

# **Multimodal Investigations of Human Cortico-Ponto-Cerebellar Connectivity**

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Supervised by Dr. Christopher J. Steele

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## Abstract

### Multimodal Investigations of Human Cortico-Ponto-Cerebellar Connectivity

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Long regarded as being uniquely involved with motor control, the cerebellum is now recognized to contribute to nearly every aspect of human cognition. The cerebellum forms reciprocal connections with much of the cerebral cortex, receiving input via the pons and sending output back through the deep cerebellar nuclei and thalamus. These topographically organized, closed loop circuits, are thought to underly the cerebellum's capacity to influence such a breadth of different processes. These connections have a rich history of study in non-human animals, but their organization in humans is largely understudied. In this dissertation we present a series of three studies that investigated the connectivity of the downstream, cortico-ponto-cerebellar, pathway in humans. In our first study (Chapter 2) we reconstructed connections between the pons and lobules of the cerebellar cortex using diffusion MRI tractography. We demonstrated topographic organizational principles broadly reflecting the segregation of motor and non-motor inputs to the cerebellum. Our second diffusion MRI tractography study (Chapter 3) mapped the corticopontine segment using methods to reconstruct gradients that reflect the continuous mappings of the cerebral cortex onto pons. In our final study (Chapter 3), we shifted to a functional connectivity approach, reconstructing gradients in the pons based on its connectivity with the cerebral and cerebellar cortices. While the first two studies serve as bridges to prior work conducted in non-human animals, the final study supports a novel perspective of the pons as a functionally dynamic integrative hub. Taken together, this work advances our understanding of cerebellar connectivity in humans and, by extension, its diverse contributions to behaviour and cognition.

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# Contributions of Authors

I am the primary author of the three manuscripts presented in this dissertation (Chapters 2-4), all of which were completed under the supervision of Dr. Christopher J. Steele. The manuscripts included in Chapters 2 and 3 have been published in peer reviewed journals, and Chapter 4 is under review at *The Cerebellum*.

## Chapter 2

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# Chapter 1. Introduction

## 1.1 An Evolving Understanding of the Cerebellum

Our understanding of brain structure and function has grown immensely over the past century, with advances encompassing both cellular mechanisms and systems-level interactions. Underlying this progress, however, has been a largely corticocentric view, one that sees the cerebral cortex as the foundation of thought and behaviour, with subcortical structures playing secondary or supporting roles (Parvizi, 2009). The cerebellum is one such structure that has been historically underappreciated or misunderstood. It is present in all vertebrates (Bell, 2002), occupies a large proportion of the brain's total volume, contains over 50% of its neurons (Lent et al., 2012), has a uniform cellular architecture, and is reciprocally connected to much of the cerebral cortex (Schmahmann, 2001). It has also shown relative evolutionary expansion in the human and primate lineage (Balsters et al., 2010). Historically, it has been viewed as being uniquely involved in motor control, a perspective that remains influential despite accumulating evidence pointing to its involvement in practically every facet of human cognition and behaviour (Buckner, 2013; Schmahmann, 2019). In fact, the more we learn about the cerebellum, the clearer it becomes that the cerebellum does not just contribute to a range of different processes, but it plays an integral role in how human brains function (Buckner, 2013; Ito, 2008; Sokolov et al., 2017).

The work presented in this dissertation is emblematic of a broader shift in our understanding of the role of the cerebellum in brain function. The paradigmatic view of the cerebellum as being involved uniquely in motor control emerged from early neurophysiology work, including that of Jean Pierre Flourens, that demonstrated motor impairment resulting from lesions to the cerebellum in animal models (Schmahmann, 2016). Early work in patients with cerebellar pathology or injuries to the cerebellum highlighted characteristic features of motor impairment (Schmahmann, 2016). A changing view towards the role of the cerebellum, recognizing its role in diverse processes, was hinted at by Leiner et al. (1986) and pushed forward by the pioneering work of Schmahmann (2010) and Middleton & Strick (1994). This shift represents the confluence of different factors, notably the mapping of cerebellar anatomical connectivity in non-human primates (Middleton & Strick, 1998; Schmahmann & Pandya, 1997b), cerebellar patient research (e.g., Schmahmann & Sherman, 1998), and the advent of structural and

functional neuroimaging (Buckner, 2013). Anatomical tract tracing studies of Schmahmann and Pandya and Strick and colleagues demonstrated rich and highly organized connections linking the cerebellum to higher order associative cerebrocortical areas (Schmahmann, 2010; Schmahmann & Pandya, 1997b; Strick et al., 2009). Work in neurological patients, including the seminal work of Schmahmann and Sherman (1998), documented neuropsychological and affective disruption associated with pathology in the cerebellum. Cerebellar abnormalities were also noted in neurodevelopmental and psychiatric disorders, perhaps most notably autism spectrum disorder (Schmahmann, 2010). A related development was the advent of functional neuroimaging: Positron Emission Tomography (PET) and later functional Magnetic Resonance Imaging (fMRI), which demonstrated cerebellar activation in a broad range of cognitive tasks (e.g., language, attention, executive functions etc.) (Buckner, 2013). Structural neuroimaging now allows us to precisely localize cerebellar damage, linking it to neurocognitive or behavioural disruption and demonstrating localized differences in cerebellar structure in neurodevelopmental disorders and psychiatric conditions (Schmahmann, 2016).

These foundational studies and innovations in non-invasive human brain imaging have opened up a new field of research that aims to understand the function of the cerebellum and its contributions to normal behaviour and pathology. This line of inquiry is important from two perspectives: it informs our fundamental understanding of how the brain functions and is influenced by normal and pathological processes, and it highlights the cerebellum as a promising intervention target for a number of neurological and psychiatric conditions (Miterko et al., 2019). Within the broader context of studying the cerebellum, understanding its connectivity is of central importance. It is key to explaining its contributions to such a wide range of processes, including language, theory of mind, and executive functions, in addition to the well-established role in motor control (Schmahmann, 2019; Stoodley & Schmahmann, 2009; Strick et al., 2009). Unlike the cerebral cortex that shows strong regional specialization (Zilles & Amunts, 2010), cerebellar cortical cellular architecture is highly stereotyped (Schmahmann, 2019). This microstructural uniformity suggests that different areas of the cerebellar cortex perform similar operations (Schmahmann, 2019). Consequently, its diverse functional contributions are likely mediated by distinct patterns of connectivity with the cerebral cortex and other subcortical structures (Schmahmann, 2019). The study of cerebellar connectivity in non-human animals has a rich history (Schmahmann, 2016), yet our understanding of these circuits in the human brain remains

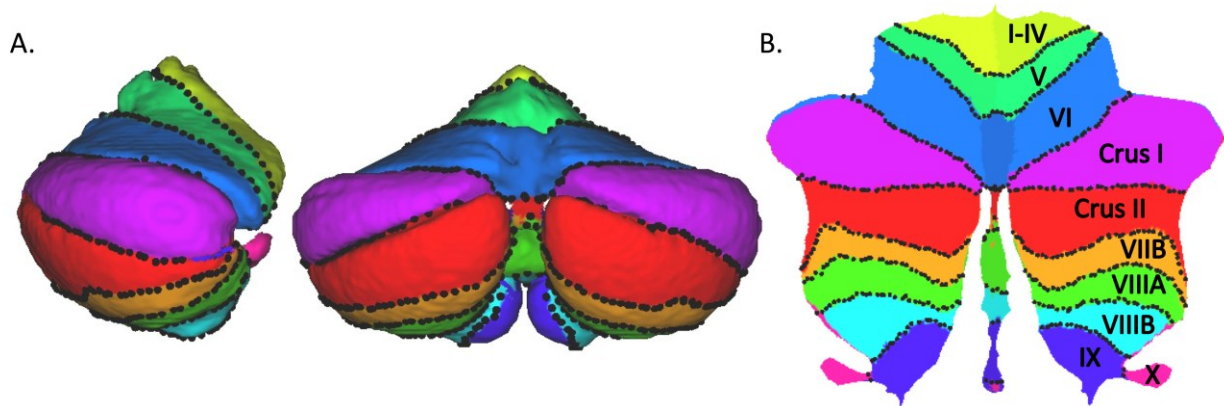
limited. The three studies in the present dissertation contribute to mapping human cerebellar connectivity and describing its underlying organizational principles. Bridging the gap between foundational non-human animal research and building on non-invasive imaging studies in humans, we employed both structural and functional neuroimaging to characterize cerebellar connectivity. The goal is to offer novel and complementary perspectives that advance our understanding of this brain structure's connectivity in humans and lay the foundation for future investigations into cerebellar connectivity and its involvement in health and disease.

## **1.2 Basic Cerebellar Anatomy and Models of Function**

To provide necessary context for the studies that follow, we start with an overview of the fundamentals of cerebellar anatomy and introduce the cerebellar microcircuit. We then present seminal models of cerebellar function, informed by its microcircuitry and anatomy, which originally emphasized its role in constructing and updating internal representations for motor control, but have since been extended to encompass non-motor functions.

### **1.2.1 Gross Anatomy of the Cerebellum**

The cerebellum, occupying approximately 10% of the brain's total volume, is situated in the posterior and inferior quadrant of the cranium, nestled in the posterior cranial fossa (Goodlett, 2008). It is comprised of two symmetric hemispheres and a narrow intermediate area running along its midline called the vermis (Roostaei et al., 2014). The cerebellar hemispheres consist of an outer layer of highly convoluted grey matter – the cerebellar cortex – and an inner core of white matter that is characterized by an intricate pattern of branching into the cerebellar cortical folia (Roostaei et al., 2014). The largest levels of subdivision of the cerebellar cortex are the anterior, posterior, and flocculonodular lobes whose boundaries are defined by the primary and posterolateral fissures, respectively (Roostaei et al., 2014). The cerebellar cortex may be further divided into ten lobules (see Figure 1.1), denoted by Roman numerals I-X (Roostaei et al., 2014). The anterior lobe is comprised of lobules I-V, the posterior lobe lobules VI-IX, and the flocculonodular lobe is lobule X (Stoodley & Schmahmann, 2018). Hemispheric portions of lobule VII can be further subdivided into Crus I, Crus II and lobule VII B (Tanabe et al., 2018). While the lobular designations are convenient and useful in offering a common language for comparing and contrasting findings across different studies, it should be noted that lobular boundaries only weakly correspond to functional boundaries (King et al., 2019).



**Figure 1.1.** Depictions of the cerebellar cortex colour-coded according to lobule designations. **A.** Three-dimensional reconstruction of the cerebellar cortex. On the left is a lateral (right) view and on the right is a posterior (back) view of the cerebellar cortex. **B.** A unfolded (flatmap) view of the cerebellar cortex with labeled lobules. The depicted three-dimensional and flatmap representations of the cerebellar cortex and the lobule parcellations are from the SUIT atlas (Diedrichsen, 2006; Diedrichsen & Zotow, 2015).

Cerebellar white matter is a complex agglomeration of efferent and afferent connections that link the cerebellum with the cerebral cortex, the brainstem, and the spinal cord (Goodlett, 2008). Embedded within the white matter of each cerebellar hemisphere are paired deep cerebellar nuclei that receive cerebellar cortical outputs but also contain connections with other structures like the pons and inferior olive (Goodlett, 2008). The largest and most laterally situated of the cerebellar nuclei is the dentate nucleus, medial to that are the interposed and fastigial nuclei (Goodlett, 2008). Information to and from the cerebellum is relayed through paired inferior, middle, and superior cerebellar peduncles, which also serve to physically connect the cerebellum to the brainstem (Goodlett, 2008). The inferior cerebellar peduncles are primarily comprised of fibres projecting from the inferior olivary nucleus, spinal cord, and vestibular nuclei to the cerebellar cortex (Stoodley & Schmahmann, 2018). They also contain a smaller number of projections from the cerebellar cortex to the spinal cord (Stoodley & Schmahmann, 2018). The cerebellar cortex receives cerebrocortical input via the pontine nuclei. Projections from the cerebral cortex synapse on the ipsilateral pontine nuclei, which send mossy fibre projections to the contralateral cerebellar cortex through the middle cerebral peduncles (Stoodley & Schmahmann, 2018). Some mossy fibres may form collateral branches that target the deep nuclei and a small number of ipsilateral pontocerebellar connections are also present (Stoodley & Schmahmann,

2018). The superior cerebellar peduncles are mostly comprised of fibres originating in the deep cerebellar nuclei and constitute the primary outputs of the cerebellar cortex (Stoodley & Schmahmann, 2018). These also contain some afferent fibres originating in the spinal cord, red nucleus, and superior colliculus – some of which project to the cerebellar cortex (Goodlett, 2008). Efferent projections synapse with an array of different subcortical targets but most prominently to the contralateral thalamus; which in turn conveys cerebellar outputs to the cerebral cortex (Stoodley & Schmahmann, 2018).

### 1.2.2 A Simplified Cerebellar Microcircuit

Unlike the cerebral cortex, whose cytoarchitecture features regional specialization (Zilles, 2018), the cytoarchitecture of the cerebellar cortex is largely uniform (Goodlett, 2008; Roostaei et al., 2014). The cerebellar cortex is comprised of three layers that are categorized based on their cellular composition and which each have different roles in cerebellar cortical processing. The innermost layer (adjacent to the white matter) is the granule cell layer, the middle is the Purkinje cell layer and the outermost is the molecular cell layer (Goodlett, 2008; Roostaei et al., 2014). Purkinje cells represent the sole output of the cerebellar cortex, inhibiting the deep cerebellar nuclei, and can be considered as a central point on which the cerebellar cortical circuit is organized. They receive excitatory input from two sources: climbing fibres originating from the inferior olive, and mossy fibres that primarily originate in the pontine nuclei, but also from the spinal cord and brain stem (Goodlett, 2008). A Purkinje cell receives strong excitatory input from a single climbing fibre, but an individual climbing fibre may innervate several Purkinje cells (Goodlett, 2008; Marr, 1969; Roostaei et al., 2014). Mossy fibres synapse with the granule cells in the granule cell layer. These are the smallest and most numerous neuron type in the brain (Ito, 2010). There is significant divergence in that a single mossy fibre will make contact with hundreds of granule cells, and a small amount of convergence in that four or five mossy fibres will contact a single granule cell (Ito, 2010). The extent of this divergence is functionally controlled by inhibitory Golgi cell interneurons (Ito, 2010). Individual granule cells give rise to an axon that enters the molecular layer and bifurcates to form parallel fibres that travel along the medial to lateral axis of the cerebellar cortex for 1 to 1.5mm in opposite directions, and excite several hundred Purkinje cells (Goodlett, 2008). A single Purkinje cell, in turn, with its characteristically large and fan shaped dendritic tree receives excitatory input from a massive number of parallel fibres (Ito, 2010). The cerebellar microcircuit also contains a number of different interneurons, in addition to the

aforementioned Golgi cells that modulate the activity of the circuit (Ito, 2010). This well-characterized cerebellar microcircuit forms the basis for cerebellar computations and its contribution to motor and non-motor processes. In addition, while our understanding of this circuit, notably the contribution of interneurons, continues to be refined, the fundamental principles hold and provide the conceptual foundation for views of cerebellar function.

### **1.2.3 Cerebellar Internal Models for Movement and Cognition**

The reconstruction of the basic circuitry of the cerebellum, along with its established role in motor control, led to the development of seminal models of cerebellar cortical computation by Marr (1969) and Albus (1971), which focused on its contribution to the learning and refinement of movement. A key point in both of these models is that the relationship between mossy fibre input and granule cell output allows for the encoding of a multitude of representations within the cerebellar cortex (Albus, 1989). Learning occurs at the level of synapses between parallel fibres and Purkinje cells, whose weights are modified in response to error signals conveyed by climbing fibres (Albus, 1989). A series of studies by Ito confirmed core predictions of these models demonstrating long-term depression at parallel-fibre Purkinje cell synapses when they were coactivated with climbing fibre input (Ito et al., 1982). Building on this work, Wolpert et al. (1998) and Kawato (1999) forwarded the notion that the cerebellum acquires, stores, and fine tunes internal models that are used for the execution of skilled movements. Inverse models translate a desired outcome into specific motor commands, while forward models predict the sensory consequences of those motor commands. The interaction between these models allows for the refinement and correction of movement (Kawato, 1999; Wolpert et al., 1998). These models are fine-tuned with experience, as suggested by Marr-Albus, allowing for highly trained movements to eventually be executed without the need for feedback from relevant body parts (Ito, 2008).

Informed by research with cerebellar patients, Ivry and Keele (1989) advanced a complementary perspective that emphasized the importance of the cerebellum for temporal processing. The cerebellar circuit is viewed as encoding precise time intervals, an essential component not only to the smooth and accurate execution of movement but for the coordination of cognitive processes. Although the authors do not explicitly refer to internal models, both forward and inverse models depend on the representation of temporal information. In the context of movement, forward models require accurate representations of when sensory events will occur,

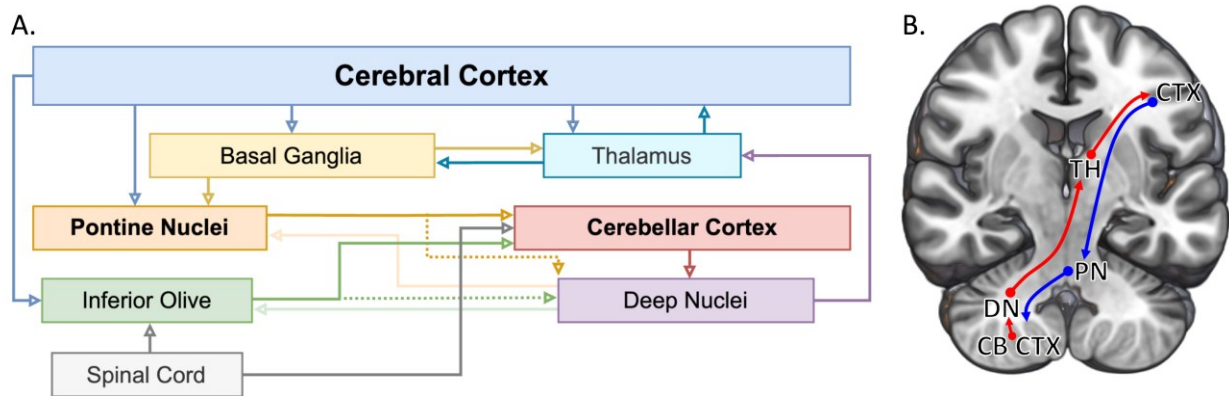
allowing for the anticipation of the consequences of motor commands. Inverse models, on the other hand, require the capacity to represent and generate precise temporal sequences of movements. The architecture of the cerebellar circuit is suited towards the representation of temporal information through conduction delays along parallel fibres – a concept first proposed by Braitenberg (1967). Taken together, these conceptual frameworks present the cerebellum as modulatory learning machine that forms internal representations, adapts them through experience, and fine tunes behavioural or cognitive output in response to specific task demands.

Based on the extensive cerebellar connections to and from higher-order association areas (reviewed in the subsequent section), in addition to some of the evidence discussed in the previous section (e.g., cerebellar lesions, fMRI) cerebellar internal models and timing functions are proposed to extend beyond motor behaviour and to the domain of mental activities (Ito, 2008). Given the homogeneity of cerebellar microcircuitry, the same principles underlying its contributions to motor control are also thought to apply to mental activities (Ito, 2008). Dynamic interactions between cerebral cortical areas, including top-down control areas in the frontal lobe, and cerebellar internal models have been proposed to contribute to virtually all cognitive processes (Ito, 2008). Understanding the nature of these interactions becomes fundamental to understanding the cerebellum's contribution to cognition and behaviour. While this has been approached from multiple different angles, understanding the substrate of corticocerebellar interactions – the wiring of corticocerebellar loops – is fundamental. This effort centres on key questions: What type of information from where in the brain is sent to the cerebellum? How is that information transformed along the different segments of the pathway? What is the nature and spatial distribution of cerebellar outputs? Our work focused on a subset of these questions, centering on the anatomical and functional organization of the downstream portion of the pathway.

### **1.3 Corticocerebellar Loops**

The study of cerebellar connectivity in non-human animal models represents the foundation of the work presented in this dissertation. In this section, we briefly introduce the methods that have been used to invasively investigate cerebellar connectivity in non-human animals, then continue with an overview of evidence regarding the organization of the main fibre systems projecting to and from the cerebellum. A simplified schematic of key afferent and efferent cerebellar pathways is presented in Figure 1.2. We emphasize the feedforward limb of the corticocerebellar pathway –

the corticopontine and pontocerebellar segments – as these represent the focus of the studies included in the dissertation. In addition to the corticocerebellar loops, the cerebellum also forms reciprocal connections with the basal ganglia and receives climbing fibre input from the inferior olive and mossy fibre input from other brainstem nuclei (Azizi, 2007; Bostan & Strick, 2018). While these connections have been characterized with neuronal tracing, this area is beyond the scope of this dissertation. We conclude this section with a synthesis of organizational principles governing these connections, setting the stage for their investigation in humans.



**Figure 1.2.** **A.** Simplified illustration of selected afferent and efferent pathways of the cerebellum. Collateral branches (e.g. pontine nuclei to deep nuclei) are indicated by dotted lines, feedback projections (e.g. deep nuclei to pontine nuclei) are indicated in pale colours. Nodes that are the subject of the presented studies are bolded. **B.** A coronal MRI slice showing the key elements and decussations of corticocerebellar loops. CTX = Cerebral Cortex, PN = Pontine Nuclei, CB CTX = Cerebellar Cortex, DN = Deep Cerebellar Nuclei, TH = Thalamus. The blue lines indicate the downstream, feedforward limb, whereas red lines indicate the upstream, feedback limb.

### 1.3.1 Lesion Studies and Neuronal Tract-Tracing

Much of our knowledge regarding the organization of corticocerebellar connectivity comes from invasive research conducted in animals (Schmahmann, 2016). Earlier studies of connectivity (e.g., Brodal, 1978; Wiesendanger et al., 1979) involved lesioning specific areas of the brain and identifying areas of degeneration upstream or downstream to that lesion (Schmahmann, 2016). In the early 1970s, neuronal tract-tracing with horseradish peroxidase was introduced, ushering a new era in the study of brain connectivity (Lanciego & Wouterlood, 2020). Since then, novel tracer molecules with distinct properties have been developed but the general procedure remains unchanged: A tracer molecule is injected into an area of the brain of the experimental animal, the molecule is taken up by neurons in the target area and is transported along the length of its axons

in anterograde and/or retrograde fashion (Lanciego & Wouterlood, 2020). After a delay period to allow transport of the tracer, the animal is euthanized and the presence and distribution of the tracer molecule is mapped with serial sectioning and histological techniques (Lanciego & Wouterlood, 2020). Because of the evolutionary proximity of non-human primates, we prioritize foundational studies conducted in these species in the following sections. While non-human studies are useful in elucidating general organizational principles of corticocerebellar connectivity, we do not know how well these findings correspond with the organization of the human brain, which has undergone significant evolutionary changes in brain structure and functional organization (Balsters et al., 2010; Buckner & Krienen, 2013; Rilling, 2014). For a number of reasons that are beyond the scope of this dissertation, tracing studies in non-human primates have also become increasingly rare (Hutchison & Everling, 2012). More recent studies conducted in rodents, leveraging innovations in neuronal tracing, are less applicable to our understanding of macro-scale organizational patterns in humans. Nonetheless, key studies (e.g., Biswas et al., 2019; Leergaard & Bjaalie, 2007) have proven valuable in illustrating the behaviour of specific neurons and neuronal populations within the corticocerebellar pathway.

