2015; Kerfoot, 1970; Mosauer, 1932; Savidge et al., 2021). In such adaptive context, the development of a high level of neuro-muscular integration, coordination and control, likely occurred to satisfy the functional demands associated with specialized ecological behaviors (Gans, 1973).

In order to assess the potential effects of locomotor adaptations on squamate brain size and shape I generated 3D brain models from a representative panel of 40 squamates, categorized in 7 different locomotor groups (see **Table1**, Materials and methods), by manual segmentation of head volumes obtained by µCT scans of iodine-stained specimens (**Study I**). This allowed to qualitatively appreciate the wide array of single brain subdivision morphologies and reciprocal spatial relationships, to quantitatively compare the whole-brain and its subdivision's shape using three-dimensional geometric morphometrics and to perform a volumetric analysis on specific brain subdivisions.

Besides confirming qualitative observations about lizard and snake brain gross organization from past neuroanatomical studies, the visual inspection of the 3D models revealed a wider and complex repertoire of squamate brain morphological traits. Both the dimension of the dataset, including 29 lizard and 11 snake species, sampled in all major squamate lineages, and the detailed representation of brain shape in the manually segmented 3D reconstructions allowed the appreciation of additional variations in squamate brain anatomy, aside from the previously described morphologies of some snake and lizard species. Especially, in addition to a certain degree of brain morphological heterogeneity among snakes and quadrupedal lizards, indeed, both limbless and limb-reduced lizards displayed transitional characteristics ranging from a snake-like pattern exhibited by burrowers to a brain organization approximating that of quadrupedal lizards in facultative burrowers **(Fig. 7; Study I)**. Furthermore, among the

major brain subdivisions, the cerebellum showed the highest level of diversification both in its size and shape across the individuals in the dataset.

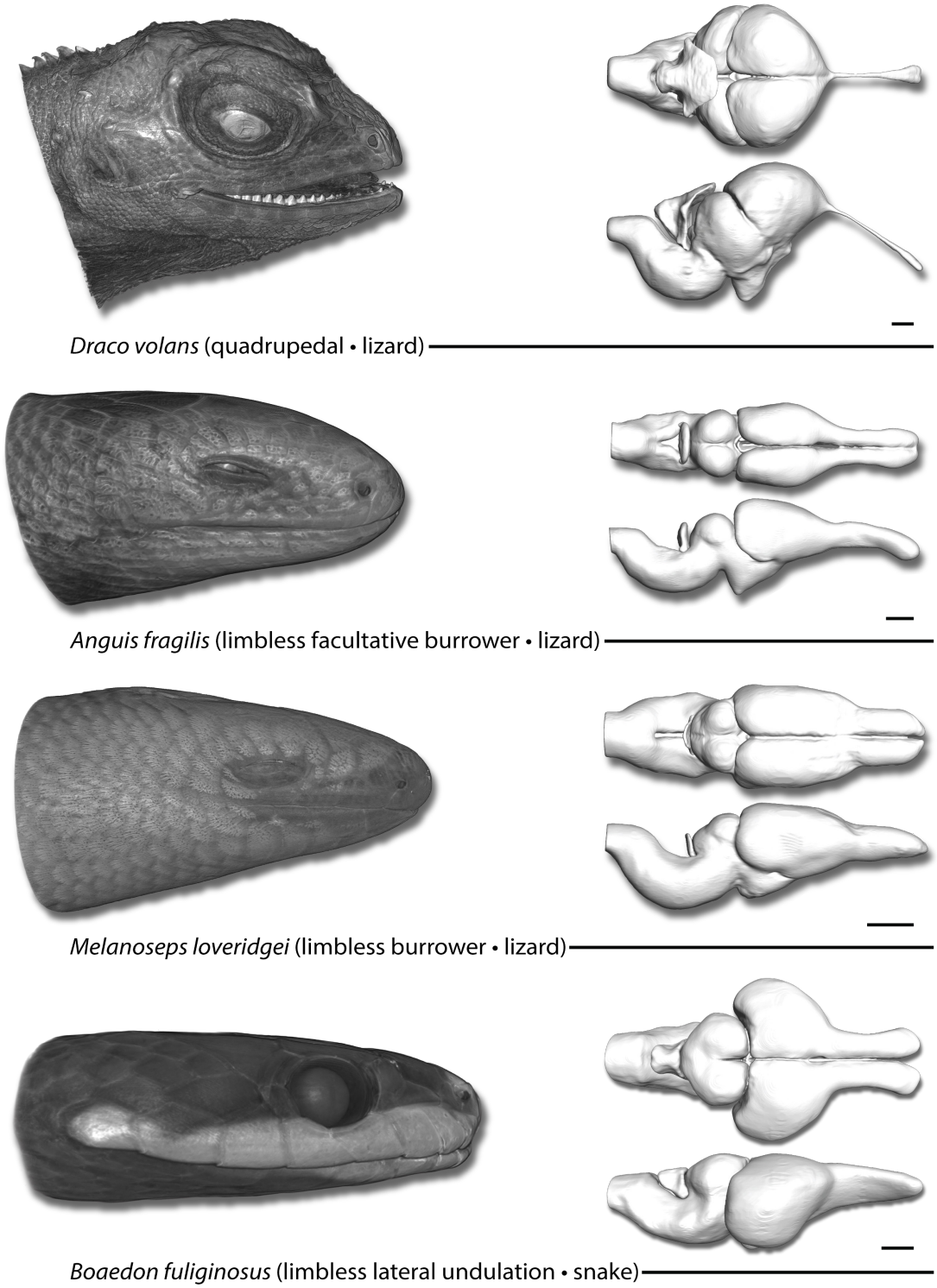

Figure 7 **Whole-brain morphological variations in squamates adopting different locomotor behaviors.** Volume rendering of iodine-stained adult squamate heads **(left column)** and corresponding 3D brain models **(right column)**. Brain models are shown in dorsal **(top)** and lateral **(bottom)** views. The locomotor specialization relative to each species is indicated between parentheses. Scale bars: 1mm.

Snakes show a small, trapezoidal cerebellum, poorly developed along the medio-lateral axis and tapering towards its posterior end whereas quadrupedal lizard cerebella are leaf-shaped and laterally expanded (**Fig. 6A**). Moreover, quadrupedal lizard and snake cerebella are tilted in opposite directions, with the former extending dorsally to overstep the optic tectum and the latter almost completely embedded in the 4th ventricle. The cerebellum of limbless and limb reduced burrowers and facultative burrowers appears smaller than the other locomotor group counterparts, and shows variation in its tilting and both medio-lateral and dorsal extent.

To ascertain potentially significant correlations between different locomotor modes and both whole-brain and single brain subdivision shape I digitized 61 landmarks, clustered in groups delineating the 3d morphology of the 5 brain subdivisions, on the 3D brain models. In addition, the segmentation of the cerebellum as a separate unit from the rest of the brain allowed me to individually landmark this brain subdivision and improve its shape representation with 5 supplementary landmarks at the interface between the optic tectum and the cerebellum, a region inaccessible on whole-brain models. Except for the cerebellum, the principal component analysis (PCA) conducted on Procrustes coordinates for the whole-brain and for any of its major subdivisions, highlighted a similar distribution pattern, with snakes and lizards generally segregating at extreme positions in the morphospace, and showed no obvious correlation with locomotor modes. The cerebellar shape variation in the morphospace, instead, suggested a potential influence of locomotion on the morphological traits of this brain subdivision. Indeed, limbless burrowers, with their thin and almost triangular cerebellum, clustered in correspondence of PC1 negative values while individuals belonging to the different multi-habitat locomotor categories occupied negative PC2 regions. Furthermore, facultative burrowers, showing mixed cerebellar morphologies, almost equally distributed in both PC2 negative and PC1 positive quadrants while all quadrupedal species, with few exceptions, populated the positive part of both PC1 and PC2 axis. Importantly, the indication about putative relationships between locomotor specialization and cerebellar morphology obtained from the shape distribution analysis were validated by phylogenetic comparative methods and statistical tests, which revealed a significant variation in cerebellar morphology between limbless burrowers and all other locomotor groups (phylogenetic ANOVA, p-values ranging from 0.0004 to 0.0216), and between any limbless or limb- reduced and quadrupedal locomotor category (phylogenetic ANOVA p-values ranging from 0.0001 to 0.0147).