### **1.3.2 The Corticopontine Projection**

The first segment of downstream connections between the cerebral cortex and the cerebellum is the corticopontine projection formed by layer V pyramidal neurons (Glickstein & Doron, 2008). The corticopontine projection is one of the most significant fibre systems in the human brain, and it is comprised of approximately 40 million fibres (Schwarz & Thier, 1999). The invasive tract tracing research has revealed both the extent of this pathway (i.e., which cortical regions contribute to it) and the principles that appear to govern its spatial organization.

The foundational studies of Brodal (1978) and Wiesendanger and colleagues (1979) in the macaque involved lesioning different areas of the cerebral cortex and identifying areas of neuronal degeneration in the pons. Both studies revealed an organization characterized by a pattern of both divergence and convergence. Divergence in that a small area of the cerebral cortex may project to several small, circumscribed areas or patches within the pons. Convergence in that distant cortical sites may project to nearby sites in the pons (although likely not to the same individual neurons). Brodal (1978) likened the general organization of corticopontine terminals to layers of an onion, with primary motor and somatosensory inputs forming the core, more rostral frontal areas

projecting more medially relative to this core, and the more caudal parietal and occipital areas projecting more laterally in a somewhat concentric pattern. While these foundational lesion studies were important in suggesting the pattern of organization of corticopontine terminals within the pons, Glickstein and colleagues (1985) provided evidence concerning the extent and distribution of the corticopontine projection. They labeled the entire pons with a retrograde tracer and identified labeled pontine-projecting neurons in the cerebral cortex. Corticopontine projections originated in approximately half of the cerebral cortex, spanning from the cingulate cortex medially to the sylvian fissure laterally, and from parietooccipital areas caudally to prefrontal areas rostrally. The densest projections were found to arise from primary motor and pre-motor areas. In contrast, primary sensory areas, most of the ventral temporal lobe, and the most rostral areas of the frontal lobe did not appear to contribute. These studies provided foundational knowledge concerning this fibre system that continues to be influential. Subsequent research, using novel methods and with different perspectives of cerebellar contributions has expanded on and refined these findings.

A series of comprehensive studies (Schmahmann et al., 2004; Schmahmann & Pandya, 1989, 1991, 1993, 1995, 1997a), used anterograde tract tracing from different cerebrocortical areas and yielded a wealth of information regarding the organization of the corticopontine pathway in non-human primates. Contrary to many of the prior studies that presupposed a primarily motor function to the cerebellum, these studies were informed by the perspective that there were putative anatomical substrates underlying the cerebellum's suspected contributions to higher cognitive function (Schmahmann, 2010). We summarize key findings from this series of studies. Parietal corticopontine projections terminate largely in lateral and central portions of the pons, with a differential pattern of terminations for projections originating in different parietal association areas (Schmahmann & Pandya, 1989). Temporopontine projections originate mostly from the upper bank of the superior temporal sulcus, and to a lesser extent the superior temporal gyrus (Schmahmann & Pandya, 1991). On the whole these projections terminate preferentially in dorsolateral areas in the pons, with multimodal association areas of the superior temporal sulcus and the auditory association areas of the superior temporal gyrus showing differential patterns in their pontine projections. More inferior temporal regions that are more interlinked with the visual system do not appear to contribute to the corticopontine projection. Projections from visual cortex originate in dorsal stream areas that form parts of the “where” stream”, with no projections originating in ventral stream areas that are more involved in object recognition (the “what” stream)

(Schmahmann & Pandya, 1993). These largely terminate in lateral portions of the pons, again with differential terminations of projections originating in different cortical areas. Prefrontal projections originate in dorsolateral, ventrolateral, medial, and polar areas and terminate preferentially in the medial half of the pons (Schmahmann & Pandya, 1997a). Similar to the other projections described, these are topographically organized such that different frontal areas showing distinct patterns of termination within the medial pons. There is no evidence for pontine projections originating in orbitofrontal and ventral areas. The most recent of this series of studies performed tracing from the supplementary motor area (SMA) and different areas along the primary motor cortex (Schmahmann et al., 2004b). Motor areas project to the caudal half of the pons with a preservation of somatotopy. The SMA was found to project the most medially of the motor regions, followed by a medial-to-lateral pattern of terminations from the face, hand, trunk, and leg/foot representations, which were found to be interdigitated rather than entirely segregated. Together these studies provide the most detailed description of the corticopontine projection in non-human primates to date, with a summary for most these studies provided by Schmahmann and Pandya (1997b). Taken as a whole, they demonstrate a complex but highly organized topography in this pathway that contains important contributions from a range of cortical association areas. Spatial relationships present in the cerebral cortex are generally conserved along this pathway, while also exhibiting patterns of convergence and divergence.

### **1.3.3 The Pontocerebellar Projection**

Pontocerebellar mossy fibres originate in the basilar pontine nuclei and reticulotegmental nuclei, the former being the principal recipients of corticopontine fibres (Cicirata et al., 2005). There are approximately half as many pontocerebellar fibres as there are corticopontine fibres (Schwarz & Thier, 1999). The majority of pontocerebellar fibres cross to the contralateral cerebellar hemisphere, but there are a smaller number of ipsilateral connections (Brodal, 1979). Additionally, a small number of corticopontine fibres form collaterals to the cerebellar nuclei, which in turn projects a small number of fibres back to the pons (Schwarz & Thier, 1999). Compared to the corticopontine projection, the pontocerebellar projection has been studied less extensively in nonhuman primates (Schmahmann & Pandya, 1997b). Foundational studies by Brodal (1979, 1982) used a retrograde tracer injected into different sites in the macaque cerebellum to identify the origin of pontocerebellar fibres within the pons. Similar to the corticopontine findings, tracer injections in different cerebellar sites results in patchy distributions of labeled cell clusters within

the pons. A general organizational principle found in these studies was that the anterior lobe – generally associated with motor function – receives projections predominantly from caudal areas of the contralateral pons. The posterior lobe, large portions of which are associated to non-motor function, receives projections from more rostral areas. Similar to the corticopontine projection, the pontocerebellar projection shows a high degree of both divergence and convergence: A small area within the pons may project to different areas of the cerebellum, with single mossy fibres potentially branching to target multiple cerebellar lobules (Biswas et al., 2019; Schmahmann & Pandya, 1997b; Wu et al., 2023). Conversely from the point of view of convergence, discrete and separate areas of the pons may project onto confined areas within the same cerebellar lobules (Schmahmann & Pandya, 1997b; Wu et al., 2023).

#### **1.3.4 Closing the Loop – Corticonuclear, Nucleothalamic and Thalamocortical Projections**

Cerebellar Purkinje cells, the unique output of the cerebellar cortex, send inhibitory projections to the deep cerebellar nuclei, which in turn send excitatory outputs to the thalamus in addition to a complex array of different targets including the pontine nuclei and the inferior olive (Chan-Palay, 1977; Schmahmann & Pandya, 1997b). The classic view holds that nucleothalamic projections are confined to motor nuclei of the thalamus, however, there is now substantial evidence that much of the thalamus receives cerebellar input (Schmahmann & Pandya, 1997b). Building on tracing work mapping connections of the motor system in non-human primates (e.g., Schell & Strick, 1984; Strick, 1985), Middleton and Strick (1994) were the first to demonstrate multisynaptic cerebellar projections to prefrontal cortical areas. A series of follow-up tracing studies focused on precisely mapping the feedback limb of the corticocerebellar circuit (Clower et al., 2001; Dum & Strick, 2003; Hoover & Strick, 1999; Kelly & Strick, 2003; Middleton & Strick, 2001). The authors were motivated to document anatomical substrates of cerebellar contributions to motor and non-motor processes. This series of studies has provided some of the most comprehensive evidence regarding the organization of this portion of the pathway in non-human primates. They documented that cerebrocortical projections originating in the dentate nucleus and relayed by the thalamus were not confined to sensorimotor cortical areas, as previously assumed by motor centric views of the cerebellar circuit. Rather, they found projections from the dentate nucleus to a number of prefrontal and posterior parietal association areas (Clower et al., 2001; Middleton & Strick, 2001). These projections were topographically organized in that different areas of the dentate nucleus projected to different areas of the thalamus that then projected to distinct cerebrocortical sites. A broad

subdivision to this topographic organization appears to be the segregation of dentate nucleus into motor and non-motor zones: The dorsal dentate preferentially projecting to motor areas of the cerebral cortex and the ventral dentate to non-motor areas (Dum & Strick, 2003). The motor projections show preservation of body somatotopy, indicating that this organization is maintained at some level across the different segments of the entire corticocerebellar circuit (Hoover & Strick, 1999). This series of studies also provided strong evidence that areas that contribute to the corticopontine projection are also the recipients of cerebellar inputs (Dum & Strick, 2003), implying that feedback projections influence the same cerebrocortical areas that contribute to the corticopontine segment. Together, these findings suggest that the system is organized in a closed-loop fashion, enabling spatially specific cerebellar modulation of cerebrocortical activity.

### **1.3.5 Insights from the Tracing Literature**

There are a few key insights that can be drawn from the lesion and tract tracing literature; these form the basis of our understanding of pathways connecting to and from the cerebellum: 1) A degree of topographic organization is preserved across the different segments of this pathway. 2) Despite the maintenance of some level of topographic organization, there is also a considerable degree of both divergence and convergence of projections that may serve to alter the way information is represented in the different nodes of the pathway. This pattern of convergence and divergence may lend itself to the combination of information from different cortical sites, and for the processing of information originating from specific cerebrocortical areas in distinct parts of the cerebellum. 3) The pathway forms a closed loop, with the cerebral cortical areas sending projections to the cerebellum via the pons also receiving feedback projections from the cerebellum through the cerebellar nuclei and thalamus. 4) The fact that much of the cerebral cortex, including association areas, sends inputs to and receives outputs from the cerebellum implies that the pathway is not limited to the control of movement. The wealth of anatomical investigations of cerebellar connectivity provides the fundamental anchoring of the work that is presented in this thesis, where we seek to expand on our knowledge to address the gap in understanding the organization of corticocerebellar loops in humans.

## **1.4 Investigating Cerebellar Connectivity in Humans**

Non-invasive investigations of cerebellar connectivity in humans are made possible with magnetic resonance imaging-based techniques. Two distinct but complementary approaches are commonly

used: diffusion MRI tractography that enables the reconstruction of white matter pathways and resting-state functional MRI that offers a proxy measure of connectivity by evaluating temporally coordinated activity in different brain areas. These approaches form the methodological basis of the studies presented in this dissertation. In the following sections we briefly introduce these techniques, present seminal studies, and identify the limitations in prior work and the specific gaps in the literature that our work aims to address.

### 1.4.1 Diffusion MRI tractography: Principles & Methodological Challenges

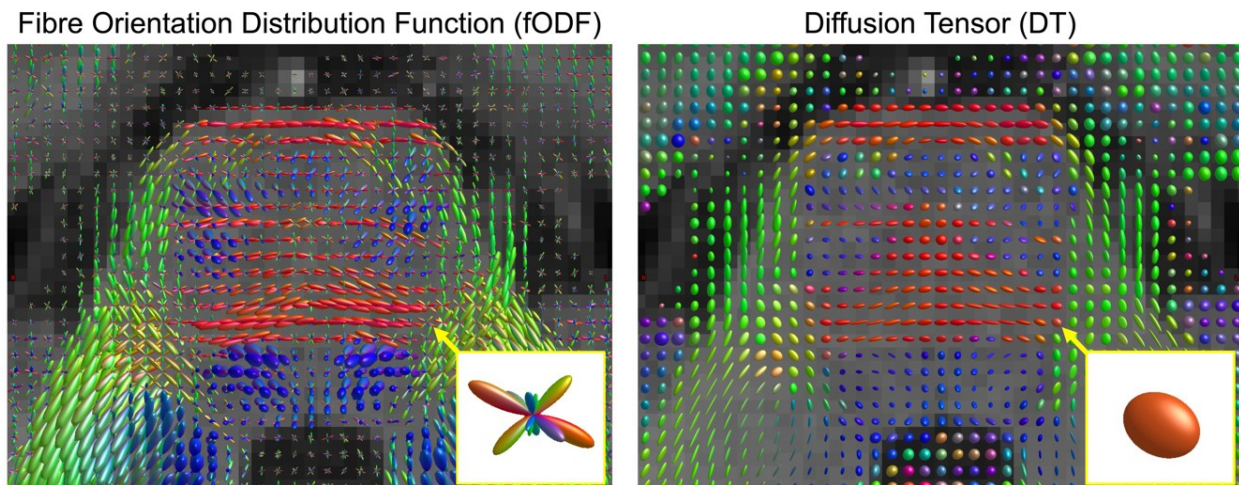
There is extensive anatomical research conducted in non-human primates and other animal species but our understanding of corticocerebellar connectivity in humans, and how it relates to animal models, remains limited. While the fundamental organizational principles outlined above are likely conserved across primate species, there have been important changes to brain structure and organization over the course of human evolution that mediate a complex array of unique behaviours and abilities, such as language, social cognition and tool use (Balsters et al., 2010). The relative expansion of prefrontal cortex in humans, for instance, is accompanied by a concomitant expansion of the cerebellar cortical areas involved in higher order cognitive function like Crus I and Crus II (Balsters et al., 2010). In living humans, *the only* way of investigating structural connectivity is with diffusion MRI tractography (Rheault et al., 2020).

Diffusion-Weighted MRI (dMRI) or Diffusion-Weighted Imaging (DWI) is sensitive to the displacement of water molecules within brain tissue (Bullock et al., 2022). Water displacement is constrained by the properties of underlying tissue (Bullock et al., 2022). Notably, in the brain's white matter, diffusion is largely restricted along the long axis of myelinated axons (Johansen-Berg & Rushworth, 2009). Multiple diffusion weighted images are acquired, each sensitive to diffusion along a different direction in space (Johansen-Berg & Rushworth, 2009). Each white matter voxel contains thousands of axons, thus the signal represents a summary of underlying fibre populations, rather than reflecting the behaviour of individual axons (Jeurissen et al., 2019; Sotiropoulos & Zalesky, 2019). Using these data, different mathematical models are used to extract the direction/s of diffusion in each brain voxel (Johansen-Berg & Rushworth, 2009). One of the most prevalent models is the diffusion tensor that models voxel-wise diffusion as an ellipsoid (i.e., the diffusion tensor) (Jeurissen et al., 2019). In cerebrospinal fluid where the direction of diffusion is not constrained, the diffusion tensor would be spherical (indicating equal diffusion along all

directions) (Johansen-Berg & Rushworth, 2009). In an area of coherently oriented fibres, such as the middle of the corpus callosum, the diffusion tensor would be elongated and oriented in the direction of the bundle (Johansen-Berg & Rushworth, 2009). The chief limitation of this model presents itself in areas with multiple crossing fibre populations (O'Donnell & Westin, 2011), where diffusion would be represented erroneously as being roughly spherical (See Figure 1.3). To help overcome this issue, “higher-order” methods of modelling diffusion have been developed to leverage DWI data acquired with high angular resolution (i.e., characterized by acquisitions of a large number of different directions to more accurately model the 3D distribution of the diffusion profile in each voxel) (Jeurissen et al., 2019). These models aim to encapsulate the directions and relative contributions of different fibre bundles within a voxel (Jeurissen et al., 2019). The most used of these methods, constrained spherical deconvolution (CSD), estimates the fibre orientation distribution function (fODF) that models the relative contribution of underlying fibre populations as a continuous distribution over all directions in a sphere (Tournier et al., 2007). Unlike the ellipsoid diffusion tensor, the fODF includes multiple peaks, each representing putative fibre orientations (Dell'Acqua & Tournier, 2019). Figure 1.3 depicts a comparison between DWI data fit with fODF and diffusion tensor models in a region of the pons. It clearly illustrates the added complexity and nuance afforded fODFs in regions with complex and intersecting pathways.

Voxel-wise models of fibre orientations are used as the inputs for tractography, where the individual pieces are linked together to form streamlines that represent putative anatomical connections (Bullock et al., 2022). There are a number of different methodological approaches to reconstructing these connections with tractography, but a major distinction is made between deterministic and probabilistic methods (Sarwar et al., 2019). With deterministic tractography, at each voxel a streamline may only have a single fixed direction (Sarwar et al., 2019). In contrast, probabilistic tractography estimates a probability distribution of possible fibre trajectories at each voxel (Sarwar et al., 2019). The approach produces a probabilistic map of likely fibre trajectories, as opposed to the single fixed path obtained using the deterministic approach. Probabilistic tractography is better suited at capturing the uncertainty in fibre orientations, particularly in areas with greater complexity in underlying fibre populations (Jeurissen et al., 2019). This comes at the expense of greater computational demands, and the potential for the generation of spurious connections (Sarwar et al., 2019). Aside from the broad choice of deterministic or probabilistic tractography algorithms, there are a number of different parameters involved in tractography.

Briefly, these include initiation and termination criteria (where to start and stop tracking), step size (the distance between successive points on a streamline), and angular threshold (how sharply a streamline can turn at each step) (Tournier et al., 2012). Anatomical constraints may also be applied that specify where streamlines are initiated and terminated, and restrict streamlines to biologically plausible trajectories (Smith et al., 2012). In the current thesis, we have used constrained spherical deconvolution along with probabilistic tractography and anatomical constraints to accurately reconstruct corticopontine and pontocerebellar connections.



**Figure 1.3.** Axial MRI slice near mid-pons, depicting fibre orientation distribution functions (fODFs) on the left and diffusion tensors on the right. In a region of crossing fibres (in the bottom right corner of the pons) the diffusion tensor looks spherical and fails to resolve distinct directions, whereas the fODF captures multiple potential fibre directions.

While the focus of this dissertation is the use dMRI to performing tractography, the method has other widely used applications. In addition to allowing us to infer information about the direction of fibre populations that can be used in tractography, local models of diffusion (like the diffusion tensor and the fODF) can also be used to estimate microstructural properties of tissue, like axonal density and myelination (Assaf et al., 2019). In other words, at each voxel, we are able to quantify the microstructural properties of underlying tissue. This is a powerful and informative technique, and there is a substantial body of research investigating how white matter microstructural properties are related to normal development, pathology, and specific interventions (Assaf et al., 2019; Le Bihan, 2003). In research, tractography is often used in conjunction with the extraction of microstructural metrics. A pathway of interest will be reconstructed with

tractography, and its microstructural properties quantified and then compared across different populations of interest, within individuals across time, or in relation to specific interventions (Assaf et al., 2019). One of the aims of our tractography studies was to provide accurate reconstructions of corticocerebellar pathways that could support this type of research.

Despite the massive appeal of these methods, there are important limitations both at the level of the estimating local fibre orientations and the reconstruction pathways with tractography. The general biases and methods for mitigating their effects have been reviewed comprehensively by others (e.g., Girard et al., 2014; Jeurissen et al., 2019; Rheault et al., 2020). There are specific limitations that are especially pertinent to the study of cerebellar connectivity, due to its small size and the complexity of its connectional anatomy; these challenges are reviewed in detail by Lundell & Steele (2024). First, there is the central issue of establishing of initiation and termination criteria for fibre tracking. In the case of the pons, dMRI data at typical resolutions does not clearly distinguish gray matter nuclei from surrounding white matter. It is therefore not feasible to accurately map out end points of streamlines entering this structure, or the start points of exiting streamlines (a critical difference with neuronal tract-tracing). Second, there is also a bottleneck effect, where there is a loss of spatial specificity when fibres coalesce on a circumscribed area and then fan out again – as might be the case if one were to track from the cerebral cortex to the cerebellum (Rheault et al., 2020). Third, there are length and curvature biases. Tractography will always take the path of least resistance, leading to an overrepresentation of streamlines with shorter and straighter trajectories in reconstructed connections (Jeurissen et al., 2019). In the example of the corticopontine pathway, this will mean an abundance of streamlines originating in sensorimotor cortex, and a lack of streamlines from frontal and parietal areas with longer and more complex trajectories. Fourth, there is also the fundamental issue of crossing fibres that is to some extent mitigated by the use of more complex higher-order approaches to modeling local diffusion (e.g., fODFs) but nonetheless affecting spatial specificity of fibre tracking (Rheault et al., 2020; Schilling et al., 2019). This issue is particularly salient in the cerebellar white matter, which is a complex agglomeration of overlapping and intersecting efferent and afferent connections (Lundell & Steele, 2024). In the context of these limitations, an important point to stress is that tractography is not equivalent or directly comparable to neuronal tracing that is a direct measure of anatomical connectivity (Johansen-Berg & Rushworth, 2009). It does, however, provide a reproducible and

robust systems-level representation of anatomical connectivity when limitations are appropriately considered (Rheault et al., 2020).

### **1.4.2 The Limitations of Existing Research**

There have been a handful of attempts at reconstructing cerebellar connectivity with diffusion MRI and tractography. Certain studies (e.g., Dell'Acqua et al., 2013; Steele et al., 2017; Takahashi et al., 2013) have focused on individual segments of the pathway while others have ambitiously reconstructed the multisynaptic cortico-ponto-cerebellar pathway (e.g., Kamali et al., 2010; Palesi et al., 2017). Reconstruction of the entire feedforward limb in one step results in a compounding of the aforementioned tractography biases, resulting in a lack of spatial precision of resulting connections. This type of approach does not lend itself to addressing questions regarding topographic organization. Specifically, it does not inform our understanding of what regions of the cerebral cortex map onto which areas of the pons and the cerebellar cortex. These works highlight a common problem with dMRI tractography research: while it is relatively trivial to reconstruct connections between any two brain regions; the bigger challenge lies in reproducing connections that are biologically meaningful and contribute to our understanding of the pathways in question. dMRI tractography is a powerful technique, but one that must be used cautiously and interpreted carefully. Reconstructing cerebellar connectivity with dMRI tractography requires a conservative approach that is mindful of the important limitations and biases inherent to the method and is informed by neuroanatomy (Rheault et al., 2020). It follows that we favour an approach that involves isolating the individual segments of the feedforward limb – specifically the corticopontine and pontocerebellar segments – and reconstructing them individually. These pathways are yet to be systemically characterized in humans, and these gaps in the literature are systematically addressed in the manuscripts presented in Chapters 2 and 3.

### **1.4.3 Functional MRI: Principles and Landmark Studies**

As alluded to earlier, functional MRI (fMRI) research has been central to reshaping perspectives towards the cerebellum in the field of neuroscience. Briefly, fMRI is an indirect measure of neuronal activity and is based on the principle of detecting changes in blood oxygenation (the Blood Oxygen Level Dependent or BOLD contrast) that accompany activity in large populations of neurons (Glover, 2011). fMRI research has demonstrated cerebellar participation in a wide range of tasks and has shown cerebellar activation to be organized topographically such that

different types of tasks engage different areas of the cerebellum (e.g., King et al., 2019; Stoodley et al., 2012). Specifically, different areas of the cerebellum are recruited in motor and non-motor tasks, a broad subdivision in its functional organization (King et al., 2019; Stoodley et al., 2012). In their seminal study, King et al. (2019) investigated the functional organization of the cerebellum using a comprehensive task battery that encompass functions such as motor control, working memory, language and theory of mind. They found distinct zones of the cerebellum associated with different functions that only weakly corresponded to lobular boundaries. The broad subdivision between motor and non-motor areas appears to mirror the anatomic organization revealed by tracing studies in non-human primates (Brodal, 1979). By leveraging task-based fMRI, however, this study revealed a complex functional organization that could not be inferred by the non-human primate anatomical data alone. Besides from the obvious species differences, tracing reflects the presence of anatomical connections, but not how these inputs are functionally integrated or dissociated according to task demands.