Because several ecological factors have been shown to play a relevant role also in defining the relative size of brain regions in most vertebrates (Allemand et al., 2017; Barks et al., 2015; Bennett and Harvey, 1985; Day et al., 2005; Hoops et al., 2017; Liao et al., 2015; Manzano et al., 2017; Montgomery et al., 2012; Symonds et al., 2014; Taylor et al., 1995; Vincze et al., 2015; Yao et al., 2021; Yopak et al., 2007), the results obtained from the quantitative analysis of the cerebellar shape lead me to perform volumetric measurements of the cerebellum to explore also the possible influence of locomotor specializations on the size of this brain subdivision (**Study I**). This comparative analysis revealed a marked divergence in cerebellar size across the various locomotor categories and a tendency for the cerebellum to occupy a relatively larger brain volume from limbless and limb reduced burrowers to quadrupedal lizards. In particular, limbless burrowers, possessing a thin and barely visible cerebellum, showed a statistically significant smaller cerebellum-to-whole-brain volume ratio when compared to all

other locomotor categories (phylogenetic ANOVA; p-values <0.05). Consistent with our findings in squamates, multiple studies on different vertebrate groups linked ecological and behavioral strategies to significant changes in cerebellar size. Indeed, specialized beak manipulative skills in crows and woodpeckers or refined limb control in squirrels and primates have been associated with larger cerebella (Sultan and Glickstein, 2007). Moreover, the cerebellum of both gorillas and frogs engaging the complex three-dimensional environment of tree branches, which demands a high degree of postural and forelimb motor control, is expanded compared to that of closely related terrestrial species (Barks et al., 2015; Manzano et al., 2017).

Altogether the results obtained from both the morphometric and volumetric analyses indicate that locomotor mode strongly defines cerebellar structure in squamates and suggest that important transitions in vertebrate motor behavior are tightly interconnected with relevant modifications in cerebellar shape and size, in addition to more obvious changes in body parts mechanistically involved in motor performance like limbs and axial skeleton. In addition, despite volumetric analyses have been the most widely used tool to assess the potential effects of ecological/behavioral variables on brain organization, these findings point out that also morphological modifications of neuroanatomical structures highly contribute to vertebrate brain evolution. Moreover, the evidences emerged from the geometric morphometric analysis underscore a mosaic pattern in squamate brain subdivision evolution with respect to locomotor modalities and suggest that vertebrate species sharing similar behaviors may feature common neuromorphological traits, either in addition or in alternative to the single brain subdivision size patterns (cerebrotypes) identified in mammals and other vertebrate lineages (Clark et al., 2001).

5.1.2 VARIATIONS IN GCP GENERATION IN A QUADRUPEDAL LIZARD AND A SNAKE (II)

One of the crucial processes in determining the size and morphology of the different tissues of an organism, including the brain, is the proliferative activity of certain regions, generally located inside or in close vicinity to the developing tissue, during embryogenesis. Brain development and its final architecture strongly relies on neuronal proliferation, differentiation and migration, and alterations of the subtle balance between these phases during CNS development can dramatically impact brain morphology, cytoarchitecture, function and evolution (Cheung et al., 2007). For instance, the appearance of secondary proliferative zones, like the subventricular zone in the telencephalon of mammals and the transit-amplifying cerebellar EGL—present also in birds, is considered to be at the base of the marked expansion of these brain districts in these groups, when compared to other vertebrates (Abdel-Mannan et al., 2008; Butts et al., 2011; Butts et al., 2014a; Cheung et al., 2007; Lui et al., 2011; Martínez-Cerdeño et al., 2006).

Owing to the relevance of proliferation patterns in defining the size of specific brain subdivisions and in order to gain insights on the potential mechanisms underlying squamate cerebellar diversity, I, then, characterized the embryonic germinative profile of the cerebellum in two squamate species displaying a pronounced difference in both the morphology and relative size of this brain subdivision, the quadrupedal bearded dragon lizard (*Pogona*

vitticeps) and the African house snake (*Boaedon fuliginosus*). Both the lizard and the snake are oviparous animals featuring a similar developmental time window and, despite early neurogenetic events have already occurred at oviposition in both species, cerebellar morphogenesis has yet to start. To follow the proliferative potential of the cerebellar germinative zones in both species I collected *P. vitticeps* and *B. fuliginosus* embryos at regular intervals spanning the entire post-ovipositional period (60 days) and performed a series of immunohistochemistry and *in situ* hybridization experiments (**Study II**). I paid particular attention to URL and GCP dynamics which have particular relevance in determining cerebellar size and shape both in anamniotes and amniotes (**Fig. 4**; Butts et al., 2011; Butts et al., 2014a; Butts et al., 2014c; Iulianella et al., 2019).

Immunohistochemical staining with an antibody against the proliferative cell antigen (PCNA) and the specific GCP/GC marker, zinc finger of the cerebellum (ZIC1/2/3; Aruga et al., 1998), showed clear differences in the germinative profile of the cerebellum in the two species. In the lizard, starting from 25 days post oviposition (dpo), double-stained cells are detected both in the URL and in GCPs forming a multilayered structure (**Fig. 8A**) which, spanning the entire rostro-caudal extent of the cerebellar pial surface, resembles the mammalian and avian EGL. These two domains, despite reducing in thickness in parallel to GCP differentiation and to a progressive accumulation of GCs in the presumptive IGL, remain site of sustained labeling for the entire embryogenesis. Furthermore, PCNA-positive cells are still present along the pial surface at 15 days post hatching (dph; **Fig. 8B**).

In *B. fuliginosus*, consistent with a relative advanced development of snake embryos at oviposition (Boback et al., 2012; Ollonen et al., 2018), ZIC/PCNA-positive cells are detected at 15 dpo (**Fig. 8C**), earlier than in *P. vitticeps*, both in the URL and along the pial surface but, in contrast to the lizard, these domains rapidly thin out and no PCNA-positive cell is found by 40 dpo. So, despite sharing similar features with the bearded dragon, the proliferative pattern of GCPs in the snake is much shorter.

Given the observed spatiotemporal changes in PCNA expression in the two squamate models, I investigated the molecular mechanisms potentially responsible for such divergent pattern. URL activation and maintenance have been shown to rely on secreted factors released by non-neural structures adjacent to the cerebellar primordium, including the 4th ventricle CP and RP (Chizhikov et al., 2006; Krizhanovsky and Ben-Arie, 2006; Liu and Joyner, 2001; Wurst et al., 2001; Yamamoto et al., 1996). In particular, BMP ligands secreted by these non-neural tissues are important in maintaining URL cells and GCPs, that tangentially migrate along the cerebellar pial surface, in an undifferentiated state (Chizhikov et al., 2006; Krizhanovsky and Ben-Arie, 2006; Qin et al., 2006; Rook et al., 2020; Tong et al., 2015). Moreover, both cell and tissue culture experiments as well as *in vivo* observations thoroughly assessed the potential of BMP family members like BMP4, BMP7 and GDF7 to mediate GCP proliferation and specification (Alder et al., 1996; Krizhanovsky and Ben-Arie, 2006; Lee et al., 1998; Salero and Hatten, 2007; Su et al., 2006). Finally, activation of the *Bmp* canonical pathway through phosphorylated forms of SMAD effectors has been documented in both mammalian URL and EGL (Fernandes et al., 2012; Owa et al., 2018; Rook et al., 2020), and SMAD1/5 have been shown to be critical factors in cerebellar development and cortical lamination (Tong and Kwan, 2013).

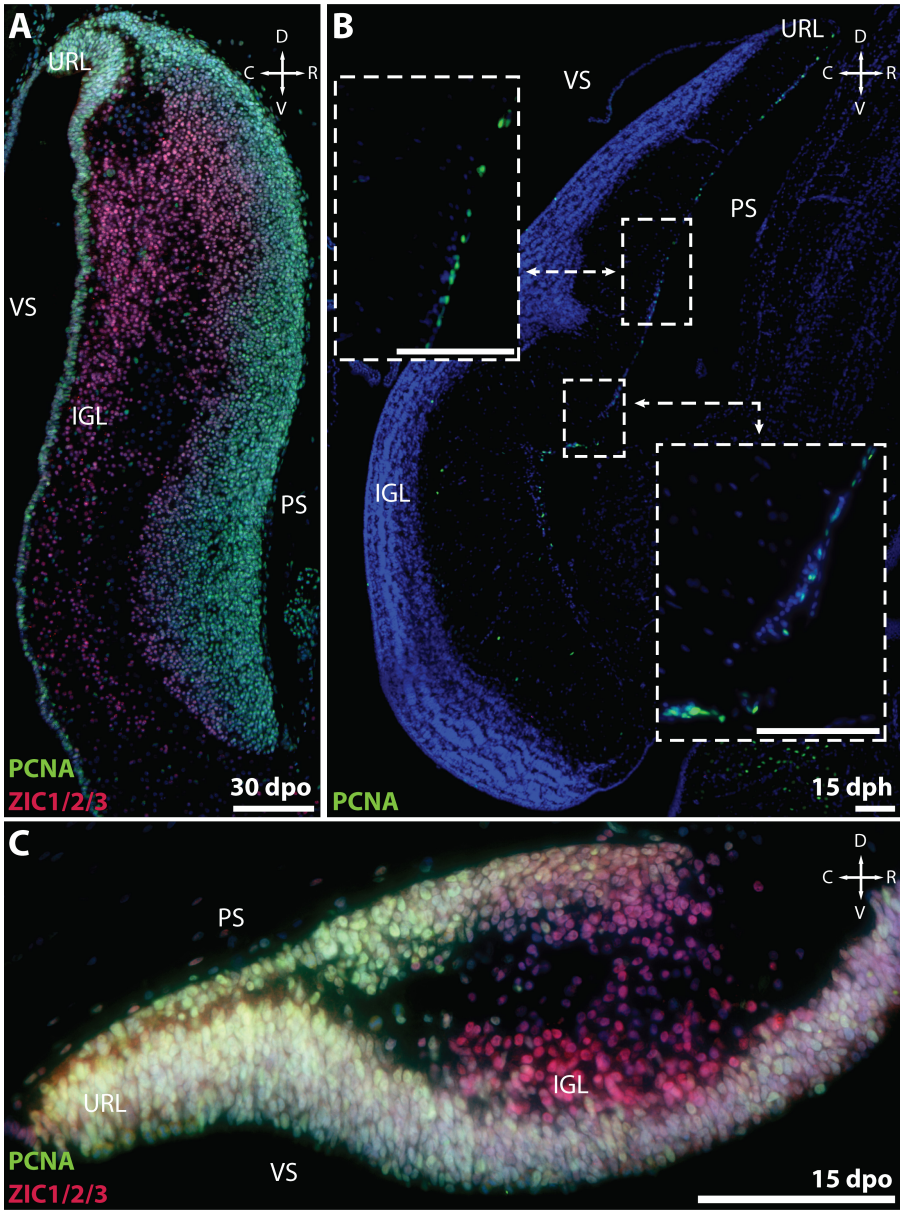

Figure 8 **URL and GCP dynamics in *P. vitticeps* and *B. fuliginosus*. (A)** Double IHC with PCNA (green staining) and ZIC1/2/3 (red staining) on a sagittal section of a 30 dpo *P. vitticeps* embryonic cerebellum. **(B)** IHC with PCNA (green staining) on a sagittal section of a *P. vitticeps* hatchling (15dph) cerebellum. **(C)** Double IHC with PCNA (green staining) and ZIC1/2/3 (red staining) on a sagittal section of a 15 dpo *B. fuliginosus* embryonic cerebellum. Cell nuclei are counterstained with DAPI (blue staining). Crossed white arrows point toward rostral (R), caudal (C), dorsal (D), and ventral (V) directions. IGL, internal granular layer; PS, pial surface; URL, upper rhombic lip; VS, ventricular surface. Scale bars: 100 µm.

I, then, analyzed both BMP ligand expression and canonical *Bmp* pathway activation to highlight variations in their spatiotemporal pattern that could explain the different PCNA labelling profile observed in the bearded dragon and African house snake.

In both animals, *Bmp4* and *Bmp7* start to be expressed in the 4th ventricle CP in coincidence with URL activation, however, while *Bmp7* transcripts are still abundant in the lizard at 40dpo, BMP ligand mRNAs are rapidly downregulated in the snake, and virtually absent soon after 30 dpo. In addition, IHC experiments with an antibody against the active (phosphorylated) forms of *Bmp* signaling effector molecules (P-SMAD1/5/9) outlined an activation pattern of the *Bmp* canonical pathway which temporally overlapped with the *Bmp* transcripts expression in the CP of the two species.

Such data indicate a tight link between BMP secretion by non-neural structures bordering the cerebellum and both GCP generation and spatiotemporal dynamics in *P. vitticeps* and *B. fuliginosus*. However, while lizard expression profile and both activation timing and domains parallel those described in other amniotes, a shortened expression of *Bmp* genes in the CP, likely results in a precocious decline of URL proliferative potential, and consequently to the disappearance of the PCNA-positive domains, in the snake.

Altogether, this comparative analysis, conducted in two models featuring highly divergent locomotor modes, revealed important differences in crucial molecular and cellular events during cerebellar morphogenesis, suggesting that the variation in cerebellar size and shape, observed in squamates adopting different locomotor strategies, could derive, at least partially, from temporal modifications of the GCP proliferation pattern. In such context, modifications in the timing/duration of inductive signals from non-neural tissues adjacent to the cerebellum, could fine tune the number of GCPs exiting the URL, and ultimately allow the cerebellum to attain a specific size, possibly in synergy with factors secreted by other developing cerebellar cell types. Unfortunately, the difficulty in obtaining embryonic series of lizards and/or snakes exhibiting locomotor modes other than quadrupedal locomotion or lateral undulation, and the impossibility, due to yet-to-overcome technical limitations, to perform *in vivo* functional experiments in squamates, prevented me to fully validate such hypothesis.

5.2 PC SPATIAL LAYOUT CORRELATES WITH LOCOMOTOR BEHAVIOR AND IS SET DURING DEVELOPMENT (I-II)

5.2.1 DIVERGENT PC SPATIAL LAYOUT AND GENE EXPRESSION PATTERN IN THE CEREBELLUM OF SQUAMATES (I)

Several studies and descriptions of squamate cerebellar cortex highlighted substantial differences in PC spatial organization across species, ranging from the almost continuous and well-ordered distribution in quadrupedal lizards to the disorganized layout displayed by snakes (Aspden et al., 2015; Hoops et al., 2018; Larsell, 1926; ten Donkelaar and Bangma, 1992; Wylie et al., 2017). However, the overall number of species collectively analyzed in these studies, corresponding to a particularly small fraction of the squamate lineages, likely provides only a limited view of the full spectrum of squamate PC layouts and prevents the identification of putative links between alterations of cerebellar cortex cytoarchitecture and different ecological and behavioral factors, which have been shown to deeply influence several

neuroanatomical features of this brain subdivision (Corfield et al., 2016; Hall et al., 2013; Iwaniuk et al., 2006; Iwaniuk et al., 2007; Larsell, 1926; Wylie et al., 2017).

With the aim to identify and characterize possible alternative PC topological patterns than those already described in lizards and snakes I performed a series of immunohistochemical stainings both on the whole cerebellum and on paraffin brain sections of different squamate species **(Study I)**. To deeply explore the extremes of the 3D PC spatial configuration range observed in squamates I carried out 3D immunostainings using an antibody against the PC specific marker calbindin-1 (CALB1) and light-sheet fluorescence microscopy on cleared whole cerebella of *P. vitticeps* and *B. fuliginosus* individuals. The results of this imaging experiments allowed me to fully visualize the highly divergent PCL in these species and to appreciate putative differences not only in PC soma distribution but also in the course and arborization of PC dendrites. Unfortunately, due to species-specific variation in antibody penetration I could not expand and quantify these observations in a large panel of squamate species and had to limit the analysis only to PC soma distribution using IHC on paraffin sections. The qualitative inspection of the immunostainings, conducted on 13 species belonging to different squamate families, revealed the existence of a large spectrum of PC arrangements both in snakes and in lizards (**Fig. 9**). In particular, the sandboa *Eryx colubrinus* (**Fig. 9B**), displays a relatively organized PC spatial layout with cell somata, though forming 1-3 rows, precisely following the outer contour of the IGL in striking contrast with other snakes showing different degree of PC scattering in the ML. In addition, the PCs of *Pseudopus apodus* (**Fig. 9C**), a legless lizard, approximate those of snakes in their organization, following no obvious distribution pattern. In light of these observations, I quantified the scattering of individual PCs and tested the existence of potential correlations between specific PC topological distribution patterns and locomotor behaviors adopted by squamates. I followed the same locomotor categorization used in the morphological and volumetric assays and the results from post hoc pairwise comparisons, performed after a significant Kruskal-Wallis test (p-value <0.0001), delineated a scenario consistent with that depicted by the morphometric analysis. The results, in fact, highlighted a strong correlation between locomotor specialization and PC spatial organization, revealing the existence of 4 significantly different PC topological patterns (**Fig. 9**): ordered monolayer (group I), ordered multilayer (II), scattered multilayer (III) and totally scattered (IV). In particular, quadrupedal lizards which all grouped in a single cluster, displayed the most ordered PC distribution followed, in order of increasing PC scattering, by burrowers, multi-habitat lateral undulation and multi-habitat other movements groups. Interestingly, the significant segregation exhibited by locomotor groups including snakes and lizards (II, burrowers and facultative burrowers vs III, multi-habitat lateral undulation) suggests that variations in PC spatial layout exists in both snake and lizard species and correlate with locomotor adaptations in a phylogenetic-independent fashion.