Resting-state functional connectivity is a related technique that is based on quantifying the temporal relationships between spontaneous fluctuations in BOLD signal in different areas in the brain when the individual is not engaged in a particular task (van den Heuvel & Hulshoff Pol, 2010). The degree of overlap between BOLD signal fluctuations between different regions – their temporal concordance as measured with Pearson’s correlation – is reflective of the strength of functional connectivity between the regions in question (van den Heuvel & Hulshoff Pol, 2010). Resting-state connectivity data can be used in a variety of different ways to address different scientific questions. For instance, one can examine the strength of connectivity between specific brain regions and relate this to group membership, a particular pathological process, or an intervention (Lee et al., 2013; Smitha et al., 2017). Resting-state connectivity data can also be used to decompose whole brain activity into networks – assemblies of brain regions that are functionally interlinked and are thought to mediate distinct cognitive processes (Smitha et al., 2017). Resting-state networks are highly replicable and include the default-mode network, dorsal and ventral attention networks, and somatomotor network (Damoiseaux et al., 2006; Yeo et al., 2011). Given the polysynaptic nature of the corticocerebellar loops, and the challenges with reconstructing these connections with dMRI tractography, resting-state fMRI (rsfMRI) studies provide a complementary perspective that has deepened our understanding of cerebellar connectivity.

Buckner and colleagues' (2011) study stands as significant contribution to our understanding of the functional organization and connectivity of human cerebellum. They used functional connectivity to replicate the well-established double somatotopic representations in the cerebellar cortex previously demonstrated with task-based paradigms (Grodd et al., 2001), and in seminal animal electrophysiology research (Snider & Eldred, 1952). They mapped connectivity between the entire cerebellar cortex and the cerebral cortex, demonstrating that the cerebellar cortex contains multiple topographically organized representations of cerebrocortical networks. Building on this foundational work, Guell and colleagues (2018) used novel connectivity gradient methods to demonstrate overlapping modes of functional organization in the cerebellar cortex. Unlike more standard functional connectivity analyses that reflect pairwise correlations between brain regions or assign discrete network memberships to regions (like that of Buckner and colleagues (2011)), the gradient approach captures more continuous or nuanced patterns of functional connectivity. Gradients reflect smooth transitions in the connectivity profiles of different brain areas. Moreover, this approach allows for the extraction of overlapping, or superimposed modes of organization, each reflecting different patterns of functional organization. In the cerebellar cortex, there is a principal connectivity gradient organized with cerebellar motor regions on one end, and default mode regions on the other. This organizational pattern is analogous to that observed in the cerebral cortex (Margulies et al., 2016). A second, superimposed cerebellar gradient distinguishes cerebellar areas associated with task-focused networks (e.g., attention) and those connected to task-unfocused networks, such as default mode network. This study demonstrated the value of gradient based approaches in capturing continuous transitions in connectivity and superimposed principles of organization within the cerebellar cortex – features that are inaccessible to more traditional parcellation based approaches.

### **1.4.4 Pontine Functional Organization**

While the functional organization cerebellar cortex has been the focus of numerous studies, the pons – the key intermediary in the downstream pathway – has remained largely unstudied from a functional perspective. Leveraging rsfMRI to questions of pontine functional organization, allows us to build on our dMRI tractography studies. It enables us to investigate the organization of pons based on its connectivity both from the cerebral cortex and to the cerebellar cortex. Our first two studies leveraged dMRI tractography to document the structural organization of the corticopontine and pontocerebellar segments separately. While this was necessary from a methodological point

of view, it undercut the integrated nature of this system. A bigger question that may be addressed with the functional approach, is to start to investigate the function of the pons in the downstream pathway. There are multiple lines of evidence – elegantly reviewed by Schwarz & Their (1999) – that form the basis of a compelling argument that the pons is not a mere relay that faithfully conveys corticopontine inputs to the cerebellum. At the very minimum, there appears to be a level of integration or consolidation occurring at the level of the pons, the nature of which is inaccessible to structural techniques. A gradient based approach to the functional connectivity of the pons allows for the investigation of superimposed organizational motifs within this structure. These organizational motifs may reflect the distinct ways in which various sources of information are functionally integrated or differentiated within the pons. In Chapter 4, we pursued these lines of inquiry with a study that reconstructed functional gradients of the pons based on its connectivity with the cerebral and cerebellar cortices.

## **1.5 The Present Work**

Our work starts with two dMRI tractography studies in live humans. In Chapter 2 we present a study that mapped the connectivity of the pontocerebellar segment of the feedforward limb. Specifically, we reconstructed probabilistic connections between the pons and individual cerebellar lobules in a large group of adults and documented topographic patterns of organization. In Chapter 3 we then took on the reconstruction of the corticopontine segment. This represented the larger methodological challenge given the extent and complexity of this pathway. Here we built on lessons from our first study and used data-driven spectral embedding methods to generate connectivity gradients that represent the different ways the cerebral cortex maps onto the pons. We performed the primary analysis in a group of healthy adults and validated our findings in a high-resolution postmortem brain sample. Together, these two tractography studies represent the most comprehensive attempts to date to assess these pathways with dMRI tractography in humans. In our final study, presented in Chapter 4, we aimed to complement and build upon our anatomical studies. We reconstructed functional connectivity gradients of the pons based on its connectivity with the cerebral and cerebellar cortices. This study provides novel insights into the function organization of the pons in humans and sheds light on its potential role in the downstream pathway.

# Chapter 2. Mapping Pontocerebellar Connectivity with Diffusion MRI

## 2.1 Abstract

The cerebellum's involvement in cognitive, affective and motor functions is mediated by connections to different regions of the cerebral cortex. A distinctive feature of cortico-cerebellar loops that has been demonstrated in the animal work is a topographic organization that is preserved across its corticopontine, pontocerebellar, and cerebello-thalmo-cortical segments. Here we used tractography derived from diffusion imaging data to characterize the connections between the pons and the individual lobules of the cerebellum and generate a parcellation of the pons and middle cerebral peduncle based on its pattern of connectivity. We identified a rostral to caudal gradient in the pons, similar to that observed in the animal work, such that rostral regions were preferentially connected to cerebellar lobules involved in non-motor function, and caudal regions with cerebellar motor regions. These findings advance our fundamental understanding of the cerebellum, and the parcellations we generated provide context for future research into the pontocerebellar tract's involvement in health and disease.

## 2.2 Introduction

The cerebellum, a structure containing approximately as many neurons as the whole cerebral cortex, has historically been considered as being chiefly involved in motor processes. Recent advances have led to a paradigm shift in our conceptualization of the cerebellum, and we are just beginning to appreciate its involvement in a breadth of higher cognitive processes (King et al., 2019; Schmahmann, 2019; Strick et al., 2009). Unlike the cortex, the cellular architecture of the cerebellum is highly invariant, this implies that it is performing similar operations albeit on different inputs with different outputs (Schmahmann, 2019). An understanding of the structural connectivity of the cerebellum is therefore essential to understanding its contribution to different cognitive processes and to the effects of cerebellar syndromes. Damage to different parts of the cerebellum and associated white matter pathways will produce specific patterns of impairments in motor and non-motor functioning (Rapoport et al., 2000). While the white matter connectivity of the cerebellum has been characterized with tract-tracing studies in non-human primates (e.g.,

Brodal, 1979; Schmahmann et al., 2004b; Schmahmann & Pandya, 1997a), our knowledge of the more fine-grained aspects of its connectivity in humans is still limited. Diffusion weighted imaging, particularly with recent methodological advancements provides an opportunity to investigate the white matter connectivity of the cerebellum in-vivo (Granziera et al., 2009; Salamon et al., 2007; Steele et al., 2017).

The cerebellar cortex receives cortical inputs via the pons and in turn projects back to the cerebral cortex predominantly via the dentate nucleus and the thalamus. These corticocerebellar loops are comprised of parallel subcircuits linking spatially and functional distinct areas of the cortex and cerebellum (Schmahmann et al., 2019). The cerebellar cortex is comprised of ten lobules based on its gross anatomy (Schmahmann et al., 1999): the anterior lobe (lobules I through V and parts of VI) in addition to lobule VIII receive projections primarily from motor regions of the cortex, in addition to projections from the spinal cord. Conversely, the remainder of lobule VI, lobules VIIA (Crus I and Crus II) and VIIB, are connected primarily to non-sensory/motor frontal and parietal association areas of the cortex (Schmahmann, 2019). Previous work has established that both the pons and the dentate nucleus exhibit topographic patterns of organization based on their afferent connections (Schmahmann & Pandya, 1997a; Steele et al., 2017).

Studying corticocerebellar connectivity with diffusion tractography presents some unique challenges. First, corticocerebellar connections are polysynaptic and it is unclear how effective diffusion tractography is at resolving these polysynaptic connections. There are also a large number of intersecting fibres in the cerebellum and brainstem, meaning that the diffusion tensor model (in which only one fibre orientation is assumed per voxel) is inadequate for studying white matter architecture in this region (Takahashi et al., 2013). High-angular resolution diffusion imaging (HARDI) data which allows for the definition of fibre orientation distribution functions (fODFs) with constrained spherical deconvolution improves the characterization of these fibre populations and affords new opportunities for studying cerebellar connectivity (Dell'Acqua et al., 2013; Tournier et al., 2007; Steele et al., 2017).

Our objective with the current study was to address the gap that currently exists in the literature and systematically characterize the white matter connectivity between the pons and lobules of the cerebellum. We first performed probabilistic tractography on the connections between the cerebellar lobules and the pons which travel through the middle cerebellar peduncle

(MCP). Our goal was to characterize their spatial distribution, and subsequently generated parcellations of the pons and MCP based on this connectivity. The current findings contribute to our understanding of the organization of cerebellar white matter connectivity in humans and may help contextualize clinical findings wherein localized damage to pontocerebellar white matter may be associated with particular impairments.

## **2.3 Materials and Methods**

### **2.3.1 Participants**

100 unrelated participants (50 females, average age = 29) were randomly selected from a dataset used in a prior study (Steele & Chakravarty, 2018) that used structural imaging data from the Human Connectome Project open-access dataset ([www.humanconnectome.org](http://www.humanconnectome.org)) (Glasser et al., 2016; Steele & Chakravarty, 2018; Van Essen et al., 2012). Written informed consent was obtained from each participant, and data was used in compliance with ethical guidelines of Concordia University and The Human Connectome Project. 100 subjects were selected to balance between the computational demands of the tractography with the requirement of a large and representative sample. Structural imaging data were acquired on a 3T Siemens Connectom Skyra scanner: T1w (0.7 mm iso, TI/TE/TR = 1000/2.14/2400 ms, FOV = 224x224 mm) and diffusion weighted imaging (1.25 mm iso, TE/TR = 89.5/5520 ms, FOV = 210x180 mm, multiband 3, b-values = 1000/2000/3000 s/mm<sup>2</sup>, 90 diffusion directions across each b-value).

### **2.3.2 Image Processing**

#### **Cerebellar Lobular Segmentation – MAGeT Brain**

Segmented cerebellar lobules were used as regions of interest for the tractography analysis. Segmentations were performed for a prior study by Steele & Chakravarty (2018) based on preprocessed T1 weighted images from the HCP using the Multiple Automatically Generated Templates segmentation tool (Chakravarty et al., 2013; Park et al., 2014). The method is described in detail in Steele & Chakravarty (2018), but to summarize: Non-linear registrations are performed between five manually labelled cerebellar lobular atlases and T1 weighted images from twenty-one participants from the dataset yielding a set of five cerebellar labels for each of the template participants. The resulting templates are then warped to each of the participants, resulting 105 cerebellar segmentations per subject. A majority vote procedure is then used to yield the final segmentation of the cerebellar lobules and vermis. The resulting parcellations comprises left and

right cerebellar hemispheres as well as the vermis. It consists of hemispheric lobules III, IV, V, VI, Crus I, Crus II, VIIIB, VIIIA, VIIIB, IX, and X in addition to the vermal I/II, III, IV, V, VI, VIIA, VIIIB, VIIIA, VIIIB, IX and X. The seed regions used in the connectivity analysis consisted of lobules III-VI, Crus I-II, lobule VIIA, lobule VIIIA, and lobule VIIIB. Lobules IX and X were excluded from the final analysis because streamlines from these regions proved to be challenging to reliably track from.

### **Segmentation of Pons and Brainstem Structures - Freesurfer**

Brainstem structures were used as regions of interest for the tractography analysis. Segmentation of brainstem structures was performed using a tool implemented by Freesurfer (v6.0) (<http://surfer.nmr.mgh.harvard.edu>). In brief, this tool uses a Bayesian segmentation algorithm which uses a probabilistic atlas of the brainstem and surrounding brain structures based on manually labeled scans to generate segmentations of the medulla oblongata, pons, midbrain, and superior cerebellar peduncle (SCP) based on T1w images (Iglesias et al., 2015). From these segmentations we used masks of the pons, medulla, and midbrain in the subsequent tractography analysis. In order to prepare ROIs for tractography, the individual subject pons masks were first dilated by three voxels. A subtraction was performed between this dilated mask and the undilated pons mask to produce a pons shell mask that was split along the midline into right and left pons shell masks. Tractography was performed from the left hemisphere cerebellar lobules and the pons mask and streamlines passing through the right hemisphere shell mask were truncated. The medulla oblongata, midbrain and SCP were used as exclusion masks for the tractography.

### **DWI Preprocessing**

3T diffusion weighted images were preprocessed by the standard HCP pipeline, the steps are delineated in detail by Glasser et al. (2016). In brief, these include intensity normalization, distortion estimation and correction and a gradient nonlinearity correction. A rigid body transformation is performed to register the resultant images to the T1w structural image.

### **Tractography between cerebellar lobules and pons**

MRtrix3 (Tournier et al., 2019) was used for the estimation of the fibre orientation distribution function (fODF) that was derived with constrained spherical convolution and to perform probabilistic tractography. To investigate whether the pons exhibits a topographic pattern of

connectivity with the cerebellum similar to that which has been observed in the animal literature (Biswas et al., 2019; Schmahmann & Pandya, 1997a), we performed tractography between lobules III, IV, V, VI, Crus I and II, VI, VIIB, and VIIIA/B in the left hemisphere of the cerebellum and the pons. We used default tracking parameters from MRtrix (tracking algorithm: IFOD2, step size: 0.625mm, angle: 45 degrees, FOD amplitude for seeding: 0.1). The segmented lobules were individually used as seed masks, and the pons used as the target mask. The medulla oblongata, midbrain, and SCP were all used as exclusion masks. All cerebellar lobules other than the one we were seeding from were also included as exclusion masks. This configuration of inclusion and exclusion masks minimized streamlines transitioning through the pons and originating from the spinal cord (inferior cerebellar peduncle fibres), originating from the cortex (corticopontine fibres) or terminating in the thalamus (superior cerebellar peduncle fibres). We note that because of the resolution of the diffusion and T1w data, the pons was treated as being entirely white matter (the pontine nuclei cannot be resolved with high confidence). This means that we cannot detect termination of streamlines within the pons, and that streamlines pass through the pons to the contralateral MCP. In order to prevent the final streamline counts from being biased by individuals with larger brains, the total number of streamlines from each lobule was constrained to be proportional to the volume of the largest lobule (Steele et al., 2017). In practice, the volume of each of lobule was computed for each participant, the number of completed streamlines originating from the largest lobule (Crus I in most individuals) were set to 50,000, and the number of completed streamlines for the remaining lobules were set as a proportion of their volume to the largest lobule. For example, if a lobule had half of the volume compared to the largest lobule, completed streamlines would be set to 25,000. The result of this procedure was that while each lobule projects a different number of streamlines, they each project the same number of completed streamlines per voxel. We note that we specifically chose the lobule seeding approach because it allowed us to directly compare streamline distributions for individual lobules, and to minimize biases in tractography favoring streamlines with shorter, more linear trajectories (Rheault et al., 2019, 2020). Subsequent to tractography, individual tracts were converted to streamline count images that were used in the group template formation and the final segmentations.

### **Group Co-registration**

In order to put the individual subject data into the same space, a group template based on the streamline count data was generated using the ANTs registration software (Avants et al., 2011). Total streamline count images across individuals (the sum of the individual streamline count images for each of the lobules) were non-linearly co-registered to one another and averaged to form a group template (antsMultivariateTemplateConstruction2, demons similarity metric). Individual native-space streamline count data for the individual lobules were then warped to the final group template. This approach to group registration reflected a need for excellent alignment in pontocerebellar white matter across participants, which could not be achieved with a whole brain, gray-matter group template.

### **Lobule-specific Visitation Maps**

In order to visualize the distribution of streamlines within the pons and MCP, individual voxelwise streamline counts for each lobule were scaled by the total number of completed streamlines from that lobule to generate comparable proportional visitation maps for each lobule. This scaling step allowed us to better account for the distribution of connectivity from the smaller lobules, and these maps served as the input for the streamline-based segmentation described in the next section. The scaled maps were then summed across individuals and normed between 0 and 1 to generate group probability maps of streamline distributions for each lobule. Intensities were then normalized according to the maximum in a slice of interest at the midline for ease of visualization.

### **Streamline Based Parcellation of the Pons and MCP**

Based on the lobule specific streamline count maps derived from the tractography analysis, subject-level and group-level parcellations of the pons and MCP were performed using a modified majority-vote procedure. A standard majority vote procedure, where a voxel is labeled according to the lobule which contributes the most streamlines, has the potential to under-represent the contributions smaller lobules to the final parcellation. In order to better characterize the spatial distribution of connectivity from smaller lobules of the cerebellum, we used a modified procedure where the individual lobule specific streamline count images for each subject were divided by the number of streamlines in that lobule. (relative strength of connectivity from each lobule). For

purposes of comparison, we present the results parcellation results from both the scaled and non-scaled streamline data.

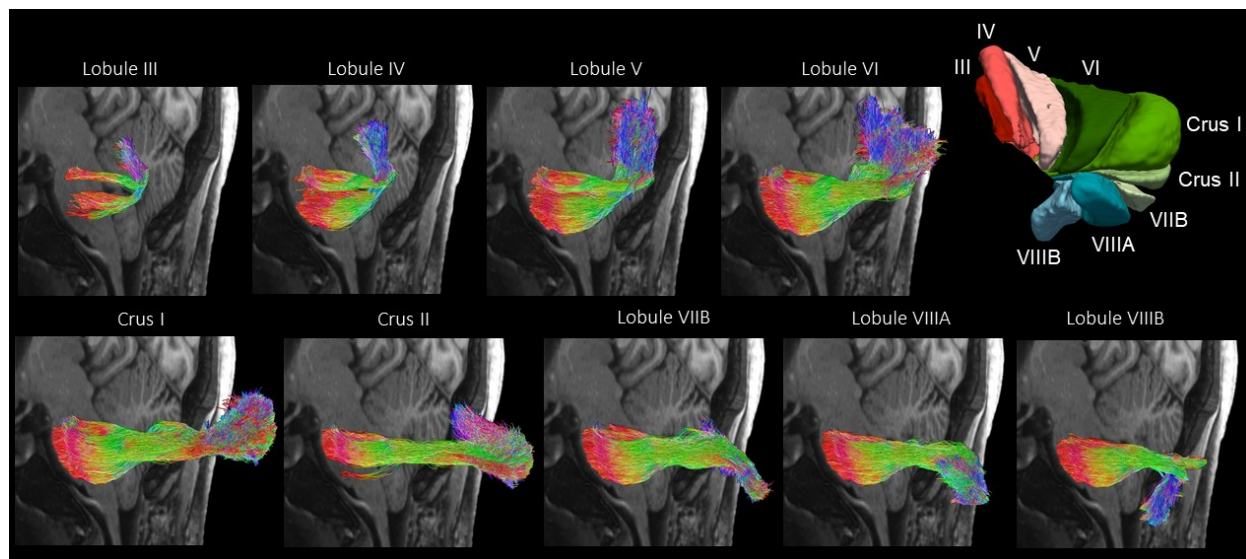
A parallel set of analyses were conducted on the group and subject level data. Using the lobule specific streamline count maps as inputs, voxels were labeled according to lobule map with the highest intensity in that position (in the lobule scaled approach), and according to the lobule contributing the most streamlines (in the simple majority vote approach). In order to estimate the consistency of the group labels across different participants we computed a cross-subject consistency metric. At each voxel in the final group segmentations, we computed the proportion of individual subject segmentations with the same label at that position. All summary maps and parcellations will be made available on NeuroVault (<https://neurovault.org/>) at the time of publication.

## **2.4 Results**

### **2.4.1 Lobule Specific Tractography between the Cerebellar Cortex and Pons**

Tractography was performed with the lobules as seeds and the pons as the target, and though axonal projections originate in the pons and terminate in the lobules, for simplicity of description we describe connectivity from the point of view of the lobules. Streamlines originating in the cerebellar lobules change direction along the superior-inferior and lateral-medial axes in their course towards the pons. Lobule specific tractography from each of the lobules in a representative participant is depicted in Figure 2.1. Streamlines from within lobules III, IV, V travel inferiorly, turn and travel anteriorly (with lateral extents matching their exit from the lobules) as they enter the region of white matter surrounding the dentate nucleus and eventually form the middle cerebellar peduncle. In contrast to the more anterior lobules, streamlines from lobules VI, Crus I, and Crus II follow a more direct and linear track as they join the white matter of the cerebellum. The majority of streamlines from VI and Crus I (and all lobules anterior to them) pass over the superior surface of the dentate nucleus, while streamlines from Crus II bifurcate to travel above and below the dentate nucleus. Streamlines from lobules VIIIB, VIIIA, and VIIIB travel in the superior direction, converge, and turn to continue anteriorly. Streamlines from lobule VIIIB bifurcate around the top and bottom of the dentate nucleus and streamlines from VIIIA and VIIIB largely travel inferior to the dentate nucleus. Streamlines from each of the lobules make up the single bundle of the MCP immediately posterior to the pons that turns to travel medially as it enters

the pons. The streamlines then bifurcate along the anterior-posterior axis to form two bundles anterior and posterior to the corticospinal tract. As streamlines enter the pons they also fan out in the rostral-caudal axis, with each lobule showing different patterns of distribution along these axes. In general, streamlines from lobules III, IV, and V had the most prominent bifurcations that form distinct rostral and caudal bundles.