The functional correlates of the different distribution of PCs in squamates are unknown and PC heterotopia in the ML is often associated with neurodegenerative disorders, generally leading to PC degeneration and death, characterized by severe locomotor impairments in humans (Borghesani et al., 2000; Bottini et al., 2012; Kuo et al., 2011; Louis et al., 2018; Pascual-Castroviejo et al., 2003). Lying outside this context of pathological conditions, the variations in squamate PC spatial organization could be, instead, an additional factor through

which the cerebellum could differentially modulate specific types of limb/body coordination in lizards and snakes adopting alternative locomotor strategies.

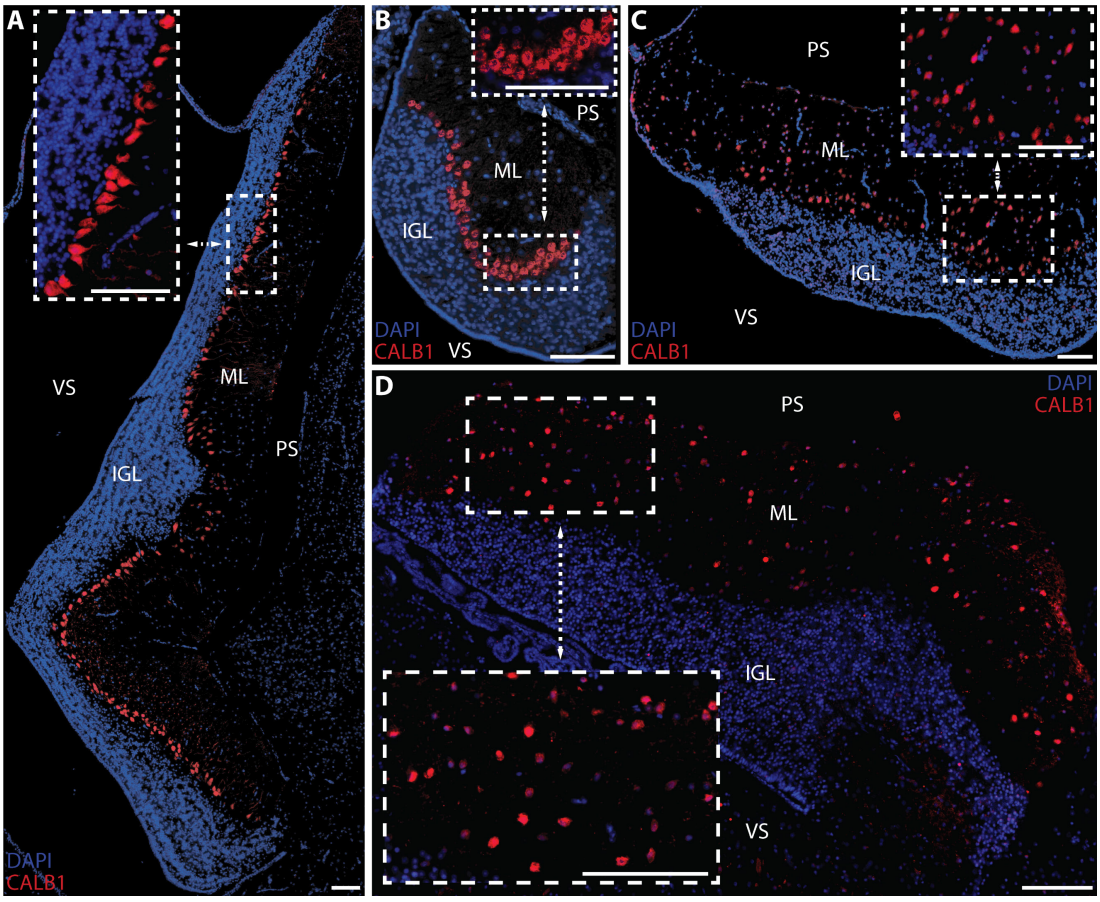

Figure 9 **Heterogeneity of PC spatial distribution in squamates.** IHC with CALB1 (red staining) on sagittal sections of the cerebellar cortex in selected species representative of the 4 distribution patterns observed: *Pogona vitticeps* **(A**, Group I**)**, *Eryx colubrinus* **(B**, Group II**)**, *Pseudopus apodus* **(C**, Group III**)**, *Dasypeltis gansi* **(D**, Group IV**)**. Insets show high magnifications of PC spatial organization. Cell nuclei are counterstained with DAPI (blue staining). IGL, internal granular layer; ML, molecular layer; PS, pial surface; VS, ventricular surface. In all sections caudal is to the left and dorsal to the top. Scale bars: 100 μm.

In such respect, data from the comparative transcriptomic analysis of the whole cerebellum, conducted in parallel to the morphometric and histological study, on a panel of 10 squamate species belonging to different locomotor groups (**Study I**), are suggestive of an association between different PC spatial layouts and divergent expression of genes involved in PC electrophysiological and metabolic properties. The hierarchical clustering on pairwise correlation of 630 orthologous cerebellar genes, by grouping species according to the similarity in their gene expression patterns, revealed a significant influence of locomotion on cerebellar gene expression patterns. The different species analyzed, indeed, clustered in the generated dendrogram according to their locomotor mode rather than to their phylogenetic relationships, indicating that differential gene expression, in addition to cerebellar morphology, size and PC distribution, might reflect the alternative locomotor patterns

featured by squamates. Importantly, the analysis of the gene cluster composed by transcripts with highly divergent expression profiles among the different locomotor groups, pointed out the presence of several genes exclusively expressed in PCs, including some with a relevant role in their electrophysiological properties and likely influencing their functions, such as the metabotropic glutamate receptor1 (*mGluR1*) and Hamartin (*Tsc1*) gene. The *mGluR1* gene product is a receptor essential for long-term depression of synaptic transmission at PC-PF synapses, a fundamental substrate for motor learning in the cerebellum (Ichise, 2000; Ito, 2001; Ito and Karachot, 1989; Ito et al., 2014), while mutations in *Tsc1* result in the tuberous sclerosis complex, a syndrome characterized by cerebellar pathology associated with increased autism spectrum disorder symptomatology in humans (Ertan et al., 2010). Moreover, the characterization of both homozygous and heterozygous mouse mutants for *Tsc1* highlighted alterations in PC firing rates, which are key determinants of DCNi activity and deeply influence the downstream motor-related neuronal networks (Gutmann et al., 2000). In addition, other genes involved in PC physiology and metabolism and associated with motor performance like striatin (*Strn*; Bartoli et al., 1999; Benoist et al., 2006), a scaffolding protein mediating signaling and trafficking at PC dendritic spines, and spatacsin (*Spt11*; Branchu et al., 2017; Varga et al., 2015), a protein modulating lipid turnover, also exhibited a differential expression profile among the different locomotor groups. These results, thus, suggest that the alternative PC layouts in squamates might correlate with different PC functional properties, mediated by expression levels modulation of genes influencing PC electrophysiology, morphology, and metabolic processes.

In the future, detailed 3D microscopic analyses coupled with electrophysiological assays, together with a finer characterization of PC expression profiles on a larger number of species, might help clarify the potential structural and functional correlates of the observed heterogeneity in squamate PC distribution.

5.2.2 DEVELOPMENTAL GROUNDS OF PC SPATIAL LAYOUTS IN SQUAMATES (I-II)

To explore the possible origins of the highly diverse PC organization exhibited by squamates I investigated, in a lizard and a snake, the embryonic cellular and molecular events shown to be crucial for a proper cortical lamination during cerebellar morphogenesis in other vertebrate groups. I characterized PC development with a particular focus on the molecular interactions and temporal dynamics involving PC migration and GC generation and differentiation. I conducted a developmental comparative study from early post-ovipositional embryonic stages to early postnatal period in the bearded dragon and in the African house snake, two squamate species which clustered in highly divergent groups (ordered monolayer and scattered multilayer, respectively) in the analysis of PC topology.

To track PC generation and development, I performed IHC experiments in both species using an antibody against LHX1 (Zhao et al., 2007), a transcription factor selectively expressed by PC, together with the proliferation marker PCNA. As already noticed for GCPs production in the URL, proliferation in the VZ starts at earlier stages in the snake compared to the lizard (12dpo and 15dpo, respectively) and the first PCs labelled by LHX1 are detected few days later in both species. After being produced, PCs leave the VZ in a radially oriented migration, likely

sliding along radial glia fibers, as documented in other vertebrates (Yuasa et al., 1991; Yuasa et al., 1996), and move towards the cerebellar PS. As development proceeds, PC distribute in sigmoidal-shaped multi-layered clusters (PCCs), in both models, occupying an intermediate position along the cerebellar ventricular-pial axis and spanning the entire rostro-caudal cerebellar extent (**Fig. 10**). Despite PCC formation occurs in advance in the snake compared to the lizard (25dpo vs 30dpo), likely reflecting the earlier onset of PC production and migration in *B. fuliginosus*, the two model PC developmental program showed no particular difference till this stage. However, the analysis of the following phases of cerebellar morphogenesis highlighted a striking divergence of PC fate in the two species. In fact, while *P. vitticeps* PCs, progressively disperse form the PCC and gradually acquire the ordered arrangement along the outer contour of the IGL, during the last third of post-ovipositional gestation, snake PCs do not reposition and remain in a multilayered configuration throughout embryogenesis, and the subtle changes observed in their arrangement from 30 dpo are likely due to both cerebellar growth and circuitry refinement rather than to PC-autonomous dynamics.

These results point out that similar events characterize the initial phases of PC development, including radial migration and PCC formation, in *P. vitticeps* and *B. fuliginosu*s and that the peculiar PCL configuration observed in the adult snake derives from PCs failing to uniformly disperse from the multilayered clusters. Furthermore, they highlight a nearly precise simultaneity between PC spatial dynamics truncation and the previously characterized GCP layer disappearance from the pial cerebellar surface *in B. fuliginosus*, suggesting a potential link between the alternative PC layouts displayed by the two models and the temporally divergent GCP dynamics they feature.

Such temporal coincidence, together with the pivotal and well-established role played by molecular interactions involving PCs, GCPs and migrating GCs in directing a proper cerebellar development, led me to verify the integrity, in both models, of the *Reln* signaling pathway, a crucial mediator of neuronal migration and both cerebral and cerebellar cortex lamination in mammals and other vertebrates (Caviness and Rakic, 1978; Costagli et al., 2002; D'Arcangelo et al., 1995; Jensen et al., 2002; Miyata et al., 1997; Nimura et al., 2019; Pesold et al., 1998; Rahimi-Balaei et al., 2018; Rodríguez-Moldes et al., 2008). I characterized the expression pattern of the main components of the *Reln* pathway by ISH and used an antibody against the phosphorylated form of DAB1 (P-DAB1), the main effector of *RELN* molecular cascade, in IHC assays, to assess its activation. In both *P. vitticeps* and *B. fuliginosus*, *Reln* transcripts are abundantly present throughout the developmental time window encompassing GC generation in the two models, both in GCPs located on the cerebellar pial surface and migrating GCs as well as in GC already settled in the progressively expanding IGL. Moreover, like in mammals, *Reln* expression is maintained also in juvenile squamate GCs. Owing to the similarities of *Reln* transcription pattern in the two models I, then, checked the existence of possible variations in the expression profile of *Vldlr* and of *Dab1,* the main *Reln* pathway receptor and effector involved in PC monolayer formation, respectively (Trommsdorff et al., 1999). In contrast to the asynchrony observed in other events of cerebellar morphogenesis between the snake and the lizard, largely due to the relative advanced development of snake embryos at oviposition, *Vldlr* and *Dab1* transcription initiates in both model PCs at 40dpo, when PCCs have already formed in the two squamates. Consistently, DAB1 activation, detected with an antibody

against its phosphorylated form, temporally matches *Vldlr* and *Dab1* transcriptional profile in *P. vitticeps* and *B. fuliginosus,* further confirming the molecular synchronization occurring between lizard and snake PCs at 40dpo. Importantly, and coherent with *Reln* expression, the active form of DAB1 is also found in juvenile squamate IGL. These data, highlighting the presence of all main components of the *Reln* pathway and their overlapping spatiotemporal expression and activation pattern in *B. fuliginosus* and P. *vitticeps*, extend the homologies observed in the early phases of PC developmental program in the two species, further suggesting that the alternative PC organization displayed by the lizard and the snake may derive from PC-non autonomous mechanisms. Moreover, the intact glial fiber organization in the snake cerebellum, assessed by immunodetection of the glial fibrillary acidic protein (GFAP), indicates that the peculiar *B. fuliginosus* PCL is not caused by alterations in the guidance system supporting PC migration.