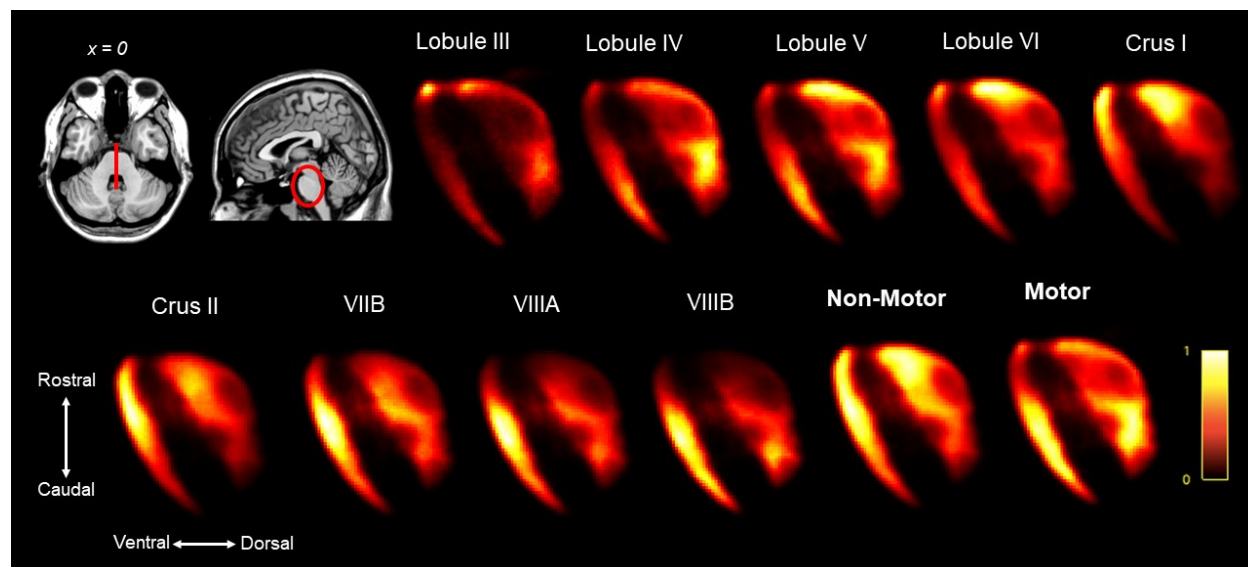
**Figure 2.1.** Tractography results in single subject for the individual lobules showing their trajectory displayed at the midline of the brain at an oblique angle. The colour of the streamlines indicates their directionality: blue is superior/inferior, red is medial/lateral, and green is anterior/posterior. The graphic in the top right is a 3D view of the cerebellar lobule segmentation shown at an oblique angle. Outlier streamlines were filtered for clarity of display using `scil_outlier_rejection.py` from the Sherbrooke Connectivity Imaging Lab toolbox in Python (Scilpy).

## 2.4.2 Group Level Visitation Maps

### Pons

Figure 2.2 depicts group average maps of normalized streamline distributions from each of the cerebellar lobules in a sagittal cross section at the midline of the pons. The dark area in the center of each cross section represents the location of the corticospinal tract. Streamlines from lobule III were distributed in two distinct areas in the rostral and caudal aspects of the pons. Lobule IV streamlines were primarily concentrated in the inferior half, whereas lobule V streamlines were more distributed in both rostral and caudal parts of the pons. Streamlines from lobules VI, Crus I and Crus II were mostly distributed in the rostral half of the pons. Lobule VIIIB and VIIIA

streamlines projected predominantly to more central parts of the pons and VIII B streamlines were distributed more caudally. To get an idea whether motor and non-motor cerebellar lobules showed differential patterns of connectivity with the pons, streamlines from the putative motor (III, IV, V, VIIIA, VIIIB) and non-motor (VI, Crus I, Crus II, VIIB) lobules were summed. Streamlines from motor lobules were distributed more in the caudal half of the pons whereas streamlines from non-motor lobules were concentrated in the more rostral aspect.

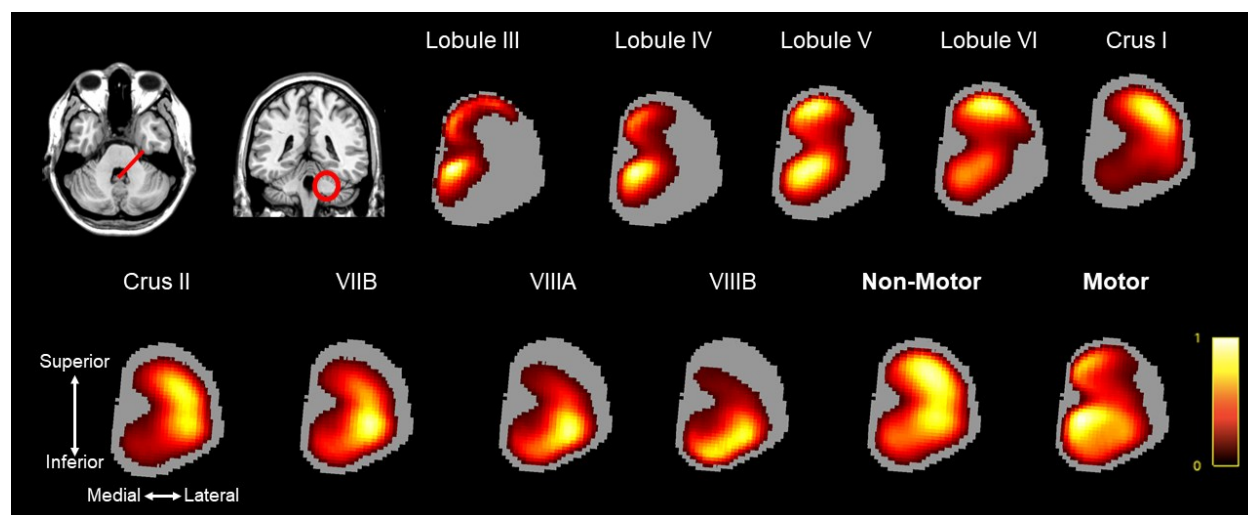


**Figure 2.2.** Normalized streamline distribution maps at the midline of the pons for each of the cerebellar lobules, and the sum streamlines of non-motor (VI, Crus I, Crus II, VIIB) and motor (III, IV, V, VIIIA, VIIIB) lobules. Brighter colours indicate higher density of streamlines. The dark region in the middle of each pons cross-section corresponds to the cortico-spinal tract.

### Middle Cerebral Peduncle

Figure 2.3 depicts group average streamline distribution maps in a cross section of the MCP as it exits the cerebellum. Streamlines from lobules III, IV and V and VI are concentrated in two distinct clusters in superior and inferior portions of the medial MCP. This reflected the bifurcations for the streamlines from these lobules described above. For Crus I, Crus II, lobules VIIB, VIIIA, and VIIIB the predominant location of streamlines are organized in a clockwise fashion around the cross-sectional center of the MCP: ranging from Crus I's concentration in the superior and lateral portion of the MCP to lobule VIIIB in the inferior and medial portion of the MCP. When streamlines from non-motor lobules (VI, Crus I, Crus II, and VIIB) were summed, they were found to concentrate in the lateral and superior quadrant of the MCP. In contrast, the summed motor

lobules (III, IV, V, VIIIA, VIIIB) were clustered primarily in the inferior and medial quadrant of the MCP and to a lesser extent in the superior and medial quadrant.



**Figure 2.3.** Normalized streamline distribution maps in a coronal cross section of the MCP for each of the cerebellar lobules, and the sum streamlines of non-motor (VI, Crus I, Crus II, VIIIB) and motor (III, IV, V, VIIIA, VIIIB) lobules. Brighter colours indicate higher density of streamlines. The angle of the coronal cross section is perpendicular to the main axis of the MCP.

### 2.4.3 Lobule Scaled Majority Vote Parcellation

#### Group Level

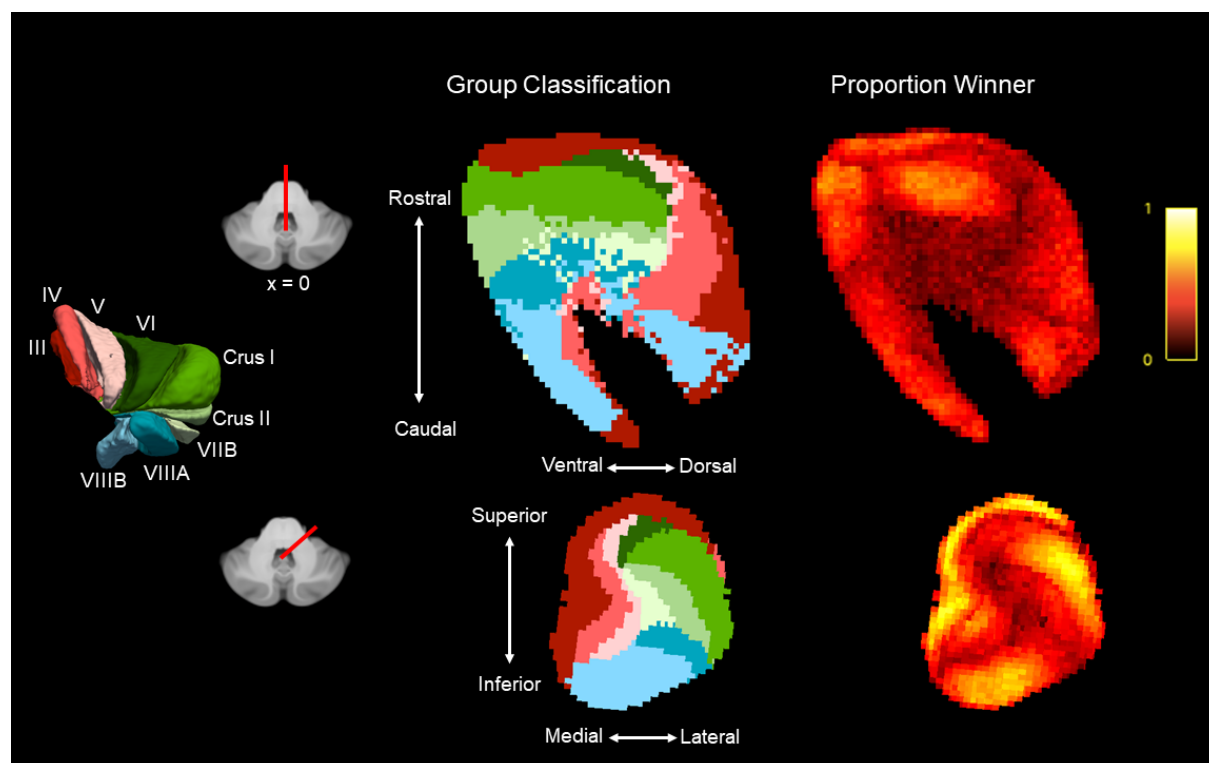
##### Pons

Figure 2.4 depicts the results of the group-level majority vote segmentation in the pons and MCP as well as the inter-subject agreement metric using the scaled streamline count data. In the ventral half of the pons, going from rostral to caudal, we note layers corresponding to lobule III, followed by Crus I, Crus II, lobule VIIIA. Lobule VIIIB is represented in much of the caudal half of the pons. In the dorsal portion of the pons, we note small lobule V and VI areas, and more inferiorly large lobule III and IV areas. The spatial configuration in the parcels in the segmented pons and MCP is similar to the configuration of lobules in the cerebellar cortex. Neighboring lobules in the cerebellar cortex are generally found in adjacent parcels the pons and MCP. Lobules III and IV are notable exceptions, appearing at multiple different locations in the pons. While they are located in the superior cerebellum, they appear to be highly connected to the caudal pons. Furthermore, while Crus I and Crus II are situated more centrally in the cerebellum, they are most highly connected to the rostral pons. Inter-subject agreement is highest in the rostral portion of the pons cross-section,

in particular in the area that corresponds to the Crus I parcel. Consistency of the labels is lowest in the center of the pons where there is a greater overlap of streamlines belonging to different lobules.

### Middle Cerebral Peduncle

In the MCP, lobule III is represented in the most medial periphery of the cross section, followed by layered medial to lateral stacking of lobules IV, V and VI towards the center of the MCP. In contrast, Crus I, Crus II, Lobules VIIA and VIIB are layered in more of a superior to inferior fashion in the lateral half of the cross section. There is a small area corresponding to lobule VIIB at the very center of the cross section. Inter-subject agreement is highest in the regions corresponding to lobule III, Crus I and VIIB parcels. As with the pons, consistency is lowest in the center where there is more overlapping of streamlines.

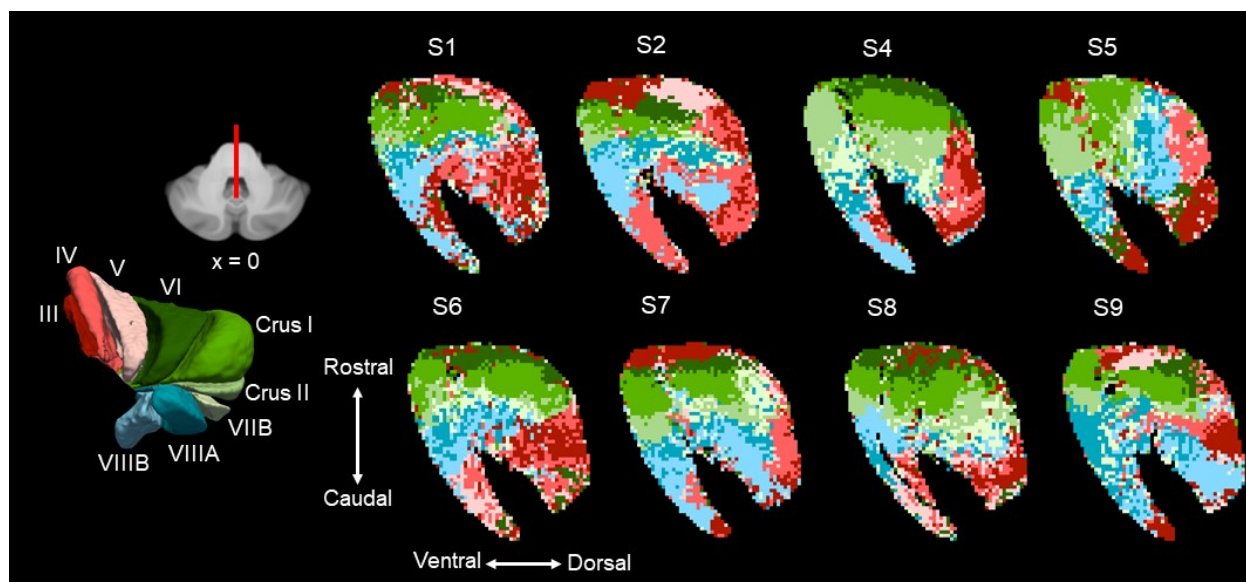


**Figure 2.4.** On the left are results of the majority vote parcellation using the scaled streamline count data for the pons (top) and MCP (bottom). On the right are the intersubject agreement maps for the pons and MCP, here brighter colours indicate higher consistency of the label across subjects.

## Individual Participant Level

### Pons

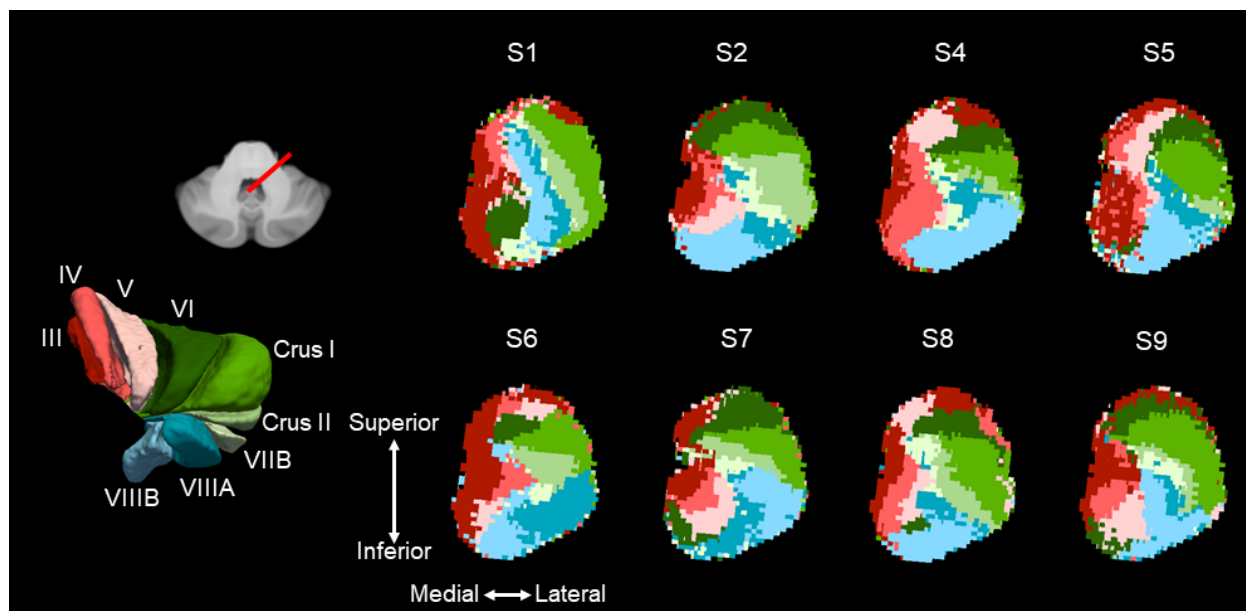
The results of the subject level parcellation in the pons using the scaled streamline data is presented for 8 representative participants (4 females) in Figure 2.5. Although there is heterogeneity in the exact position and size of specific labels, there are a number of consistent observations between individuals and with the group segmentation. Labels show a similar layering pattern which, for the most part, recapitulates the spatial configuration of the cerebellar lobules.



**Figure 2.5.** Results of the majority vote segmentation of the pons using the scaled streamline count data in 8 representative participants.

### Middle Cerebral Peduncle

In the MCP, depicted in Figure 2.6, we observe consistency in the general spatial configuration of the individual parcels, with some variability in their exact position and size. Similar to the group segmentation: In the medial portion of the MCP cross section, towards the midline of the brain, we observed areas corresponding to lobule III, IV and V. In the lateral half of the MCP cross section, going from superior to inferior, we observed layers corresponding to Crus I, Crus II, lobules VIIIA and VIIB as identified in the group map. However, we also noted that there was variability in the orientation of the layers, with some participants exhibiting a more medial to lateral organization.



**Figure 2.6.** Results of the majority vote segmentation of the MCP using the scaled streamline count data in 8 representative participants. Shown in a coronal cross-section of the MCP located proximal to the pons.

#### 2.4.4 Simple Majority Vote

##### Group Level

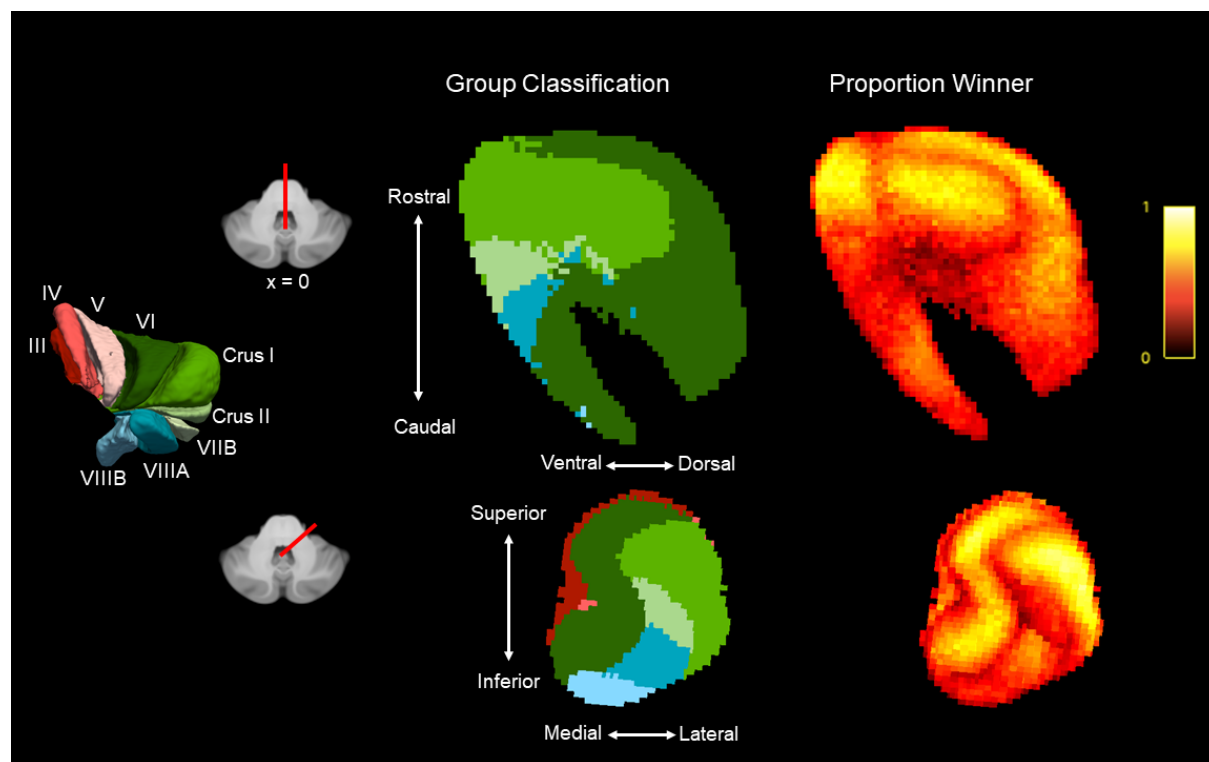
##### Pons

Presented in Figure 2.7 are the results for the group-level majority vote in a cross-section of the pons and MCP, as well as the inter-subject agreement metric using the non-scaled data. The segmentation is generally consistent with that performed with the scaled data, but as expected it only includes the larger lobules of the cerebellum. In order of size, there are areas corresponding to lobules VI, Crus I, Crus II and VIIIB. The lobule VI area starts in the dorsal part of the rostral pons and then wraps around to cover much of the caudal half the pons. The Crus I area is in the rostral half of the pons, and the Crus II and VIIIB areas are immediately caudal to this and are focused in the ventral portion. The agreement metric shows that the Crus I label, and Lobule VI labels were broadly consistent across participants, in the rostral half of the pons. The Crus II and lobule VIIIB labels were less consistent across participants.

##### Middle Cerebral Peduncle

At the level of the MCP, the majority vote results again parallel those from the scaled analysis. Again, however, we note that the larger lobules are overrepresented in the segmentation. In a cross-

section most proximal to the body of the pons, we note a small area in the most lateral portion corresponding to lobule III and a large area in the lateral half corresponding to Lobule VI. Going clockwise we find smaller areas corresponding to Crus I, Crus II, VIIIA, and VIIIB. With the agreement metric, similar to with the pons, we note high consistency of the Lobule VI and Crus I labels, but considerably more heterogeneity for the other labels.