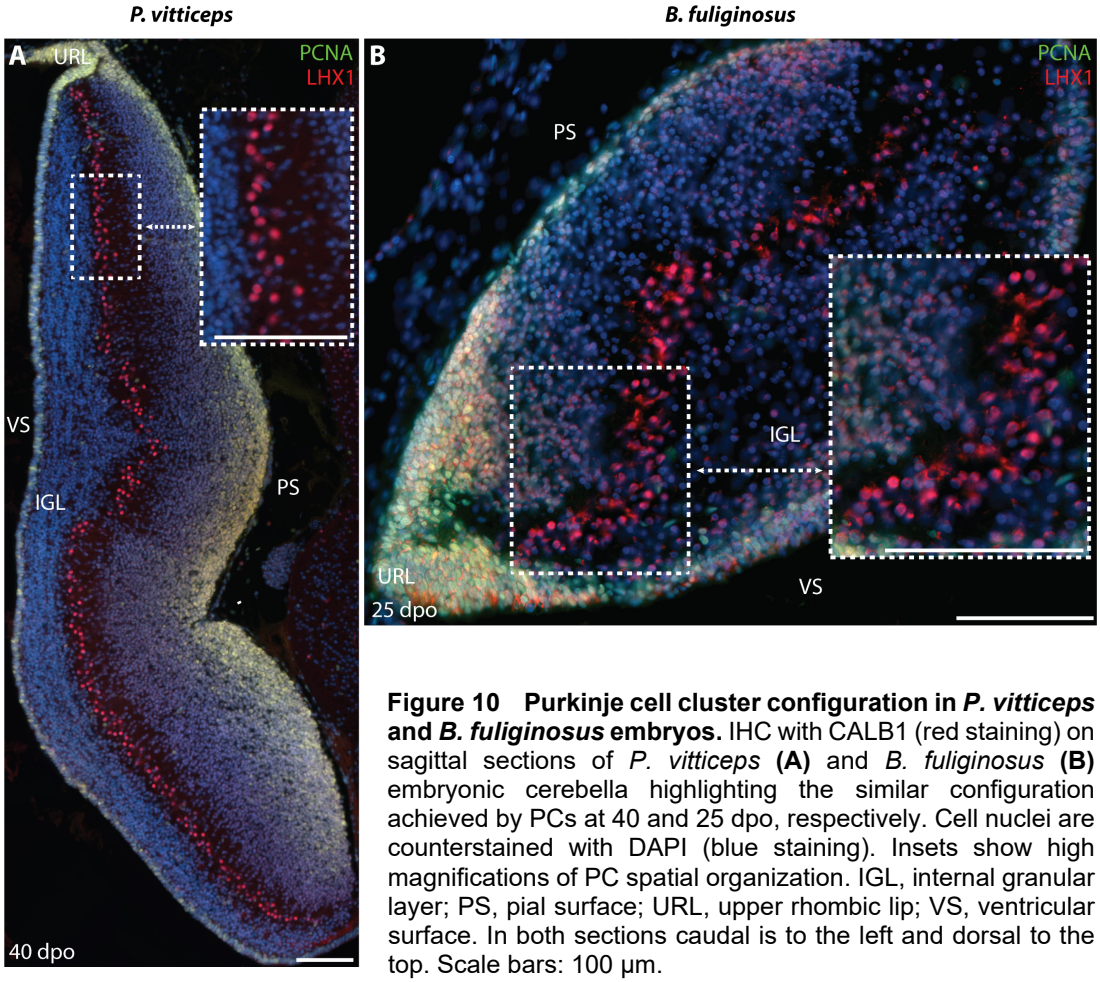

Figure 10 Purkinje cell cluster configuration in *P. vitticeps* and *B. fuliginosus* embryos. IHC with CALB1 (red staining) on sagittal sections of *P. vitticeps* **(A)** and *B. fuliginosus* **(B)** embryonic cerebella highlighting the similar configuration achieved by PCs at 40 and 25 dpo, respectively. Cell nuclei are counterstained with DAPI (blue staining). Insets show high magnifications of PC spatial organization. IGL, internal granular layer; PS, pial surface; URL, upper rhombic lip; VS, ventricular surface. In both sections caudal is to the left and dorsal to the top. Scale bars: 100 μm.

Despite the synchronous activation of DAB1, the 3D extracellular environment and morphogenetic context experienced by PCs is radically different in *P. vitticeps* and *B. fuliginosus* at 40 dpo. In *P. vitticeps*, at this stage, the strong *Reln* expression detected in the several-cell-thick EGL and in a conspicuous number of GCs delaminating from it, likely providing a permissive extracellular milieu, allows PCC dispersal. Moreover, as development

progresses *Reln* expression gradually become exclusively expressed in lizard IGL GCs, in coincidence with the progressive reduction of GCPs on the pial surface. *P. vitticeps* PCs are, thus, exposed to a *Reln* expression gradient which varies over time, progressively shifting its concentration peak from the pial side during the PCC dispersal phase to the IGL at late developmental stages and postnatal life. In contrast, snake PCs are exclusively exposed to a fixed source of Reln, the IGL GCs, owing to the disappearance of the pial surface GCP layer prior to DAB1 activation. Furthermore, this divergence in *Reln* spatial distribution parallels the different level of maturation of the cerebellar cortex in the two models at the onset of DAB1 activation. At 40 dpo, in fact, the layers of the cerebellar cortex are only barely delineated in *P. vitticeps*, whereas *B. fuliginosus* cerebellum displays almost a mature configuration, featuring an expanded ML hosting an entangled network of GC ascending and parallel fibers. The joint effect of these divergent molecular and spatial conditions experienced by PCs in the two models could, thus, be at the base of the radically diverse PCL phenotype in *B. fuliginosus* and *P. vitticeps*. Though RELN mechanism of action in PCC dispersion and PCL formation have not been fully elucidated, this hypothesis is in line with previous results highlighting the pivotal role of GC delaminating from the pial surface in promoting PC reorganization (Jensen et al., 2002; Miyata et al., 1997). Moreover, both *in vitro* and *in vivo* observations suggest a shift in RELN localization and concentration from GC somata, where RELN is abundant as GC differentiate and migrate through the PCC, to PF segments when GC settle in the IGL and the glycoprotein levels decrease (Miyata et al., 1996). This shift in RELN extracellular distribution and concentration could correlate with the transition from its role in promoting PC spatial rearrangements in early phases of cerebellar morphogenesis to a modulatory function in PC dendrite maturation at later stages (Miyata et al., 1996). At the onset of DAB1 transcription and activation snake PCs, then, are likely exposed to a low RELN concentration which, accumulating on their dendrites, might be only capable of inducing limited responses in PC, insufficient to drive their somata relocation. In addition, snake PCC dispersal could also be prevented by the physical barrier constituted by the dense network of AGAs and PFs, likely present in the mature ML of *B. fuliginosus* upon DAB1 activation, when all GC have already settled in the IGL. Phenotypic analyses of human cerebellar tissue and the developmental characterization of mouse models for ataxia-telangiectasia (Borghesani et al., 2000; Bottini et al., 2012; Vinters et al., 1985), a systemic syndrome characterized by ectopic Purkinje cell in the ML, have suggested that the tight packing formed by PFs around improperly migrated PCs could preclude, by firmly anchoring them in place, PC somata compensatory spatial rearrangements at later stages. Moreover, embryonic graft experiments in rodents have further highlighted the relevance of a precise spatiotemporal patterning of GCs and PCs demonstrating the direct correlation between the ratio of donor-PC ectopically positioning in the host developing cerebellum and the degree of cortical maturation of this latter (Carletti et al., 2008; Sotelo and Alvarado-Mallart, 1986; Sotelo and Alvarado-Mallart, 1987a; Sotelo and Alvarado-Mallart, 1987b).

5.3 SQUAMATES FEATURE THE BASE PLAN OF AMNIOTE CEREBELLOGENESIS (II)

The analysis of GC developmental dynamics strongly suggested possible homologies between the layer of PCNA positive cells on the pial surface of both the snake and lizard cerebellum and the proliferative EGL featured by the avian and mammalian developing cerebella. The EGL is a transient, secondary germinative zone defined not only by its highly proliferative nature but also by other molecular features, including the sustained expression of the bHLH transcription factor *Atoh1* (Akazawa et al., 1995; Ben-Arie et al., 1996; Ben-Arie et al., 1997), which is essential to preserve GCP identity and allow their transit amplification (Butts et al., 2014b; Flora et al., 2010; Klisch et al., 2011). In addition, GCP amplification in the EGL requires the mitogenic factor SHH which, secreted by PCs, is crucial for the cerebellum to achieve a proper size and degree of foliation (Corrales et al., 2004; Corrales et al., 2006; Dahmane and Ruiz, 1999; Lewis et al., 2004; Wallace, 1999; Wechsler-Reya and Scott, 1999). To clarify whether such features are a distinctive trait of bird and mammal cerebellogenesis or represent a developmental blueprint common to all amniotes, I conducted a series of IHC and ISH experiments in different stage lizard and snake embryos with a set of specific markers (**Study II**).

Immunohistochemical labelings using the antibody against the phosphorylated form of histone H3 (PH3), a universal mitotic marker, in combination with PCNA highlighted the occurrence of cell divisions in both *P. vitticeps* and *B. fuliginosus* embryos (**Fig. 11A,C**). In particular, dividing cells were detected in proximity of the URL and both in superficial and, as recently observed in birds (Hanzel et al., 2019), also more internal layers of the subpial stream of tangentially migrating GCPs in the two models. The direct comparison of the spatiotemporal evolution of the EGL, however, pointed out relevant difference in its proliferative potential in *B. fuliginosus* and *P. vitticeps*. In contrast to lizard embryos, which at 60 dpo, together with a 1-to-4-cell thick EGL, display mitotic GCPs located even far from the URL (**Fig. 11B**), snake embryos show a progressive and quick reduction of both EGL thickness and cell division number. At 30 dpo only few PH3-positive cells, exclusively found in vicinity of the snake embryo URL, are labeled and at 40 dpo no proliferative cells are detected (**Fig. 11D**). These results point out that squamate GC developmental program, like in avian and mammalian cerebellogenesis, features a transit amplification phase in a secondary germinative domain, aimed to expand the GCP pool. Moreover, they confirm and further detail the significant differences already observed between *B. fuliginosus* and *P. vitticeps* URL and GCP proliferation potential that could influence both the final size and cortical structure of the two squamate species.

To further explore the potential homologies between squamate, mammalian and avian cerebellar morphogenesis at a deeper level, I checked the expression of the proneural gene *Atoh1*, a fundamental molecular prerequisite for GCP transit amplification in the EGL, and of the mitogenic protein SHH by PCs. ISH experiments revealed *Atoh1* transcripts in the EGL of *B. fuliginosus* and *P. vitticeps*. At all stages tested bearded dragon embryos displayed an *Atoh1* spatiotemporal expression pattern overlapping with that of the proliferating URL and EGL, showing a sustained level of transcription, likely protracting till early postnatal life, as suggested by the proliferation marker analysis. Immunolabelings for SHH further confirmed

the existence of a common blueprint for cerebellar morphogenesis in amniotes, this protein being detected both in the lizard and snake PCs by 40 dpo (**Fig. 11B,D**) onwards, further suggesting, as already indicated by *Reln* pathway characterization, a synchronization of PC developmental program in the two models at this stage. The timing of SHH production, was confirmed by the expression pattern analysis of *Rora*, a thyroid hormone-regulated transcription factor directly modulating *Shh*, involved in PC dendritogenesis, survival and lifelong physiological integrity (Boukhtouche et al., 2010; Chen et al., 2013; Dussault et al., 1998; Gold et al., 2003; Gold et al., 2007; Hamilton et al., 1996; Sidman et al., 1962; Takeo et al., 2015), which highlighted a perfect temporal match with SHH immunodetection.

Altogether, these results clearly point out that squamate cerebellogenesis features hallmarks, like GCP transit amplification in the EGL and SHH secretion by PCs thought, so far, to be an exclusive trait of birds and mammals, outlining that such cellular and molecular processes represent developmental milestones shared by amniotes. In addition, they provide further indications on the critical importance the temporal coupling between the different cerebellar cell type development/maturation takes on in determining the cortical phenotype and final size of the cerebellum in lizards and snakes (**Fig. 12**).

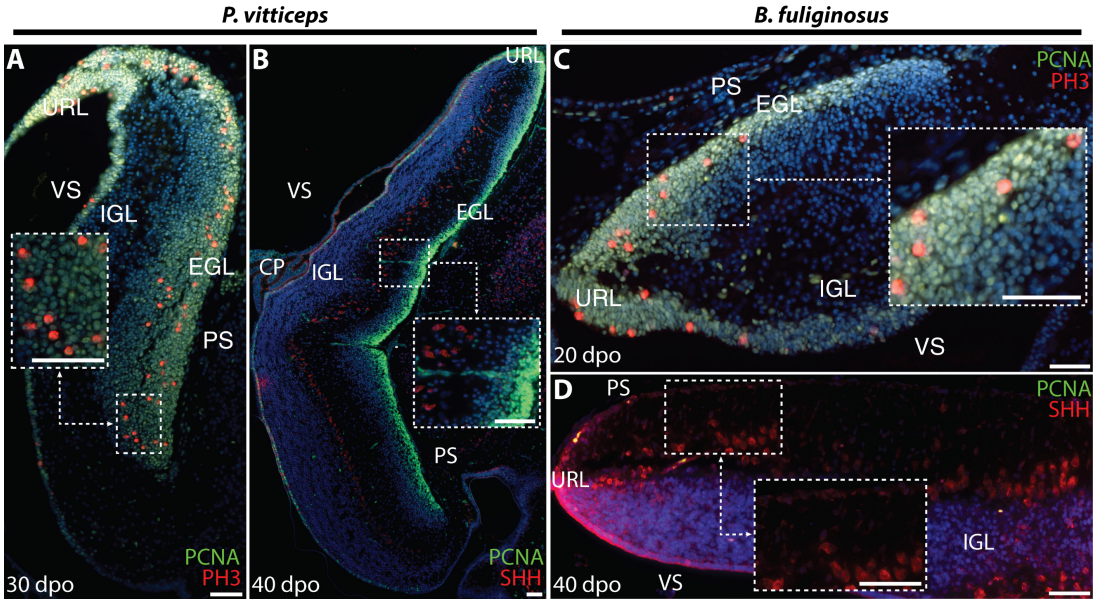

Figure 11 **Squamates feature hallmarks of avian and mammalian cerebellogenesis.** (**A**,**C**) Double IHC for PCNA (green staining) and PH3 (red staining) in the embryonic cerebellum of *P. vitticeps* (**A**) and *B.fuliginosus* (**C**) at 30 and 20 dpo, respectively. (**B**, **D**) Double IHC for PCNA (green staining) and SHH (red staining) in the embryonic cerebellum of *P. vitticeps* (**B**) and *B.fuliginosus* (**D**) at 40 dpo, in both species. Insets show high magnifications of mitotic GCPs in the EGL **(A,C)** and SHH-positive PCs **(B,D)** in both species. Cell nuclei are counterstained with DAPI (blue staining). CP, choroid plexus; EGL, external granular layer; IGL, internal granular layer; PS, pial surface; URL, upper rhombic lip; VS, ventricular surface. In all sections caudal is to the left and dorsal to the top. Scale bars: 100 µm.

As already noted in the context of RELN cascade activation, and despite the synchronization of SHH expression in terms of dpo, the two model EGL state is completely different at 40 dpo.

In fact, when SHH begins to be produced and secreted by PCs, the EGL thickness ranges from three to five layers of cells in the lizard, while GCPs are virtually absent from *B. fuliginosus* pial surface at 40 dpo. In a similar way to RELN, then, SHH might exert differential and context dependent functions in the two models. In addition and independently from its role in GCP amplification, SHH maintains a niche of heterogeneous and functionally divergent cerebellar progenitor cells important for motor learning and cognition in mice prospective white matter (Fleming et al., 2013).

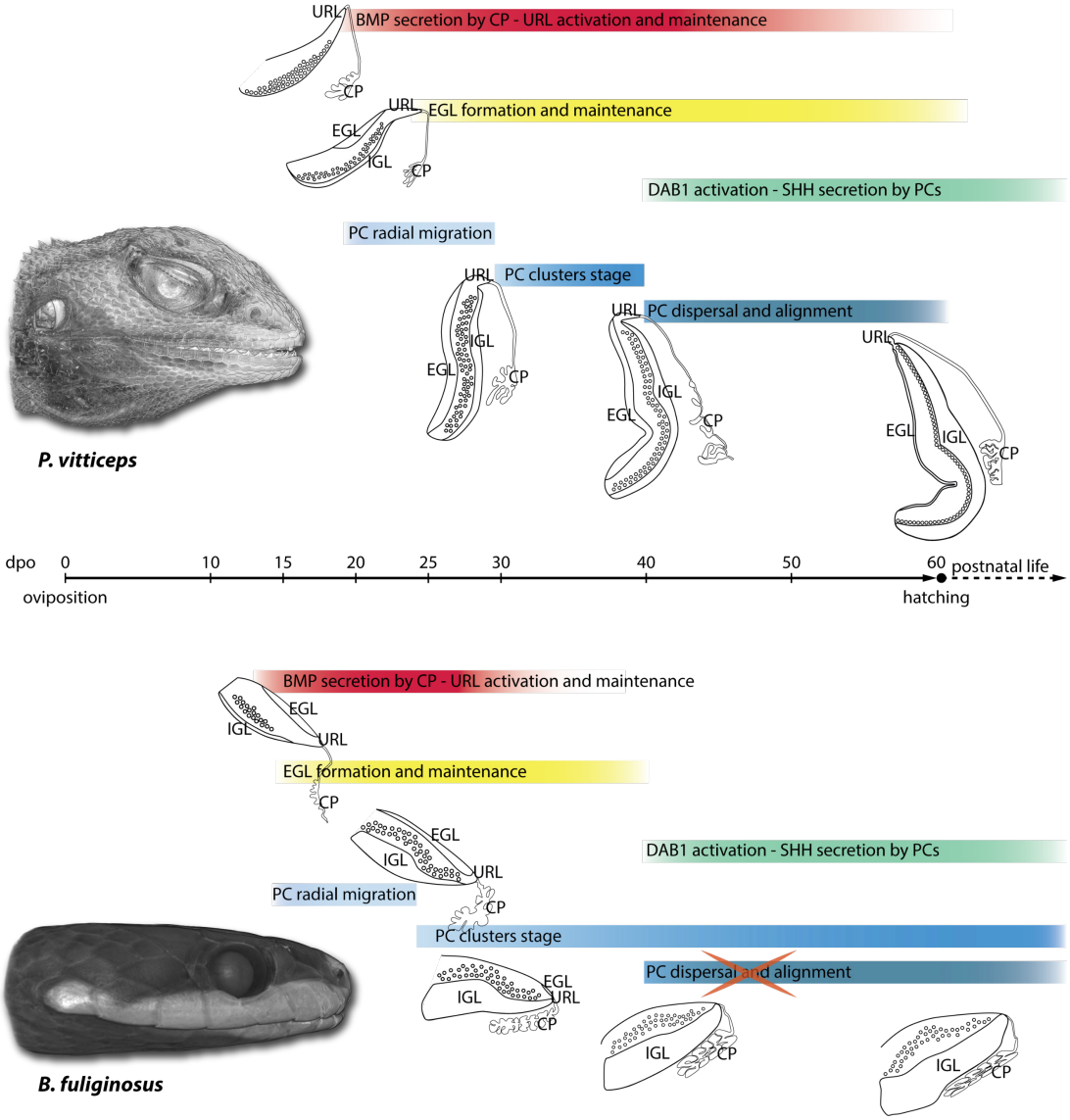

Figure 12 **Overview of cerebellar development in squamates.** Schematic and chronological representation of the main molecular and cellular events at the origin of the divergent cortical lamination in *P. vitticeps* **(top row)** and *B. fuliginosus* **(bottom row)** cerebellum. The absence of major developmental processes is marked by a red cross. Purkinje cells are represented as empty circles. EGL, external granule layer; IGL, internal granule layer; URL, upper rhombic lip; CP, choroid plexus.