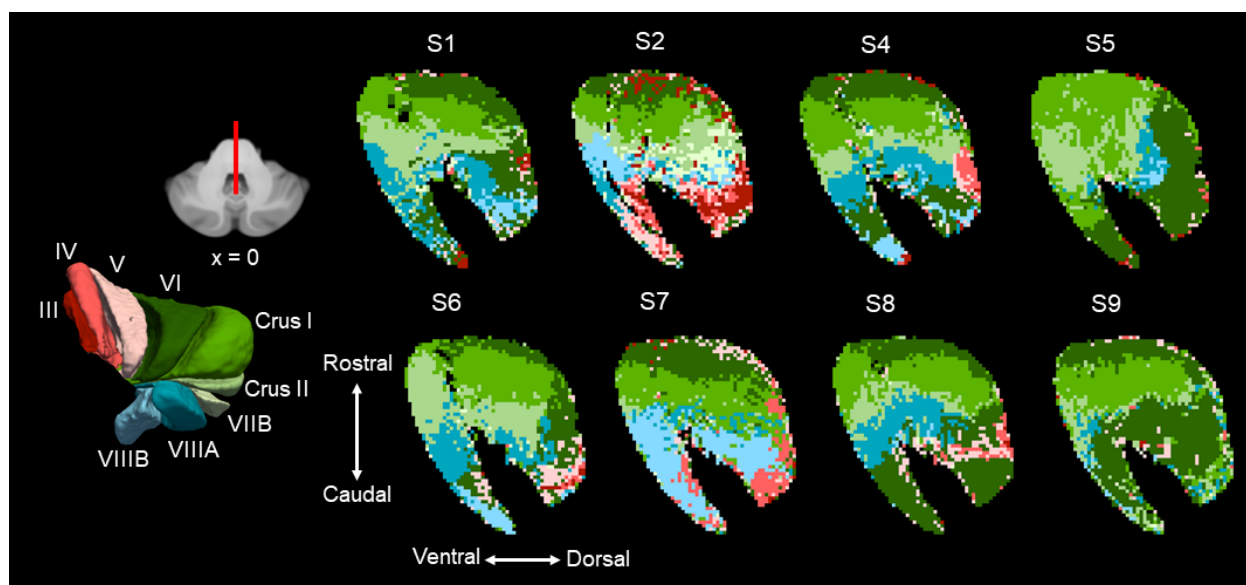
**Figure 2.7.** On the left are results of the majority vote parcellation using the raw streamline count data for the pons (top) and MCP (bottom). On the right are the intersubject agreement maps for the pons and MCP, here brighter colours indicate higher consistency of the label across subjects.

## Individual Participant Level

### Pons

The results of the subject-level pons segmentations for the same 8 participants using the non-scaled majority vote data are depicted in Figure 2.8. Similar to the group analyses, the segmentation is dominated by a small number of lobules. As with the scaled analysis, there is considerable heterogeneity across individuals, but some consistent patterns – the areas corresponding to Crus I and Crus II streamlines occur in the rostral half of the pons, whereas the area corresponding lobule VI streamlines occur in most rostral and caudal parts of the pons. This likely reflects the bifurcation

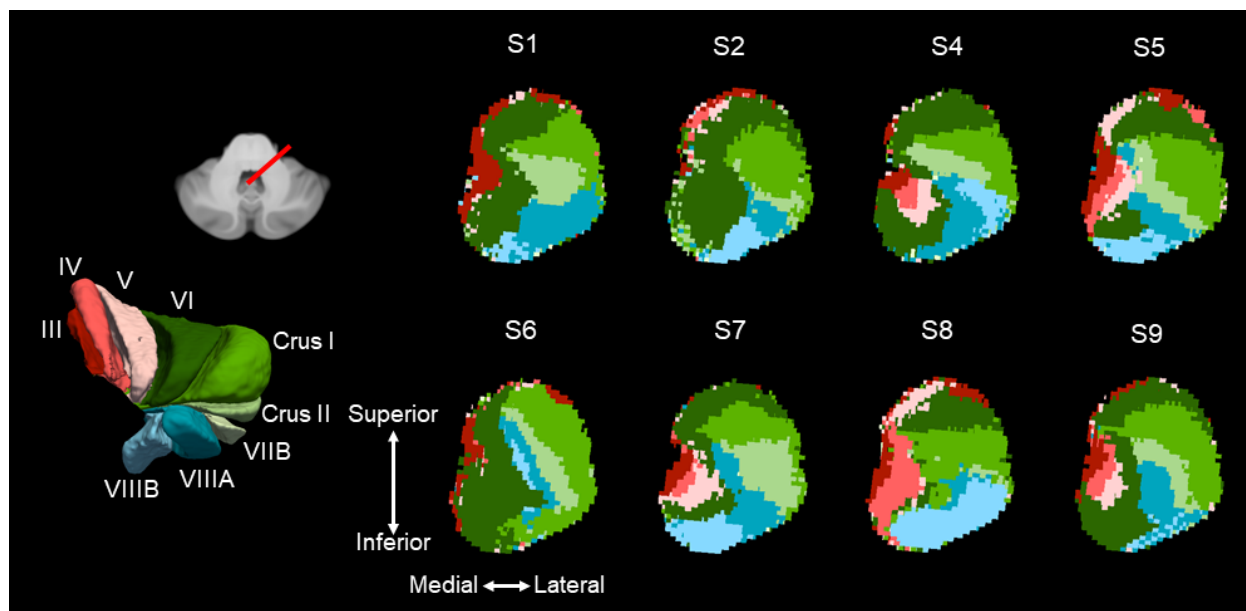
noted in the tractography and two distinct clusters of streamlines noted in the average streamline distributions in the MCP cross-section.



**Figure 2.8.** Results of the majority vote segmentation of the pons using the raw streamline count data in 8 representative participants. Shown the midline of the brain ( $x = 0$ ).

### Middle Cerebral Peduncle

Figure 2.9 depicts the results for the majority voting procedure in the same 8 participants in a cross section of the MCP. In most participants, we observe a small area corresponding to lobule III in the most medial portion of the cross-section. We then note a large area in the medial half corresponding to lobule VI. Crus I, Crus II, VIIIA, VIIIB are organized in a layered fashion in the lateral half of the pons. As with the scaled data, these can be layered on more of a medial to lateral axis in some participants.



**Figure 2.9.** Results of the majority vote segmentation of the MCP using the raw streamline count data in 8 representative participants. Shown in a cross-section of the MCP located proximal to the pons.

## 2.5 Discussion

We used in-vivo diffusion imaging to reconstruct pontocerebellar projections and demonstrated segregated patterns of connections between the pons and cerebellar cortex. Consistent with animal anatomical work, we find a distinct pattern of connectivity such that the motor lobules of the cerebellum appear to be preferentially connected to the caudal pons and non-motor lobules are more connected to rostral pons. Our observations parallel observations from stroke data in humans which shows different patterns of behavioural disturbances depending on lesion location in the pons (Schmahmann et al., 2004a). To our knowledge, our study is also the first to classify the pons and MCP based on their connectivity to the cerebellar lobules. Our novel approach yielded a parcellation of the pons and MCP which provides further evidence for the topographic organization in these structures. The parcellation also provides future researchers with a framework for disentangling the contributions of different regions of the pons and MCP to these different functions.

### 2.5.1 Spatial Configuration of the MCP

The spatial configuration and trajectory of the MCP has previously been described using dissections in a small sample of human brains (Akakin et al., 2014; Perrini et al., 2013; Thomas,

1897; Jamieson, 1910). Our lobule specific tractography approach recapitulated the prominent organizational principles noted in these studies. Thomas (1897) described two white matter bundles in the pons which contribute to the MCP: a more superficial one which originates in the rostral pons and travels to the inferior cerebellum, and a deeper bundle originating more caudally which travels to the superior cerebellum. Jamieson (1910) built on this work and noted the existence of three distinct white matter bundles in the pons: a more superficial bundle originating in the rostral pons traveling to the superior posterior lobe, a deeper bundle mostly originating in the caudal pons whose more superficial part travels to the inferior posterior lobe, and the deepest part which travels to the anterior lobe. We noted a similar overall pattern in our results: the rostral pons seemed to have stronger connections to the superior posterior lobe (primarily Crus I and Crus II), whereas the caudal pons was connected to the superior lobe. Furthermore, streamlines connecting the pons to the posterior lobe tended to be concentrated in the lateral portion of the MCP, while those connecting to the superior lobe were concentrated in the medial MCP. A similar distinction between deep and superficial bundles of the MCP was also made by Akakin et al. (2014) who studied its anatomy in relation to the dentate nucleus. They observed one bundle with a more lateral course (projecting to the posterior lobe of the cerebellum) and another travelling along a more medial course parallel to the midline. For the latter bundle, the authors did not specify where its fibres terminated in the cerebellar cortex. The first observation is in line with our findings concerning the distribution of streamlines in the MCP, where streamlines connecting the pons to lobules in the posterior lobe were more concentrated in its lateral aspect. Taken together, these dissection studies lend validity to our tractography analysis in that the larger scale aspects of connectional anatomy of this brain region are reflected in our results. We did note a bifurcation in the bundles corresponding to lobules III, IV, and this is possibly due to the intersection between these and other bundles such as those originating in Crus I and II and reflects a limitation of tractography at this resolution.

### **2.5.2 Topographic Organization of the Pons**

We demonstrated that connections between the pons and the cerebellum follow a rostral-caudal gradient, with the rostral pons being more highly connected to lobules more involved with cognitive and emotional processing and the caudal pons with areas more involved with motor control. This is likely to reflect segregation that occurs within the cerebral cortical inputs to the pons. Based on anterograde tracing work done in animals, corticopontine projections appear to be

characterized by patterns of divergence and segregation. Small cortical areas tend to project throughout the rostro-caudal extent of the pons, and to distinct regions which form patchy clusters with minimal overlap (Brodal & Bjaalie, 1997). Keeping in mind the fractured topology that appears in these projections and terminations in the pons, we note some larger organizational principles and their relevance to the present study. Anterograde tracing studies in macaques found that corticopontine projections from primary motor cortex terminate primarily in the caudal half of the pons, whereas projections from associative cortical areas are distributed throughout the pons but primarily in the rostral half (Schmahmann et al., 2004b; Schmahmann & Pandya, 1997a). Earlier lesions studies, also in macaques, found a similar overall pattern of a superior to inferior gradient in non-motor and motor cortical inputs to the pons (Brodal, 1978; Wiesendanger et al., 1979). In humans, there is a smaller body of research to draw on, but it is consistent with the notion of spatially segregated cortical inputs to the pons. Ramnani et al. (2006) used diffusion imaging and tractography to demonstrate segregation in cortical inputs to the cerebral peduncle. Using high-resolution (7T) functional MRI data and functional connectivity between cortical areas and the pons, Karbasforoushan et al. (2022) demonstrated rostral-caudal topography such that frontal areas were more highly connected to rostral pons and sensory motor areas to caudal pons. While our findings are different in the respect that they demonstrated a rostro-caudal gradient in terms of pontocerebellar connections we expect these to show spatial correspondence to the corticopontine projections at the gross level (Biswas et al., 2019; Brodal, 1982) .

Pontocerebellar projections have been less extensively researched, but the existing literature is consistent with the notion of distinct regions of the pons projecting to motor and non-motor areas of the cerebellar cortex. Recent task-based and resting state functional imaging studies have demonstrated functionally distinct areas in the cerebellum (Buckner et al., 2011; King et al., 2019), it follows that white matter input to the cerebellum from the pons should follow a similar level of segregation. Studies using horseradish peroxidase retrograde tracing found that the anterior lobe of the cerebellum receives fibres primarily from the caudal pons, whereas the posterior lobule received fibres mostly from the rostral part of the pons (Brodal, 1979, 1982). More recently, Biswas and colleagues (2019) reconstructed the trajectory of individual pontocerebellar axons in mice. Notably, they observed a core-shell organization wherein the central part of the pons and the surrounding areas projected to specific zones within the cerebellum. While they found that single axons diverged into multiple collaterals these tended to innervate particular combinations of

lobules. These patterns of divergence may account for how a single motor representation in the caudal pons gives rise to multiple motor representations in the anterior lobe and inferior portion of the posterior lobe of the cerebellum. In fact, similar to our observations, they found that the caudal pons projected both to lobules II-V as well as lobule VIII. This provides further context for our observation that inputs to motor and non-motor areas of the cerebellum originated in spatially distinct areas of the rostral and caudal pons, and that adjacent lobules tended to connect to similar areas in the pons.

In humans, the rostral to caudal functional gradient in terms of non-motor and motor function within the pons has been demonstrated in lesion studies which have found cognitive and affective disturbances resulting from damage to the rostral part of the pons (Kim Jong S. et al., 1995; Schmahmann et al., 2004a). The impairments noted here may result from damage to cerebrocerebellar circuitry originating in cortical areas involved in cognitive and emotional processing. Our study offers new context to these findings in demonstrating the preponderance of connections to regions of the cerebellum implicated in higher order processes including language, emotional regulation, and theory of mind originating in the rostral pons.

To our knowledge, structure function relationships within the MCP itself have not been explicitly studied in the previous literature. Studies have consistently found degradations in cerebellar white matter in multiple sclerosis (Wilkins, 2017), and Tobyne et al. (2018) found white matter changes in the MCP to be related to cognitive impairment in this population. The latter study found that groups of cognitively impaired and non-cognitively impaired patients did not differ in terms of cortical and cerebellar lesion load but did differ in terms of the spatial distribution of white matter lesions in cerebellar white matter. Our approach to characterizing the distribution of connections from different parts of the cerebellum offers additional perspective to understanding the nature of these impairments in multiple sclerosis as well as other lesions affecting cerebellar white matter. In the near future, methods of modelling whole brain dysconnectivity like those described by (Karnath et al., 2018; Kuceyeski et al., 2013; Zayed et al., 2020) with diffusion MRI based models could also provide insights into cognitive and behavioural disturbances arising from lesions to cerebellar white matter.

### **2.5.3 Lobule Specific Parcellation of the Pons and MCP**

Diffusion tractography has been previously used to segment the thalamus and the basal ganglia based on their connectivity to the cortex (Draganski et al., 2008; Johansen-Berg et al., 2005; Traynor et al., 2010). Our study is the first to apply this technique to the segmentation of the pons based on its cerebellar connectivity. This presented some unique challenges: compared with the thalamus and the basal ganglia, where different areas of the cortex project to more spatially distinct regions of these nuclei, in the pons there is more complex pattern of organization with more convergence and divergence (Brodal & Bjaalie, 1992). In this case, a standard-majority voting approach is less valuable in that it results in a segmentation which underrepresents the smaller cerebellar lobules that project fewer streamlines. By scaling the streamline counts according to the size of the lobules in each individual, we were able to produce segmentations of the pons which better reflect the spatial configuration of the of the connections to individual lobules within the pons.

### **2.5.4 Limitations and Future Directions**

We based our tractography analysis and subsequent segmentation on cerebellar lobular subdivisions. While the functional territories of the cerebellum do not map perfectly on to the lobules, as is evidenced by a growing body of resting-state and task based functional imaging research (Buckner et al., 2011; King et al., 2019), these do provide a proven means of subdividing the cerebellum across individuals. Future research could consider more data driven methods of parcellating the cerebellum based on its white matter connectivity to the pons, such as independent component analysis (Hale et al., 2015). Other approaches could involve clustering streamlines into distinct bundles (Garyfallidis et al., 2018) or using clustering algorithms based on the local orientation distribution function data (Najdenovska et al., 2018).

Further, we noted some discrepancies between our observations and our expectations based on known anatomy. In the tractography analysis, we noted that streamlines from lobules III, IV, V (motor lobules) showed a prominent bifurcation as they entered the pons (Figure 2.1). This was reflected in the final group parcellation where we found rostral and caudal parcels corresponding to these regions (Figure 2.4). It is likely that this is due to the close proximity and intersection between different white matter bundles originating in the posterior cerebellum. In light of this, the finding of rostral parcels corresponding to lobules III, IV, and V should be interpreted with caution.

While our study shows that fine grained anatomical details of the white matter architecture of the cerebellum can be resolved with high quality 3T data in-vivo, our results could be further supported with higher resolution diffusion data and ex-vivo techniques. There are several limitations inherent to diffusion MRI acquisition and tractography, most prominently tissue classification within the pons, and bottleneck effects from tracking between the cerebellum and pons (Rheault et al., 2020). Because of the resolution at which the data was acquired, and the lack of a notable difference of fODF amplitude between MCP white matter and regions of the pons likely corresponding to pontine nuclei grey matter, we could not detect termination of streamlines within the pons. This means that while we could obtain information about relative streamline distribution along rostro-caudal and dorsal-ventral gradients, we could not make any inferences about streamline distributions along the medial-lateral direction. Furthermore, there are other biases in tractography which could have influenced our results. Notably, there is a bottle neck effect such that streamlines originating in the cerebellar lobules converge onto the MCP potentially resulting in poorer accuracy as the streamlines diverge as they enter the pons. This means that the results in the MCP may reflect a closer approximation of the actual anatomy compared to the results obtained within the pons.

We initially explored using the 7T diffusion data available from the HCP, but the considerable signal drop-off in the inferior regions of the cerebellum made it unusable for tractography. Even using a cerebellum specific acquisition Steele et al. (2017) encountered similar issues of poor signal in the most inferior lobules of the cerebellum. While higher in-vivo MRI does offer interesting possibilities for studying the white matter of the cerebellum, future research will have to overcome this constraint. Ex-vivo diffusion and novel techniques like tractography based polarized light imaging microscopy, which offers spatial resolutions of up to 100 $\mu$ m, can offer new perspectives into meso-scale connectional architecture (Axer et al., 2011b). Using these techniques, we should be able to resolve the pontine nuclei and therefore be able to make more specific inferences about pontocerebellar white matter trajectories.

## 2.6 Conclusion

The cerebellum's involvement in a wide range of cognitive, affective and motor processes is mediated by its connectivity to the rest of the brain. Our study contributed to our understanding of its connectivity by demonstrating that the connections between the cerebellar cortex and the pons

follow a topographic pattern of organization that is similar to observations from non-human primates. Specifically, we provided evidence that areas of the cerebellum involved with motor and cognitive/emotional processing process are connected to spatially distinct areas in the pons, via different parts of the MCP. As well as contributing our fundamental understanding of the cerebellum, our description of its pattern of connectivity as well as the parcellations of the pons and MCP provide additional context for studying the relationship between damage to these areas and functional impairments.

# Chapter 3. Multiscale Gradients of Corticopontine Structural Connectivity

## 3.1 Abstract

The cerebellum's involvement in a range of cognitive, emotional, and motor processes has become increasingly evident. Given the uniformity of the cerebellar cortex's cellular architecture its contributions to varied processes are thought to be partially mediated by its patterns of reciprocal connectivity with the rest of the brain. A better understanding of these connections is therefore fundamental to disentangling the cerebellum's contribution to cognition and behaviour. While these connections have been studied extensively in non-human animals using invasive methods, we have limited knowledge of these connections in humans. The current work reconstructed the corticopontine projection, the first segment of downstream connections between the cerebral and cerebellar cortices, with diffusion MRI tractography in human in-vivo whole brain data and an independent higher resolution postmortem brainstem dataset. Dimensionality reduction was used to characterize the pattern of connectivity of cerebral cortical projections to the pons as two overlapping gradients that were consistent across participants and datasets: medial-to-lateral and core-to-belt. Our findings align with invasive work done in animals and advance our understanding of this connection in humans – providing valuable context to a growing body of cerebellar research, offering insights into impacts of damage along the pathway, and informing clinical interventions.

## 3.2 Introduction

The historical paradigm of the cerebellum as being a motor control structure has been upended by evidence highlighting its involvement in a range of cognitive and emotional processes (Schmahmann, 2019). The cytoarchitecture of the cerebellar cortex is relatively uniform compared to that of the cerebral cortex, effectively being comprised of the same computational units repeated along its surface (Schmahmann et al., 2019; Walter & Khodakhah, 2006). This suggests its contributions are at least partially mediated by its pattern of reciprocal connectivity with the cerebral cortex (Schmahmann et al., 2019). Accurate characterization of cerebellar connectivity is therefore fundamental to disentangling its roles in cognition and behaviour and has potential implications for clinical interventions (Baker et al., 2023; Wessel & Hummel, 2018). The first

segment of downstream connections between the cerebral and cerebellar cortices, the corticopontine projection, has been extensively studied in non-human animals using invasive methods (Brodal & Bjaalie, 1997; Glickstein et al., 1985; Leergaard & Bjaalie, 2007; Schmahmann & Pandya, 1997b) but is almost completely overlooked in humans.

Based on studies conducted in non-human animals, corticopontine projections are characterized by convergence and divergence: disparate cerebral cortical areas project to distinct and fractured patches within the pontine nuclei. Patches are organized in a lamellar fashion, reminiscent of the layers of an onion. Projections originating in sensorimotor areas terminate in a central core interspersed with passing corticospinal fibres and terminations from adjacent cortical regions form external layers (Brodal, 1978). Superimposed on this onion-like organization, cortical areas anterior to sensorimotor cortex project medially whereas more posterior cortical areas project laterally (Brodal, 1978). There is also a rostrocaudal organization, wherein motor areas project preferentially to the caudal pons and association areas to the rostral pons (Schmahmann et al., 2019). We can conceptualize these patterns as reflecting three overlapping spatial gradients of connectivity: core-belt, medial-lateral, and rostro-caudal gradients.

Region-of-interest approaches have been used to investigate corticopontine connectivity in humans (Doron et al., 2010; Engelhardt et al., 2010; Karbasforoushan et al., 2022; Ramnani et al., 2006). While these approaches have demonstrated general spatial mappings between the cerebral cortex and the cerebral peduncle (an intermediate segment of the corticopontine pathway) and pons, discrete regions of interest undercut the more nuanced nature of these connections apparent in the non-human animal literature. Other work has applied non-linear dimensionality reduction techniques to diffusion weighted imaging tractography data to delineate fine-grained gradients of connective topography of different cortical regions (Blazquez Freches et al., 2020, 2021). Similar approaches have been used in resting state functional connectivity to characterize gradients of functional organization (e.g., Guell et al., 2018; Katsumi et al., 2023; Margulies et al., 2016). They utilize nonlinear dimensionality reduction techniques (e.g., diffusion-map embedding, spectral embedding) to generate lower-dimensional and overlapping representations of high-dimensional data that encapsulate the dominant modes/gradients of connectivity across space (Belkin & Niyogi, 2001; Blazquez Freches et al., 2021; Margulies et al., 2016). Given the nature of corticopontine projections, with their overlapping organizational principles, patterns of

segregation and integration, gradient methods are ideally suited to disambiguating these principles in neuroimaging data.

We hypothesized that human corticopontine projections would follow analogous organizational principles to those identified in non-human animals across both scales of dMRI data and on the individual and group levels. Specifically, we expected to find these projections to be organized in a core-belt, and medial-to-lateral fashion (Leergaard & Bjaalie, 2007; Brodal, 1978). Given the inability of dMRI tractography to resolve terminations within the pons (Lundell & Steele, 2024), we did not expect to find a rostro-caudal organization. To investigate the organizational principles of the corticopontine pathway in humans we used diffusion MRI tractography and spectral embedding in two complementary datasets: a set of participants from the Human Connectome Project (Glasser et al., 2016) and a single high-resolution acquisition of postmortem human brainstem (Sitek et al., 2019). The Human Connectome Project data allowed for investigation of individual participant level data and the creation of group averages while the complementary high-resolution sample served as a more granular confirmation of the organizational principles identified in the in-vivo data. With both datasets we 1) generated connectivity fingerprints within the pons that represent the patterns of cerebral cortical projections and then 2) projected the fingerprints to the cortical surface (in-vivo data) or the cerebral peduncle (postmortem data) for visualization. To further compare across the two sets of results we projected the in-vivo pontine gradients along the entire corticopontine tractogram.

### **3.3 Materials and Methods**

#### **3.3.1 In-Vivo Dataset**

##### **Participants**

10 unrelated participants (5 females, average age = 29.7) were randomly selected from a dataset used in our previous study (Rousseau et al., 2022) that employed T1w and diffusion weighted imaging data from the Human Connectome Project open-access dataset ([www.humanconnectome.org](http://www.humanconnectome.org)) (Steele & Chakravarty, 2018; Van Essen et al., 2012; Glasser et al., 2016). Individual IDs of participants from the dataset will be provided by the authors upon reasonable request. Informed consent for each participant was obtained by the HCP Washington University - University of Minnesota Consortium and all methods were performed in accordance with the relevant guidelines of the Washington University Institutional Review Board (IRB).

Structural imaging data were acquired on a 3T Siemens Connectom Skyra scanner. T1w (0.7 mm iso, TI/TE/TR = 1000/2.14/2400 ms, FOV = 224 × 224 mm) and diffusion weighted imaging (1.25 mm iso, TE/TR = 89.5/5520 ms, FOV = 210 × 180 mm, multiband 3, b-values = 1000/2000/3000 s/mm<sup>2</sup>, 90 diffusion directions across each b-value). Ten participants were used due to the large computational demands of the tractography procedure implemented in the present study.

### **dMRI Preprocessing**

Diffusion weighted images were obtained preprocessed by the HCP data processing pipeline (Glasser et al., 2016). Preprocessing steps included intensity normalization, distortion estimation and correction and a gradient nonlinearity correction. Diffusion weighted images were aligned to the T1w structural images with a rigid body transformation. MRtrix3 (Tournier et al., 2019) was used to obtain fibre orientation distribution function (fODF) images that were derived with constrained spherical convolution to perform probabilistic tractography (Tournier et al., 2007, 2012).

### **Corticopontine Tractography**

The processing pipeline for the in-vivo dataset is depicted in Figure 3.1a. Our tractography approach involved seeding small parcels (i.e., ROIs) distributed across the cerebral cortical surface and performing tractography between each of these parcels and the pons on a parcel-by-parcel basis. This approach was designed to minimize spurious connections, reduce the impact of tractography biases, and to ensure that each area of the cerebral cortex projected a comparable number of completed streamlines directly to the pons. The alternative, wherein the entire cerebral cortex is seeded in one step, would result in an over-representation of streamlines from easier to track regions (i.e., regions with a more linear trajectory to the pons and regions that are closer to the pons)(Girard et al., 2014).

### **Generation of Subcortical ROIs**

Hand-drawn regions of interest (ROIs) of the left and right pons and cerebral peduncle for each of the participants were manually created by PNR using MRtrix3's *mrview* and the T1-weighted and overlaid fODF images (Tournier et al., 2012, 2019). *Mrview* was also used to manually delineate exclusion ROIs: a sagittally oriented plane at the midline spanning the corpus collosum (to prevent tracking of fibres to the contralateral cortical hemisphere), coronally orientated plane immediately

posterior to the pons (to prevent the tracking of fibres of the middle cerebral peduncle), a small axial region posterior to the middle cerebral peduncle and covering the ascending white matter tracts (i.e. medial lemniscus). An anatomical atlas of the brainstem and cerebellum was used as reference for the delineation of all ROIs of interest and exclusion (Naidich et al., 2009).

### **Generation of Cerebral Cortex ROIs**

The cerebral cortical surface was generated from the T1w images with the Freesurfer pipeline (7.3.2) as presented in detail in our previous work (Rousseau et al., 2022). All analyses were conducted on the left-hemisphere. The boundary between the cerebrum's grey matter and white matter was converted to a single-voxel thick volume and then subdivided into ROIs of the frontal, somatosensory, parietal, insular, and cingular cortices. These served as the base ROIs that were further subdivided for subsequent tractography. Occipital and temporal cortices were excluded based on previous tract tracing studies in macaques indicating very few white matter projections to the pons from these regions (Schmahmann & Pandya, 1997b). Freesurfer's white matter parcellation (*wmparc*) was used to generate ROIs of lobar white matter to be used as exclusion ROIs in the cortical lobar specific tractography. The single-voxel thick cortical lobar volumes were subdivided into small and approximately equally sized and contiguous parcels using Nighres' *intensity\_propagation*, which grew each parcel from voxel seeds distributed uniformly across the lobar volumes (Huntenburg et al., 2018). The total number of parcels across the frontal, somatosensory, parietal, insular and cingular cortices was set to 500. A total of 500 parcels was selected to balance between the spatial specificity of the subsequent parcel specific corticopontine tractography, while keeping the computational demands low enough that tractography could complete within a reasonable amount of time. To prevent tracking of invalid streamlines that originate in the seeded parcel and travel to other areas of the cortical white matter surface, a large exclusion ROI consisting of all other white matter surface ROIs was generated for each parcel.

### **Cerebral Cortex Parcel to Pons Tractography**

Probabilistic tractography was performed between each of the cerebral cortical parcels and the left pons, with the cerebral peduncle set as an intermediary inclusion ROI. Tractography was performed with Mrtrix3's *tckgen* (Tournier et al., 2019) using the following parameters: iFOD2 algorithm, 1000 completed streamlines, maximum length of 150mm, step size of .625mm, maximum angle of 45 degrees between steps, initiation, and termination FOD amplitude threshold

of 0.1. Tractography was performed independently for the frontal, somatosensory, parietal, insular and cingular cortices and resulted in an approximately equal distribution of streamlines across the cortical surface. This approach allowed for the inclusion of lobe-specific exclusion ROIs to minimize the amount of tracking of spurious streamlines between cerebral cortices and the pons. Frontal parcels included exclusion ROIs of parietal white matter, and parietal parcels included exclusion ROIs of frontal white matter to prevent tracking of superior longitudinal fasciculus fibres. The strict definition of exclusion ROIs was necessary due to the longer maximum streamline length that was used as a requirement to connect more distant regions of the cerebral cortex and the pons.

### **Spectral Embedding**

For each participant we created individual single voxel parcellations of the pons (where each voxel received a unique index label) that were combined with the 500-node cortical parcellations used in the tractography. The resultant node parcellation image was used along with the tractography results (combined across all cortical parcels) to generate a connectome matrix with MRtrix3's *tck2connectome*. Individual cells in the matrix represented the number of streamlines connecting pairs of parcels (cortex-pons, cortex-cortex, pons-pons). This matrix was further constrained to remove cortex-cortex and pons-pons connections prior to spectral embedding to ensure that our results were specific for cortex-pons connections. In order to mitigate the effect of spurious connections on subsequent spectral embedding the connectivity matrix was thresholded such that pons parcels that received less than 10 streamlines were excluded from the analysis.

Spectral embedding was then used to construct gradients representing the connectivity of the cerebral cortex and the pons, similar to previous work (Blazquez Freches et al., 2020, 2021). Scikit-learn's *SpectralEmbedding* function was used to perform spectral embedding on the streamline connectivity matrix (Pedregosa et al., 2011). Briefly, the algorithm first transforms the connectivity matrix to an affinity matrix that represents the similarity in streamline connectivity between pairs of pons nodes. Spectral decomposition was then performed on the corresponding graph Laplacian, similar to the procedure adopted by Blazquez Freches and colleagues (Blazquez Freches et al., 2020, 2021) and the top four gradients that represented the dominant patterns of corticopontine connectivity were extracted. Based on our hypothesis that we could identify two gradients within the pons, we analyzed and interpret the first two gradients. For completeness we

also visually inspected the third and fourth gradients – finding that they contained much more noise and were more inconsistent in their spatial patterns across individuals. For each gradient, each of the nodes within the pons receives a value along a continuous gradient based on the similarity of its connectivity to the cortex compared to other pons nodes, which we remapped to range between 1 and 10 to facilitate display as in previous work. To project gradients back to the cerebral cortex and visualize the spatial correspondence between the pons and cortical gradients, we performed the dot product of the embedding values within the pons and the original pons by cerebral cortex connectivity matrix, and then again remapped the values between 1 and 10. The approach of weighing a connectivity matrix by embedding values has previously been described by others who applied the same general method to gradients derived from functional connectivity (Guell et al., 2020; Katsumi et al., 2023; Zhang et al., 2019). The final result was that each of the 500 cerebral cortical parcels received a value that represents a weighted average (by number of streamlines) of the embedding values of connected pons voxels. This effectively allows us to visualize spatial changes in corticopontine connectivity patterns across the cerebral cortical surface. The cerebral cortical projections were viewed on the Freesurfer white matter surface for each participant.

### **Group Analysis**

Individual participants' data were combined to generate group embeddings in a template space based on HCP data (Zayed et al., 2020; Tremblay et al., 2024). In brief, the group template was generated from 1001 participants from the Human Connectome Project (Glasser et al., 2016). As described by Tremblay and colleagues (2024), a subset of 200 participants were used to generate an initial fODF based template using MRtrix3's *population\_template* function and then individual participant data were registered to template space using *mrregister*. The fODFs were used to drive registration within white matter to prioritize white matter correspondence and ensure that alignment was not unduly influenced by grey matter cortical differences.

For the group analysis in the present study, the ten-participant space corticopontine tractograms from the individual participant analysis were transformed to group space with *tcktransform* using the warps generated from the fODF registration (pipeline depicted in Figure 3.1b). The average of all ten participants' T1 weighted images was processed with Freesurfer's *recon-all* pipeline to generate cortical surface segmentations as done previously at the individual

level. The volumetric representation of the cortical surface (including frontal, parietal, insular and cingular cortices) was parcellated into 250 equally sized parcels using the same procedure as in the individual participant analysis. Because of the multiple transforms applied to the parcellation (see below), 250 parcels were used instead 500 to prevent the loss of parcels when transforming between group and individual spaces. A hand drawn ROI of the pons in group space was also created in the same manner as described above, and parcellated such that each voxel was assigned a unique label. In order to obtain a common cortical surface parcellation across participants, the parcellated cortical surface was transformed into individual subject space, dilated such that each voxel took on the value of the closest parcel (Nighres' *intensity\_projection*, 10mm dilation), and then masked by the cortical gray/white matter interface volume and projected back into group space. The result was individual subject parcellations in group space that were unique to the subject (reflecting their cortical anatomy) but comparable across subjects because parcel indices were maintained and projected to/from the same anatomical location across subjects. For each subject we generated a group space connectivity matrix based on the unique but analogous cortical parcellations and the common parcellation of the pons. The resultant connectivity matrices were summed and thresholded such that pons parcels with fewer than 45 streamlines were excluded from the analysis. The threshold here was set so the resulting connectivity matrix would contain a comparable number of completed pons nodes to that in the individual participants analyses. Spectral embedding and cortical projection were performed on the group total connectivity matrix in the same manner as for the individual subject analyses.

### **3.3.2 Postmortem Brainstem and Thalamus Dataset**

#### **Data Acquisition and Preprocessing**

A preprocessed postmortem diffusion imaging dataset of the human brainstem and thalamus from a 65-year-old male was obtained from a previous study by Sitek and colleagues (2019). The acquisition of the original dataset (Calabrese et al., 2015) was approved by the Duke University Health System Institutional Review Board, and all methods were performed in accordance with relevant guidelines. Tissue preparation, MRI acquisition, and preprocessing parameters are described in detail by the authors. In summary, 3D-gradient echo and diffusion weighted Magnetic resonance imaging data was collected on a small-bore Magnex/Agilent scanner. Diffusion-weighted images were acquired with 200 micrometre spatial resolution (120 diffusion directions at  $b = 4000 \text{ s/m}^2$ ,  $TR = 100\text{ms}$ ,  $TE = 33.6\text{ms}$ ,  $FOV = 90 \times 55 \times 45 \text{ mm}$ ). DIPY 0.14 was used to was

used to obtain fibre orientation distribution function (fODF) images that were derived with constrained spherical convolution.

### **Tractography**

Probabilistic tractography was performed between hand drawn ROIs of the left cerebral peduncle and left pons. Due to the incompatibility of the older version of DIPY ODF images with MRtrix3, SCILPY tools (<https://github.com/scilus/scilpy>) were used to perform tractography on the postmortem dataset (postmortem pipeline is depicted in Figure 3.1c). Seeding was performed in the cerebral peduncle (1000 seeds per voxel, step size 0.1 mm), with an inclusion ROI in the left pons and a large exclusion ROI surrounding the pons and cerebral peduncle. Following the generation of the initial tractogram, the ROI of the cerebral peduncle was then parcellated into 500 approximately equal sized parcels using Nighres *intensity\_projection* and the oversampled tractogram was then filtered with MRtrix3 *tckedit* function such that each parcel included 200 randomly selected completed streamlines. This procedure assured that each parcel in the cerebral peduncle projected an equal number of streamlines to the pons.

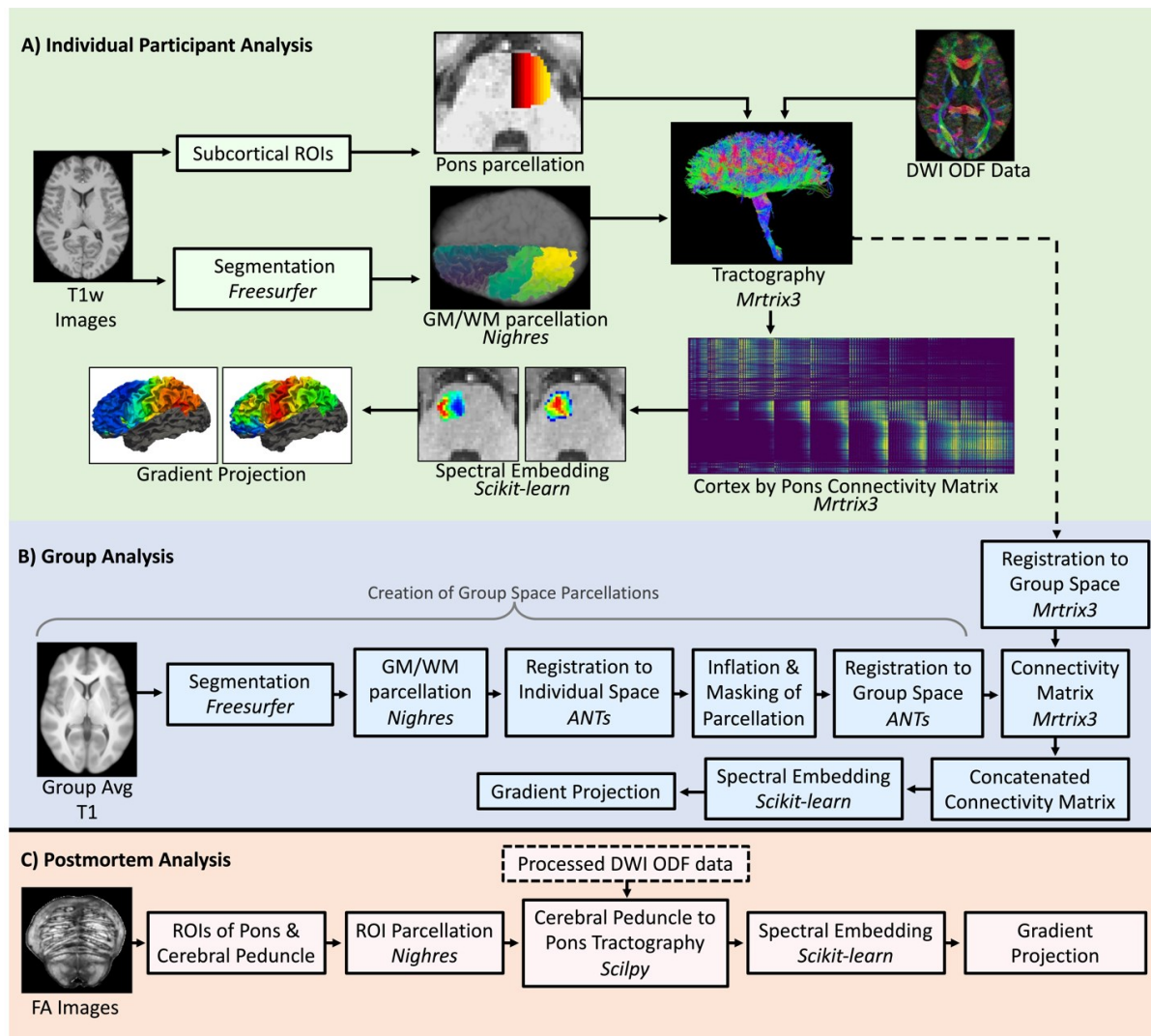
### **Spectral Embedding and Cerebral Peduncle Projection**

To reduce computational load and facilitate comparison at similar spatial resolutions, instead of the voxel-wise pons parcellation used in the in-vivo analysis, the pons ROI was parcellated into 1000 equally sized parcels using Nighres *intensity\_propagation*. Mrtrix3's *tck2connectome* function was then used to obtain a connectivity matrix that included the number of streamlines between each of the parcels in the cerebral peduncle and pons. The resultant matrix was constrained to only contain the pons to cerebral peduncle connections (not pons-pons or peduncle-peduncle). Spectral embedding, and projection of embedded values in pons to the cerebral peduncle were performed using the same procedure as in the in-vivo analyses.

### **3.3.3 Whole Tractogram Projection of In-Vivo Pons Gradients**

In order to evaluate the correspondence between the in-vivo and postmortem gradients, we also implemented an additional approach to project in-vivo pontine gradients along a group average tractogram. This allows for the inspection of in-vivo gradients along the entire corticopontine tractogram, including in axial slices of the cerebral peduncle that are comparable to the postmortem analysis. We first combined all individual participant tractograms in average space and then filtered them to include only streamlines traversing the cerebral cortical surface, the cerebral

peduncle, and the pons. We also used a hand drawn shell around the pons to exclude any stray streamlines exiting the pons towards the MCP. A subset of 100,000 streamlines were randomly selected to reduce computational load and facilitate display. Dipy's *values\_from\_volume* function was used to project the average of the embedding values from all voxels in the pons traversed by each streamline onto the streamline for display. The result is a single value per streamline representing the average of the embedding values in the pons voxels it passes through. Mrview was then used to display the tractogram with streamlines colour coded according to the range of average embeddings.



**Figure 3.1.** Processing pipelines, including relevant software packages for the individual participant analysis (A), group analysis (B), and postmortem analysis (C).

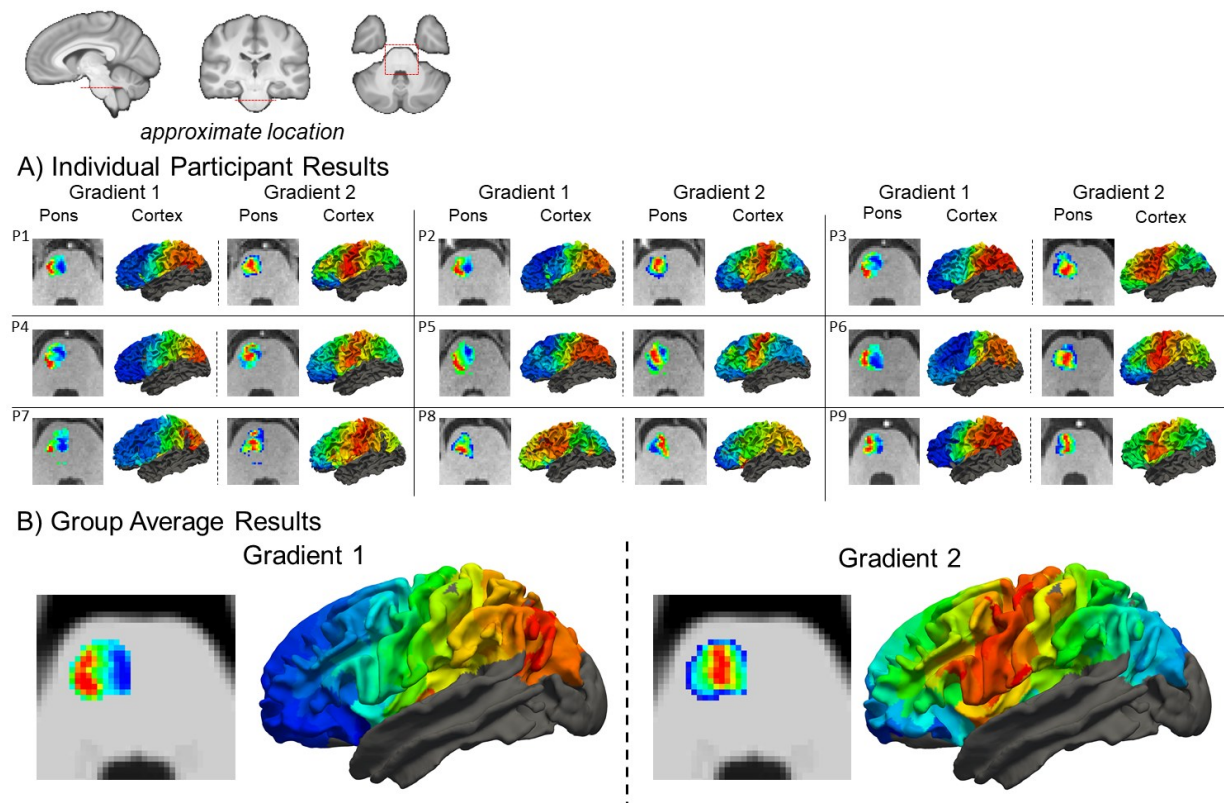
## 3.4 Results

### 3.4.1 In-Vivo Corticopontine Connectivity Gradients

Corticopontine tractography and spectral embeddings were successfully completed in 9 of the 10 HCP datasets. In one participant gradient results were discrepant with the rest, specifically there was greater noise evident in the second gradient, and it did not contain continuous transitions seen in other participant's gradients. This appeared to be driven by anomalies in their tractography that resulted in streamlines originating in sensorimotor cortex entering the pons in a more diffuse manner in comparison to other participants. For the 9 successfully completed participants, the first two components reflected a (1) medial-to-lateral and (2) core-to-belt gradients when viewed in an axial plane close to the centre of the pons (Figure 3.2a). There was remarkable consistency in the gradients across participants. The first component was a medial-to-lateral gradient for all but one of the participants, where the order of components was reversed. The medial-to-lateral gradient did not show a smooth gradation but was primarily dominated by two zones (medial and lateral) with a small area of gradation between the two. This indicates that the connectivity showed strong segregation between streamlines traveling to the medial and lateral parts of the pons. The core-to-belt gradient was more variable across participants with some displaying more of a banded gradient, as opposed to more of a concentrically organized gradient (for instance participants 6 and 9). The central area of this gradient corresponds well with regions of the pons occupied by the corticospinal fibres, which is in agreement with tract tracing work in non-human primates (Schmahmann et al., 2004b). The group analysis reflected very similar medial-to-lateral and core-to-belt gradients found in the individual participants (Figure 3.2b).

When projected back to the cerebral cortex, the medial-to-lateral pontine gradient appears as an anterior-to-posterior gradient (Figure 3.2a). In most participants, there is limited gradation within the frontal and parietal lobes and a more pronounced gradation starting at the motor cortex and extending posteriorly. This parallels our observations of the spectral embedding within the pons and may indicate that convergence of cerebral cortical streamlines in the pons results in a loss of spatial specificity. In the cerebral cortex, the core-to-belt pontine gradient appears as a gradient radiating outward from primary motor cortex. This illustrates good correspondence with the results in the pons, in which the core of the gradient appears to be dominated by corticospinal fibres. The cerebral cortical gradient projection in the group data showed similar results (Figure 3.2b), albeit with smoother gradations compared to the gradients of the individual participants. We

noted missing data in one of the cerebral cortex parcels in the dorsal aspect of the post-central gyrus. After group registration, this parcel did not have any streamlines connecting to the it the pons, resulting in a null value for the dot-product at this node.