Moreover, both *Shh* and its pathway component detection in adult rodent PCs and other cerebellar cells (Corrales et al., 2004; Lewis et al., 2004; Traiffort et al., 1998; Traiffort et al., 1999; Vaillant and Monard, 2009; Wallace, 1999) has been associated with a key role in synaptic plasticity, by controlling cerebellar astrocyte complex molecular signature and both glutamate and adenosine triphosphate (ATP) trafficking (Farmer et al., 2016; Okuda et al., 2015). In the context of squamate cerebellogenesis, SHH could promote GCP expansion in the EGL till the acquisition, in early postnatal life, of a mature cortical organization in the lizard, before switching to a synaptic plasticity mediator role afterwards. Owing to the precocious EGL disappearance in the snake, accompanied by an advanced degree of cortical maturation already established at 40 dpo, SHH secretion by *B. fuliginosus* PCs could, instead, reflect the indispensable function played by this molecule in adult cerebellum physiological dynamics.

The absence of a SHH-sustained GCP amplification phase in *B. fuliginosus* is likely, at least in part, linked to the relatively small cerebellar size achieved by the snake cerebellum compared to the lizard one. The analysis of mouse mutants, conditionally expressing different levels of SHH, have demonstrated a direct correlation between the amount of this secreted mitogen and the degree of cerebellar foliation (Corrales et al., 2006). In particular, the complete ablation of *Shh* from PCs causes a precocious termination of GCP amplification in the EGL, producing a severely reduced and unfoliated cerebellum, while increasingly complex cerebellar morphologies parallel rising levels of *Shh* pathway activation. Considering these experimental observations, the asynchrony between *Shh* pathway activation and EGL presence in *B. fuliginosus* developing cerebellum can be considered as functionally equivalent to *Shh* complete abrogation in mouse mutants. Indeed, the cerebellum of these knockout mice shows homologies with the snake one. Both are small, smooth and show no foliation, all characteristics linked to the absence of a SHH-driven GCP amplification phase in the EGL. *P. vitticeps* cerebellum, on the other hand, featuring a second transit-amplifying phase of GCPs promoted by PC-secreted SHH, achieves a larger size relative to the whole-brain and a more elaborated morphology compared to the snake. However, though being more complex than the snake counterpart, *P. vitticeps* cerebellum doesn't even approach the sophisticated morphologies exhibited by mammals, suggesting that the smaller number of PCs residing in the lizard cerebellum, when compared to mouse (Bakalian et al., 1991; Bakalian et al., 1995; Frederic et al., 1992; Wetts and Herrup, 1982; Zanjani et al., 1992; Zanjani et al., 2004; and personal observations), can only provide low intensities of SHH signaling. As a whole, though additional data and functional assays are needed to validate the hypothesis of a SHH-mediated transition from the morphologically simple cerebellum of squamates to the more elaborated forms exhibited by birds and mammals, this comparative analysis provide new details on the possible mechanisms promoting cerebellar phenotypic evolution within squamates and across amniotes.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this thesis work I first explored the potential relationships between a major evolutionary transition—locomotor behavior—and variations in brain organization in squamates, with a particular focus on the cerebellum. I also investigated the embryonic development of the cerebellum in a lizard and a snake species to expand the current knowledge on the mechanisms underlying the extreme phenotypic diversification of this brain subdivision across vertebrates and within squamates.

One of the main goals of evolutionary biology is to understand the forces and processes leading to deviations from phenotypic regularities. Historically, studies on brain evolution have analyzed adult brain or major brain subdivision size variations in relation to behavioral and ecological niches (Jerison, 1973; Lefebvre and Sol, 2008; Lefebvre et al., 2002; Sayol et al., 2016; Sol et al., 2005; Stephan et al., 1981; Striedter, 2005), while only few investigations have evaluated the influence of such factors on multiple aspects of brain biological organization. Phenotypic diversification is, in fact, a complex and multifaceted phenomenon and demands the integration of multiple experimental approaches to fully appreciate its nuances.

The results of this thesis work pointed out significant correlations between squamate cerebellar size, morphology, cortical organization, gene expression patterns and specific locomotor behaviors in a phylogenetic independent fashion, unveiling that important behavioral transitions can influence not only whole-brain or brain subdivision size, but also a broad range of brain features in vertebrates. Furthermore, they revealed that squamates display features thought to be exclusive of bird and mammal cerebellogenesis and suggested that modifications in the timing and/or duration of specific events during cerebellar development could be responsible of the observed variations in both cerebellar size and cortical arrangement in squamates.

However, additional work is needed to better clarify the role played by locomotor specializations on squamate brain evolution. Moreover, the hypothesized causal relationships between variations in the temporal expression pattern of specific factors as well as in the degree of cerebellar cortex maturation during development and the large phenotypic heterogeneity of squamate cerebella need to be thoroughly tested.

The morphometric analysis showed a significant influence of locomotor mode on squamate cerebellar morphology. Owing to the spatial segregation of cerebellar corticonuclear and efferent projections in squamates, the neuroanatomical characterization of DCNi, red nucleus (in lizards only) and nuclei of the reticular formation, could be helpful to assess the impact of squamate locomotor specialization on the extended cerebellar network. Past descriptive studies highlighted a remarkable variability in the number and size of certain reticular nuclei in a restricted number of lizard and snake species (Newman and Cruce, 1982), suggesting potential relationships with their locomotor pattern, but the qualitative nature of these data precluded any experimental validation of this hypothesis.

In addition, a deeper characterization of PC morphological and electrophysiological properties in species adopting different locomotor strategies, could provide insights on the putative functional significance of the different PC layouts displayed by squamates and help refine the scope of the effects that relevant behavioral traits can have on brain evolution.

The characterization of cerebellar morphogenesis in a snake and lizard model highlighted features so far considered evolutionary milestones of avian and mammalian cerebellogenesis, like the formation of a proliferative EGL and the secretion of SHH by PCs. Moreover, they suggest the relevance of GCP and PC spatiotemporal patterning and molecular interactions in determining cerebellar size and cortex configuration within squamates.

Currently, one limitation posed by the use of non-canonical model organisms, like squamates, is the difficulty to perform *in vivo* embryo manipulations to validate functional hypothesis. Overcoming this experimental limitation is of crucial importance for evolutionary-developmental studies owing to the key position squamates occupy in the vertebrate phylogenetic tree. In the context of this thesis research work, for instance, it would be of interest to modulate EGL persistence in snakes and lizards to generate cerebellar phenotypic variants displaying different levels of PC organization and relative size. The cerebellum is a relatively well-accessible brain area in squamate embryos and beads, soaked with factors to either upregulate (BMPs, SHH) or downregulate (dorsomorphin homolog 1 or cyclopamine, a BMP and SHH inhibitor, respectively) GCP production/amplification, could be implanted at different developmental stages to alter the EGL formation and persistence. Furthermore, precocious PC maturation could be induced by thyroid hormone administration in snake organotypic cerebellar slice cultures to reproduce a lizard-like developmental context and evaluate possible modifications in PC spatial distribution.

In addition to *ex vivo* organ cultures and potential *in ovo* manipulations, the developmental characterization of the cerebellum of burrower and/or facultative burrower species could both corroborate the data obtained in this thesis work and provide further important insights on the mechanisms responsible for squamate cerebellar diversity.

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