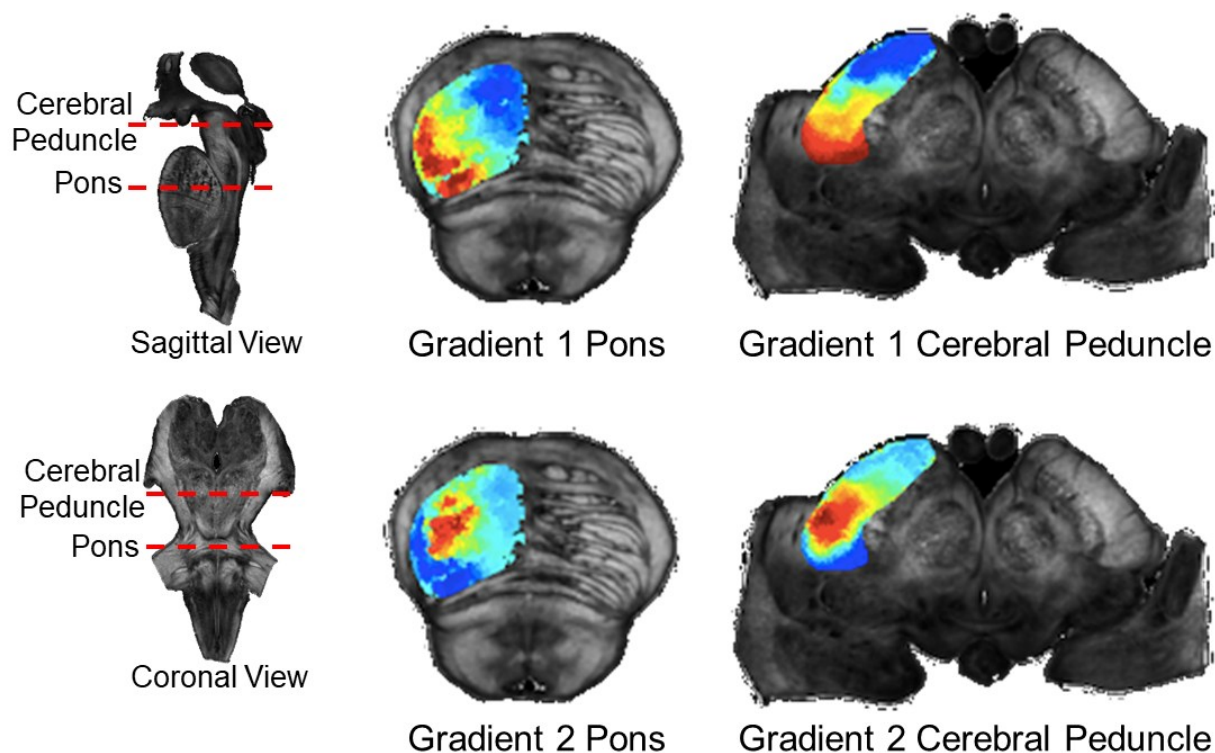


**Figure 3.2** Spectral embedding results and cerebral cortex projection for individual HCP participants (A) and the group average (B). Results show first two components of spectral embedding in an axial slice in the middle of the pons and the associated cerebral cortical projections on the left white matter surface.

### 3.4.2 Postmortem Cerebral Peduncle to Pons Connectivity Gradients

In the postmortem data, we observed two very similar gradients to those identified in the in-vivo analysis (Figure 3.3). When viewed in an axial slice in the center of the pons, the first component reflected a medial-to-lateral gradient, and the second a core-to-belt gradient. Compared with the in-vivo results, the medial-to-lateral gradient exhibited finer gradation and was angled more obliquely. This more oblique configuration is partially the result of the postmortem sample being oriented in such a way that the pons was more closely aligned to the vertical axis than in the in-vivo data. In the core-to-belt gradient, the core was smaller and more circumscribed compared to

the in-vivo results, likely reflecting increased spatial specificity because of higher resolution and decreased partial voluming. When projecting the first gradient to the cerebral peduncle, we observed an oblique anterior-to-posterior gradient. In combination with the in-vivo results reflecting an anterior-to-posterior gradient, these findings highlight the lateral twisting of corticopontine fibres as they transition through the internal capsule, the cerebral peduncle, and enter the pons (Ross, 1980; Sullivan et al., 2010). When projecting the second, core-to-belt, gradient to the cerebral peduncle we observed a core-to-belt gradient whose centre was in a lateral and inferior portion of the cerebral peduncle – corresponding well with the cortical sensory motor projections previously identified by Ramani and colleagues (2006a).

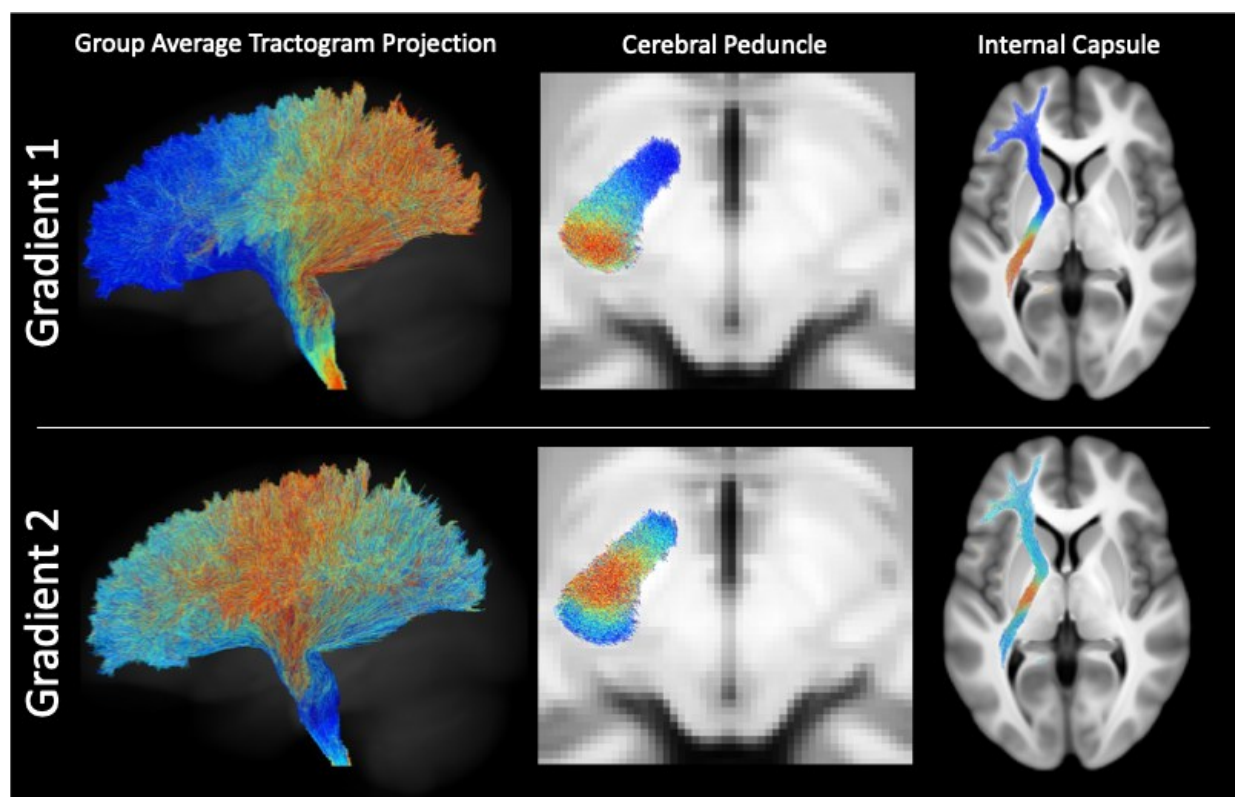


**Figure 3.3** Spectral embedding results and cerebral peduncle projection for postmortem brainstem dataset. Figure depicts first two components of spectral embedding in an axial slice in the middle of the pons and the associated cerebral peduncle projections.

### 3.4.3 Projection of In-Vivo Pontine Gradients

The projection of the pontine gradients along the whole tractogram and in axial slices in the cerebral peduncle and internal capsule is depicted in Figure 3.4. In the cerebral peduncle, we confirmed a strikingly similar topographical organization of gradients to the postmortem data.

With the first gradient oriented on the axis intermediate to the medial-lateral and anterior-posterior axes observed within the pons and cerebral cortical projections, respectively. The second gradient was also remarkably similar to the cerebral peduncle projection in the postmortem data, though exhibiting a somewhat larger central zone that is likely a result of summing across multiple participants' individual data. When inspecting the projection of the first gradient in the internal capsule, the medial portion of the pons is projected to uniformly from a large segment of the anterior limb of the external capsule that extends slightly past the genu. The central zone of the second in-vivo pons gradient projects to a circumscribed area posterior to the genu, corresponding well with the area of the internal capsule demonstrated to contain passing corticospinal fibres (Ross, 1980).



**Figure 3.4.** Projection of in-vivo pontine gradients along the corticopontine tractogram. On the left is a three-dimensional representation of the colour-coded group sum tractogram. The middle depicts the colour-coded gradient projection in an axial slice in the cerebral peduncle, and on the right is the projection in an axial slice of the internal capsule. Streamlines were displayed with 75% opacity and a slab thickness of 1mm.

### 3.5 Discussion

We reconstructed the corticopontine pathway using diffusion MRI and tractography in two complementary datasets with different levels of resolution; demonstrating topographic patterns of organization similar to those observed in non-human animal work. Our work is the first to use a data-driven decomposition approach, as opposed to region of interest, to characterize the corticopontine pathway in humans and demonstrates two gradients of connectivity that capture core organizational principles. In the pons, we demonstrate both medial-to-lateral and core-to-belt connectivity gradients that are present in both the in-vivo and postmortem high-resolution datasets. In the in-vivo data, the medial-to-lateral gradient corresponded to an anteroposterior gradient in the cerebral cortex whereas the core-to-belt gradient corresponded to a cerebral cortical gradient radiating anteriorly and posteriorly from primary motor cortex. We also projected the in-vivo gradients along the entire tractogram to confirm that our two datasets shared the same spatial organization within a region that was common to both, the cerebral peduncle, and explore the organization of connectivity within the internal capsule.

The first gradient, accounting for the largest proportion of the variability in the connectivity data, was organized in a medial-to-lateral fashion in the pons. In the in-vivo data set, cerebral cortical areas anterior to the central sulcus project preferentially to the medial pons, whereas areas posterior to the central sulcus project more laterally. This configuration (medial-to-lateral mapping in pons corresponding to an anterior to posterior mapping in the cerebral cortex) may reflect in part the lateral rotation of corticopontine fibres as they traverse the internal capsule and enter the cerebral peduncle. The observed organization in the pons is consistent with and extends the findings of invasive lesion and tract tracing work in macaques that shows frontal cortical areas projecting medially in the pons and parietal areas projecting more laterally (Brodal, 1978; Koziol et al., 2014; Schmahmann et al., 2019). Within the pons, we observed uniform lateral, central, and medial zones or bands as opposed to a smooth gradation in our in-vivo results. Previous tract tracing work indicates that corticopontine fibres from different cerebral cortical areas terminate in discontinuous patches within the pons (Koziol et al., 2014; Schmahmann et al., 2019; Schmahmann & Pandya, 1997a). The patches representing terminations from frontal, sensory-motor, and parietal areas follow the medial-to-lateral organization that we identified but also exhibit significant overlap (Koziol et al., 2014; Schmahmann et al., 2019). Our observations of more clearly demarcated regional projections are likely a function of the lower resolution of the

in-vivo dMRI data and convergence of cerebral cortical streamlines on the small cross section in the pons – both resulting in lower spatial specificity than tract tracing. Importantly and supporting this interpretation, the higher resolution postmortem dataset exhibited a smoother gradient that followed the same medial-to-lateral organization (Figure 3.3) and helps to serve as a mesoscale bridge between in-vivo dMRI and tract tracing results.

The secondary core-to-belt gradient that we observed reflects a funnel-like convergence of projections originating from across the cortical mantle onto the pons. Here we observed a prominent central zone in the pons that, when projected back to the cortex, corresponded to primary motor and somatosensory cortex. The spatial extent of the projection of this central zone was broadly consistent across individual participants (including primary motor cortex in all), however in some individuals it was highly circumscribed on primary motor cortex and in others included somatosensory and/or premotor cortical areas. The position of the projection of this central zone to the cerebral peduncle in the postmortem dataset – along with its link to the primary motor cortex in our in-vivo results – is consistent with previous tractography-based parcellations of the sensorimotor projections (Ramnani et al., 2006). It is likely that the white matter bundles circumscribed within this central zone in the pons consist of both corticospinal and corticopontine sensorimotor fibres. The corticopontine tracing and lesion work in non-human primates has demonstrated that fibres originating in sensorimotor cortex terminate in areas adjacent to corticospinal fibres (Brodal, 1978; Schmähmann et al., 2004). At the resolutions used in the current study, we were unable to identify terminations within the pontine nuclei or differentiate between corticospinal and corticopontine fibres. Given that corticopontine fibres originate as corticospinal fibres early in development, they are likely in very close spatial correspondence and would be very difficult to differentiate with dMRI (Heffner et al., 1990).

With dMRI we were limited to detecting gradients along the mediolateral and dorsoventral axes within the pons. Even at 200 micrometre resolution it is not feasible to resolve and differentiate corticopontine white matter and pontine nuclei gray matter with sufficient resolution to reliably detect termination of streamlines. As a result, we were unable to recapitulate another organizational principle within the pons that has been demonstrated in the animal literature: the termination of corticopontine fibres originating in non-sensory motor frontal and parietal areas in the rostral pons (i.e., superior aspect in the human brain) and termination inputs from sensory

motor inputs in the caudal pons (inferior aspect)(Brodal, 1978; Schmahmann & Pandya, 1997a; Wiesendanger et al., 1979). However, previous work has indirectly demonstrated this using dMRI tractography between the pons and motor and non-motor areas of the cerebellar cortex (Rousseau et al., 2022), a finding that is corroborated by older postmortem lesion work in humans (Beck, 1950).

Our analyses of the cortico-ponto-cerebellar pathway with dMRI tractography highlight important limitations in detecting streamline terminations along the rostrocaudal axis in the case of the corticopontine segment, and the mediolateral axis in the case of the pontocerebellar segment (Lundell & Steele, 2024). These limitations, in addition to the complex patterns of convergence and divergence of white matter along the whole cortico-ponto-cerebellar pathway, emphasize the value of our approach of assessing corticopontine and pontocerebellar segments individually (Rousseau et al., 2022). Reconstruction of the entire corticocerebellar connection in a single step with dMRI tractography (effectively representing this pathway as being monosynaptic), while algorithmically possible, results in a compounding of tractography biases and a lack of spatial specificity of resulting maps. In general, it stresses the value of a more conservative approach to tractography, like the one adopted here, that considers known anatomy while being mindful of limitations of the method (Rheault et al., 2020; Schilling et al., 2019).

Finally, it is increasingly clear that the pons doesn't function as a simple relay from the cerebral cortex to the cerebellum, and that it likely serves a computational role that includes filtering and integration of cerebral cortical signals (Schwarz & Thier, 1999). Our demonstration of overlapping structural gradients and, by extension, organizational principles within the pons, hints at substrates underlying integration of information from disparate cortical areas. Functional work has demonstrated areas in the cerebellum that correspond to the default mode network (Buckner et al., 2011), which may imply that there is integration of inputs from frontal and parietal default mode areas at some point along the corticocerebellar pathway. An overlap in the two gradients that we have observed, and in frontal and parietal inputs to the pons depicted in the core-belt gradient may reflect this integration at the level of the pons. Applying gradient decomposition to functional MRI data may shed further light on a possible integrative function of the pons. For instance, they may help situate how putative pontine functional connectivity gradients map onto

well described cerebrocortical and cerebellar connectivity gradients (Guell et al., 2018; Margulies et al., 2016).

### **3.6 Conclusion**

The corticopontine connection, the first step in the downstream pathway connecting the cerebral cortex and the cerebellum, has been studied extensively in non-human animals. Employing two diffusion MRI datasets at different levels of spatial resolution, anatomically grounded tractography, and data-driven gradient methods we are able to demonstrate analogous organizational principles to those seen in animal studies: results that were consistent across individuals and at different levels of granularity. This work informs our anatomical understanding of the corticopontine pathway in humans and lays the groundwork for future research studying more fundamental aspects of this connection and how it may be impacted by different normative and pathological processes.

## Chapter 4. Pontine Functional Connectivity Gradients

### 4.1 Abstract

The cerebellum contributes to a wide range of motor and non-motor functions through its reciprocal connections with much of the cerebral cortex. The pons is a key intermediary in these connections. It receives extensive projections from the cerebral cortex, and in turn projects to cerebellar cortex. The anatomical organization of corticopontine and pontocerebellar connectivity has a rich history of study, but the functional organization of the pons in humans remains largely unexplored. We sought to characterize the functional organization of the pons based on its functional connectivity with the cerebral and cerebellar cortices. To this end, we used diffusion map embedding to reconstruct gradients that capture the dominant patterns of its functional connectivity. Our results reveal a primary gradient in the pons that is organized along rostrocaudal and mediolateral axes. This gradient mirrors the unimodal-to-transmodal gradient previously described in the cerebral and cerebellar cortices. The observed patterns recapitulate core findings from invasive tract-tracing in non-human animals and extend them to demonstrate that pontine functional organization can be characterized by a complex pattern of segregation and integration. These findings highlight that, rather than serving as a simple relay, the pons may function as an integrative hub within the cortico-ponto-cerebellar pathway.

### 4.2 Introduction

It has become increasingly evident that the cerebellum plays a role in nearly every facet of cognition and behaviour (Sokolov et al., 2017). Given that the cellular architecture of the cerebellar cortex is relatively uniform, its contributions are at least in part mediated by precisely organized reciprocal connections with the cerebral cortex (Schmahmann et al., 2019). Understanding the organization of corticocerebellar loops is therefore essential to furthering our understanding of the function of the cerebellum. Bridging the downstream connections between the cerebral cortex and the cerebellum are the pontine nuclei of the pons (Schmahmann et al., 2019). The organization of corticopontine and pontocerebellar connections has been characterized over decades of invasive animal research (e.g., Brodal, 1978, 1979; Glickstein et al., 1985; Leergaard & Bjaalie, 2007; Schmahmann et al., 2004; Schmahmann & Pandya, 1997b). More recently, key findings from the animal literature demonstrating topographic organizational principles within these connections

have been recapitulated with non-invasive diffusion MRI tractography studies in humans (Rousseau et al., 2022, 2025). Despite these recent insights, our knowledge of how the pathway is organized in humans and of its role in transmitting, and potentially transforming (Schwarz & Thier, 1999) cerebrocortical information destined for the cerebellum remains limited.

Much of the cerebral cortex contributes to the estimated 40 million axons forming the corticopontine projection, one of the most substantial projection systems in the brain (Leergaard & Bjaalie, 2007; Schwarz & Thier, 1999). Corticopontine terminals are organized in a complex topographic manner, with different cerebrocortical sites projecting to demarcated patches within the pons (Schmahmann & Pandya, 1997b). The projection is characterized by both convergence – distant cortical areas convergence on nearby pontine patches – and divergence whereby a discrete cortical area may project to diffusely distributed patches in the pons. While the spatial relationships between different cortical areas are partially preserved, the distinctive arrangement of corticopontine terminals results in an interdigitation of patches from disparate cortical areas (Schmahmann & Pandya, 1997b). Approximately half as many fibres that enter the pons exit as mossy fibres that target the cerebellar cortex in a complex convergent and divergent manner (Kratochwil et al., 2017; Schwarz & Thier, 1999). A specific area of the pons may project to multiple cerebellar cortical areas while, conversely, a circumscribed area of cerebellar cortex may receive projections from several pontine regions (Biswas et al., 2019; Kratochwil et al., 2017). Cerebellar cortical outputs are conveyed via the deep cerebellar nuclei to the thalamus and then back to the cerebral cortex in a topographically organized manner, thereby creating a closed loop (Dum & Strick, 2003). Overall, the cortico-ponto-cerebellar projection is characterized by the partial preservation of the cerebral cortex’s topography, but with additional integration or recombination of different cerebrocortical inputs.

Diffusion MRI (dMRI) tractography investigations have informed our understanding of different portions of the cortico-ponto-cerebellar pathway in humans, but these methods do not lend themselves to reconstructing a complete mapping of the entire circuit in a single step. First: this is a polysynaptic connection, which cannot be accurately resolved with dMRI tractography. Second: at typical dMRI resolutions the pontine nuclei are indistinguishable from surrounding white matter, and therefore fibre tracking cannot reliably detect terminations of corticopontine fibres or the origin of pontocerebellar fibres within the pons (Lundell & Steele, 2024). Independent reconstruction of corticopontine and pontocerebellar pathways partially addresses these

limitations, but yields an incomplete picture of the organization of this connection (Rousseau et al., 2022, 2025). Methodological challenges make it difficult – if not impossible – to accurately reconstruct the entire cortico-ponto-cerebellar pathway in a single step with dMRI. Resting state fMRI (rsfMRI) offers a promising alternative for mapping these multi-synaptic connections and revealing functional topographies that reflect anatomical organization (Buckner et al., 2011).

In addition, while there are multiple lines of evidence suggesting that the pons is more than a relay and plays a role in filtering and integrating cortical inputs on their way to the cerebellum (Schwarz & Thier, 1999), the methodological limitations of dMRI tractography in humans mean that anatomical investigations of afferent/efferent pontine connections are restricted to demonstrating macroscale organization. Though useful for characterizing the overall coarse mappings of structural connections, this approach cannot contribute to our understanding of the pons as a functionally dynamic structure. Functional MRI, particularly with large high-quality datasets like those of the Human Connectome Project (Van Essen et al., 2013), provides an alternate method for non-invasive investigations of pontine connectivity that can characterize pontine function within the context of the entire cortico-ponto-cerebellar circuit.

Functional connectivity based on resting state functional MRI provides complementary and novel perspectives to prior anatomical and dMRI studies. Functional connectivity can be used to investigate connectivity across the multiple levels of the cortico-ponto-cerebellar pathway. Furthermore, rsfMRI allows us to ask questions regarding the principles governing functional organization within the pons. Seed based connectivity has provided clues as to corticopontine functional organization, showing that different cerebrocortical areas connect to the pons in a topographically organized manner, resembling patterns observed in non-human primate work (Karbasforoushan et al., 2022). Other methods are needed, however, to determine if the functional organization of the pons, with respect to its primary afferent/efferent structures, reflects a composite of the overlapping organizational principles of structural connectivity demonstrated in both non-human animal work (Biswas et al., 2019; Leergaard & Bjaalie, 2007) and targeted tractography in humans (Rousseau et al., 2022, 2025). Functional connectivity gradients represent a data-driven approach that has successfully been used to address fundamental questions of cerebral (Margulies et al., 2016) and cerebellar cortical (Guell et al., 2018) functional organization. This approach allows researchers to derive mappings of continuous transitions in connectivity signatures across the brain, which are expressed as gradients. Gradients are anchored by two poles,

where each pole represents different anchor points characterized by distinct connectivity profiles with a transitional area that blends between them. Given that the pons sits between the cerebral and cerebellar cortices and is hypothesized to have a dynamic functional architecture that supports brain function (Schwarz & Thier, 1999), this approach is directly applicable to teasing apart potentially overlapping organizational principles and distinct patterns or modes of connectivity within the structure.

In the present study, we aimed to uncover the principles of functional organization of the pons to better characterize its role in the corticocerebellar pathway. To this end, we reconstructed functional connectivity gradients within the pons based on its connectivity from the cerebral cortex and to the cerebellar cortex in a large resting state functional magnetic resonance imaging dataset of 1003 individuals from the Human Connectome Project (Van Essen et al., 2013). Given the well documented challenges of fMRI in the brainstem (e.g., Beissner, 2015), the averaging of a large number of datasets available from the HCP is required to improve the signal to noise ratio and allow a reliable delineation of connectivity. We projected the principal pontine gradient to its input/output structures (cerebral and cerebellar cortices) to map their functional correspondence and contextualize the putative integrative role of the pons. The present study advances our understanding of organization of the human cortico-ponto-cerebellar pathway and contributes to evidence that the pons is not a simple relay but serves to integrate information from disparate cortical areas.

## **4.3 Materials and Methods**

### **4.3.1 Connectivity Dataset**

The Human Connectome Project's (Van Essen et al., 2013) dense connectome dataset, downloaded from Connectome-DB (<https://db.humanconnectome.org>), was used for all analyses in the present study. Very briefly, this is a correlation matrix that represents the average correlation between all pairs of brain coordinates (cerebral cortical vertices and subcortical voxels, matched across individuals) for 1003 individuals from the HCP's s1200 release (WU-Minn HCP Consortium, 2017). The raw data was based on 1-hour of resting state fMRI data that were concatenated across four fMRI runs for each of the individual participants. The data collection and preprocessing parameters are described in detail by (Glasser et al., 2016). ((TR) of 720 ms, echo time (TE) of 33.1 ms, and 2 mm isotropic resolution). The procedure used to generate the dense connectome is

detailed in HCP Documentation (Human Connectome Project, 2017). Using HCP’s Workbench Command tools (<https://www.humanconnectome.org/software/workbench-command>), we constrained the whole brain correlation matrix to include only the pons, cerebral cortex, and cerebellar cortex. While the cerebral cortical coordinates were already defined, we utilized a hand-drawn mask of the pons, and a mask of the cerebellar cortex based on FSL’s cerebellar atlas (Diedrichsen et al., 2009) as regions of interest to identify appropriate voxels. The final connectivity matrix included connectivity between 1213 pons grayordinates, 59412 cerebral cortical grayordinates, and 17709 cerebellar grayordinates. Note that we explicitly did not include intrinsic pontine or cerebellar cortical connections, nor did we include cerebellar to cerebral cortex connections. This final connectivity matrix was used as the basis for all subsequent analyses.

#### **4.3.2 Diffusion Map Embedding**

Diffusion map embedding was used to identify five gradients within the pons based on its connectivity with the cerebral and cerebellar cortices. As described above, intrinsic pontine, cerebrocortical, and cerebellocortical connectivity were not considered in the analysis. Analyses were performed using procedures and code adapted from Guell et al. (2018) and available at [https://github.com/xaviergp/cerebellum\\_gradients](https://github.com/xaviergp/cerebellum_gradients), which implements diffusion map embedding with the `mapalign` package (<https://github.com/sensein/mapalign>). Diffusion map embedding is a non-linear dimensionality reduction technique initially introduced by Coifman & Lafon (2006) and applied to human connectivity data in a seminal study by Margulies et al. (2016). Diffusion map embedding allows for the representation of high-dimensional connectivity data, in our case the connectivity between 1213 pons nodes and 77121 cerebral and cerebellar cortical nodes, in a lower dimension space that encapsulates the dominant patterns or gradients of connectivity. Prior to diffusion map embedding, the correlation matrix was transformed into an affinity matrix using the cosine similarity metric. The affinity matrix effectively represents the similarity of connectivity between pairs of pontine nodes. Diffusion map embedding was then performed on the affinity matrix to extract the modes (or components) that represent the connectivity gradients within the pons (ordered by decreasing amount of variance accounted for).

#### **4.3.3 Projection of Pontine Gradients to the Cerebral and Cerebellar Cortices**

In order to investigate the correspondence between pontine gradients and connectivity with the cerebral and cerebellar cortices we then “project” pontine gradients by performing the dot product between the embedding values within the pons (a 1213 by 1 array) and the pons by cerebellar

cortex and pons by cerebellar cortex connectivity matrices (1213 x N nodes arrays) separately as in previous work (e.g., Guell et al., 2020; Katsumi et al., 2023). Each node in the cerebral cortex and cerebellar cortex thereby receives a value representing the relative strength of its connectivity to the pontine voxels, weighted by pontine gradient values. Cerebral cortical projection maps were visualized using Connectome Workbench (Marcus et al., 2011). Cerebellar projection maps were first converted from volumetric space to cerebellar surface-based flatmaps using SUI (Diedrichsen, 2006; Diedrichsen & Zotow, 2015) and then visualized with Connectome Workbench.

#### 4.3.4 Residuals Analysis

We conducted a residuals analysis to directly compare our cerebrocortical and cerebellar cortical gradient projections with the published gradients of Margulies (2016) and Guell et al. (2018). This approach allowed for the quantification of regional differences between these gradients and our gradient projections. The residuals reflect shifts along the unimodal-transmodal continuum in our gradient projections relative to the previously published gradients. In each case, a linear regression was performed with the previously published gradient being predicted by our corresponding projection. Residuals were plotted either on the cerebrocortical surface, or on a volume of cerebellar cortex that was converted to a cerebellar flatmap using SUI (Diedrichsen, 2006; Diedrichsen & Zotow, 2015). All results were visualized with Connectome Workbench.

## 4.4 Results

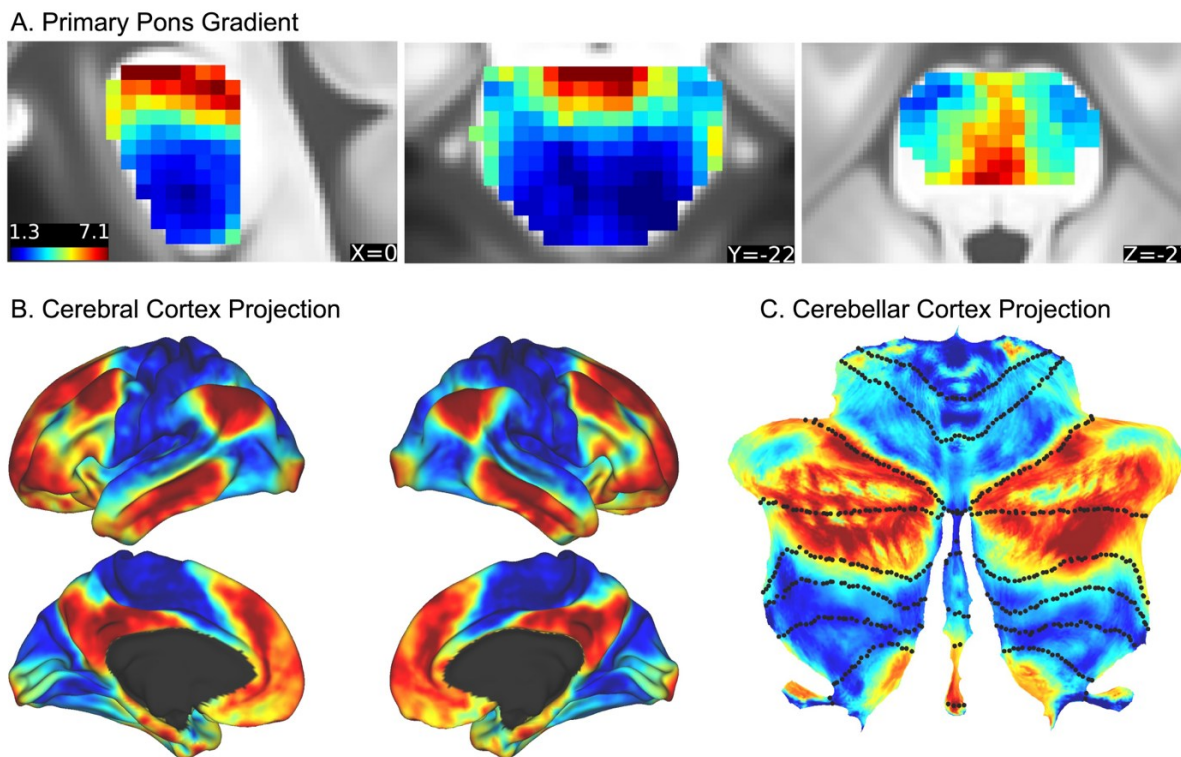
### 4.4.1 Pontine Connectivity Gradients

The first gradient, depicted in Figure 4.1a, explained over 70% of the variability in cortico-ponto-cerebellar functional connectivity (see Supplementary Figure 4.1 for a graph of variance accounted for by each gradient). Similarly coloured voxels exhibit similar patterns of functional connectivity jointly with the cerebral cortex and the cerebellum. We observed two directions of transition within the three-dimensional structure of the pons. First, there is a transition along the rostral-to-caudal axis. Second, in the rostral pons we identified a medial-to-lateral transition. The gradient shows a sharp transition just below the rostral third of the pons, beyond which it remains largely uniform throughout the caudal pons.

When this gradient was projected to the cerebral cortex (see Figure 4.1b) rostral/medial pons shows stronger weighted connectivity to higher-order, association areas, notably areas of the

default mode network (depicted in warm colours). Caudal/lateral pons corresponds to primary and secondary sensorimotor areas including primary motor cortex, pre-motor and cortex and supplementary motor areas, auditory cortex, and secondary visual areas (depicted in cool colours). In addition to primary and secondary sensorimotor areas, we also see correspondence with dorsal and ventral attention network areas. In the cerebellum (Figure 4.1c), rostral/medial pons shows stronger weighted connectivity to Crus I and II and lobule IX (warm colours), whereas lateral/caudal pons corresponds more to lobules I-VI and VIIB-VIIIB (cool colours). The cerebellar projection roughly corresponds to a non-motor versus motor functional organization previously demonstrated in resting-state and task-based work (Buckner et al., 2011; King et al., 2019).

Gradients 2 and 3 (see Supplementary Figure 4.2) appear to be dominated by laterality effects, with sharp transitions of embedding values at the midline of the pons. Gradients 4 and 5 are organized in a medial to lateral and ventral to dorsal manner, respectively. However, given the strong laterality effects observed in Gradient 2 and Gradient 3, and the small proportion of variance accounted for by subsequent gradients (less than 5% each), we do not discuss these further in the present study.

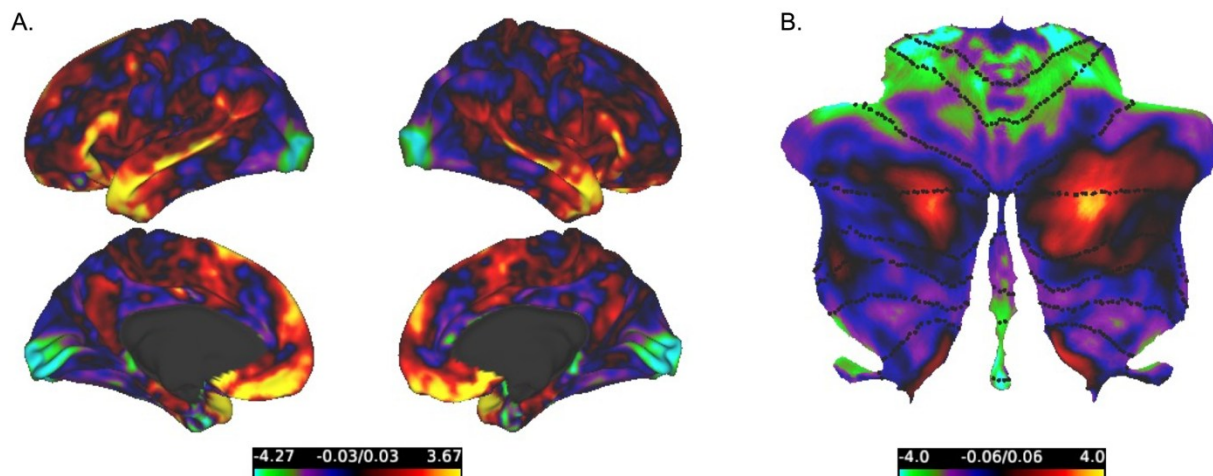


**Figure 4.1.** Pontine connectivity gradient and corresponding cerebral and cerebellar cortical projections. **A.** The primary gradient in the pons based on its connectivity to cerebral and cerebellar cortices, shown on sagittal, coronal, and axial slices ( $x = 0$ ,  $y = -22$ ,  $z = -27$ ). **B.** The cerebral cortex projection of the primary gradient. **C.** The cerebellar cortical projection of the primary gradient, depicted on a cerebellar flatmap generated with SUIT tools (Diedrichsen & Zotow, 2015).

#### 4.4.2 Residuals Analysis

Results of the residuals analysis are presented in Figure 4.2. Residual values reflect shifts in the organization of our gradient projections relative to those of Margulies (2016) and Guell et al. (2018), both of which are interpreted as reflecting a unimodal-transmodal organization. Negative residuals indicate a shift in our projection toward the transmodal end of the gradient (i.e., Default Mode Network), whereas positive residuals indicate a shift towards the unimodal end (i.e., sensorimotor areas). In the cerebral cortex (Figure 4.2a), we identified the most prominent negative residuals in areas of visual cortex, and positive residuals in inferior frontal gyrus, middle temporal gyrus, temporal pole, and medial prefrontal cortex. In the cerebellar cortex (Figure 4.2b) we identified the most prominent negative residuals in areas of lobules IV-VI, vermal portions of VIIIb and IX, and positive residuals in areas of Crus I and Crus II and hemispheric portions of IX.

Residuals appear to show some lateralization effects, in that we identified higher positive residuals in left inferior frontal gyrus, and right Crus I and Crus II.



**Figure 4.2.** Residual analysis comparing our gradient projections to previously published cerebrocortical and cerebellar gradients. **A.** Residuals between our cerebrocortical gradient projection and the principal gradient of Margulies (2016). **B.** Residuals between our cerebellar cortical projection and the principal gradient of Guell et al. (2018). Positive residuals indicate regions where the referenced gradients show higher gradient values compared to ours; negative residuals indicate regions where our gradients projections show higher values.

## 4.5 Discussion

We reconstructed the functional connectivity gradients of the pons based on its connectivity with the cerebral and cerebellar cortices to uncover principles governing its functional organization. We found that the primary functional gradient of the pons, accounting for 70% of the variance, was broadly organized in a rostral-to-caudal and medial-to-lateral fashion that, when projected onto the cerebral and cerebellar cortices, formed gradients that corresponded closely with the unimodal-to-transmodal organization seen in prior work (Guell et al., 2018; Margulies et al., 2016). Rostral and medial areas of the pons were preferentially connected to default mode and frontoparietal network areas, whereas caudal pons aligned with somatomotor, dorsal and ventral network areas of the cerebral and cerebellar cortices. While our results align closely with previously published gradients, the residuals analysis revealed localized differences in our gradient projections in several cerebrocortical and cerebellar cortical areas including visual cortex, inferior frontal gyrus, middle temporal gyrus, and portions of Crus I and II in a manner that may suggest multimodal integration or reconfiguration of cerebrocortical information in the pons. Together, these findings

provide supporting evidence for the notion that the pons has a complex functional organization and serves as an integrative hub in the downstream corticocerebellar pathway.

A rostral-caudal and medial-lateral functional connectivity gradient in the pons is remarkably consistent with anatomical connectivity work in non-human primates (Schmahmann et al., 2004; Schmahmann & Pandya, 1997b) and our recent dMRI tractography work in humans (Rousseau et al., 2022, 2025). Specifically, motor projections to the pons have been shown to terminate preferentially in the caudal half of the pons (Schmahmann et al., 2004), which is also the origin of pontocerebellar fibres projecting to cerebellar motor areas (Brodal, 1979; Rousseau et al., 2022). Prefrontal cerebrocortical areas target the medial pons (Schmahmann & Pandya, 1997a). Specifically, projections originating in medial prefrontal cortex target rostral pons whereas projections originating in dorsolateral prefrontal cortex are distributed along its rostral-caudal extent (Schmahmann & Pandya, 1997a). Different areas of the parietal cortex also show differential patterns of projection to the lateral pons, with the inferior parietal lobule projecting rostrally and superior parietal lobule projecting more diffusely along the rostro-caudal extent (Schmahmann & Pandya, 1989). These anatomical findings are mirrored in our primary gradient, which shows sensorimotor areas clearly localized to the caudal pons and a more complex, and distributed pattern for non-sensorimotor regions. Specifically, medial prefrontal areas and the inferior parietal lobule – both nodes in the default mode network – correspond to the medial and rostral portions of the pons in our gradient. Conversely, the superior parietal lobule and portions of dorsolateral prefrontal cortex show a wider distribution of connectivity with the caudal two thirds of the pons. Broadly, our findings correspond with organizational patterns demonstrated in the anatomical literature.

Our findings differ from the anatomical literature in ways that highlight the complexity of comparisons between structural and functional connectivity. For instance, detailed work in macaques has demonstrated that temporal lobe corticopontine projections arise predominantly from the upper bank of the superior temporal sulcus and the superior temporal gyrus (Schmahmann & Pandya, 1991). However, in our gradient, we found that the rostral maps onto large areas of middle and inferior temporal gyri. Without excluding the possibility that the anatomical connectivity is different in humans, this is more likely to reflect indirect connections mediated at the level of the cerebral cortex (Damoiseaux & Greicius, 2009). These temporal regions are highly interconnected with other cortical areas with known projections to the pons in non-human primates

(e.g., inferior parietal lobule). It follows that the functional connectivity of the pons, and by extension our gradient, is shaped by a combination of direct and indirect cortical inputs. In addition, though we excluded pons-pons connectivity from our analysis we cannot rule out potential indirect contributions that could be mediated by pontine interneurons (Mihailoff et al., 1992) and/or projections from the deep cerebellar nuclei (Schwarz & Thier, 1999).

Our findings also differ from those of Margulies (2016) and Guell et al. (2018) in a manner that may suggest a possible integrative function in the pons. In our residual analysis, when comparing our gradient projections with the primary gradient of Margulies (2016), we found strong negative residuals in visual cortex. This finding suggests that (relative to the canonical gradient presented by Margulies (2016)) visual areas in our gradient projection are situated towards the middle of the gradient, whereas in Margulies (2016) these same areas clearly correspond to the unimodal end of their primary gradient. The anatomical tract-tracing work provides compelling evidence that ventral stream visual areas project along the rostro-caudal extent of the pons (Schmahmann & Pandya, 1993). With our dataset, connectivity from visual areas to the pons do not appear to be spatially specific, in line with these anatomical findings. This results in the projection to this area sampling values from both ends of the pontine gradient, leading to more intermediate values. By extension, this also implies that visual inputs to the pons are in proximity to both motor inputs and inputs from associative cortical areas, thus providing the spatial proximity necessary for integration (Schwarz & Thier, 1999). In our residual analyses, we also found strong positive residuals in the inferior frontal gyrus, middle temporal gyrus, and regions of the cerebellar cortex that are recruited during language production tasks (King et al., 2019). This pattern suggests that, in our gradient projection, these language related regions are shifted towards the unimodal end of the functional hierarchy relative to the cerebrocortical and cerebellar cortical gradients of Margulies (2016) and Guell et al. (2018). It may imply that at the level of the pons, language and sensorimotor channels may be more integrated or overlapping than in the cerebral or cerebellar cortices. This interpretation aligns with our structural gradient findings (Rousseau et al., 2025) showing connections from primary motor cortex and inferior frontal gyrus terminating in nearby areas of the pons and is compatible with tract-tracing work done in non-human primates (Schmahmann, Rosene, et al., 2004; Schmahmann & Pandya, 1997a).

The anatomical tracing work shows that corticopontine terminations from different cerebrocortical areas are interdigitated with one another, bringing inputs from different

cerebrocortical areas (e.g., visual and motor) in close proximity to one another. Schwarz and Thier (1999) propose that this arrangement complements filter-like characteristics of pontocerebellar neurons to allow the pons to integrate signals from different regions before projection to the cerebellum as mossy fibres. They suggest that depending on context or specific environmental demands, different combinations of corticopontine inputs can be integrated and relayed to the cerebellum. Our findings align with and support portions of this theory by demonstrating the convergence of different functionally relevant inputs to overlapping zones within the pons. Thus, our results provide evidence that visual information may be integrated with motor related signals (e.g., for visually guided reaching) or coupled with non-motor signals at the level of the pons. Similarly, language-related inputs may be integrated with sensorimotor information or with signals from multimodal association areas depending on contextual demands. This fits with the theoretical work suggesting that the pons is a structure with a dynamic functional organization that evolves with behavioural demands (Schwarz & Thier, 1999). This interpretation should, however, be considered in light of the small size of the pons and the level of spatial resolution of the dataset employed in this study, which may limit spatial specificity and contribute to the overlap of different inputs

The fact that cortical, pontine, and cerebellar nodes of the cortico-ponto-cerebellar pathway appear to be organized in a similar hierarchical fashion may be interpreted as the cortex imprinting its functional architecture on downstream areas. Alternatively there may be more of a reciprocal relationship such that the pons and cerebellum may together influence integration at the level of the cortex (Schwarz & Thier, 1999). Developmentally, the spatial organization of pontine neurons is established early, and these neurons provide cues that guide the termination patterns of corticospinal axons (Kratochwil et al., 2017). This suggests that the pons actively reshapes the configuration of cerebrocortical inputs, rather than merely reflecting their organization. Additionally, McAfee and colleagues (2022) argue that cerebellar outputs to the cerebral cortex shape connectivity patterns by modulating the spatial and temporal coherence of neuronal populations based on specific situational demands. They draw on the highly influential communication through coherence framework that holds that effective communication between different cortical areas is facilitated by coherence in their oscillatory activity (Fries, 2015). Cerebellar outputs to the cerebral cortex (relayed by the thalamus) have been shown to modulate cortical coherence to facilitate the functional coupling between different cortical regions (McAfee

et al., 2022). Cerebellar outputs mediate coherence between primary motor and somatosensory cortices in rodents (something first demonstrated by Popa et al. (2013)) and cerebellar stimulation in humans modulates the coherence between homologous prefrontal areas, which is associated with better working memory performance (McAfee et al., 2022). The extent of thalamocortical projections, arising from cerebellar nuclei, that target prefrontal and parietal association areas (Dum & Strick, 2003) suggests that this modulatory role may extend to the coordination of large scale cerebrocortical networks like the default mode network (DMN). In fact, transcranial magnetic stimulation of Crus I and II of the cerebellum is associated with increased DMN resting-state connectivity (Halko et al., 2014). Given compelling evidence for the cerebellum's role in coordinating cerebrocortical activity, we, along with others (e.g., Schwarz & Thier, 1999), hypothesize that the pons contributes to the integration of cerebral inputs prior to cerebellar processing.

Relative to prior gradient work (e.g., Guell et al., 2020; Katsumi et al., 2023; Margulies et al., 2016) with the same or similar sample size, the cerebral cortex and cerebellar cortical projection gradients presented here exhibit more “noise”. This can be appreciated as a relative decrease in spatial smoothness across the gradient projections. Even with this large sample of high-quality fMRI data used in the present study, we still face important limitations related to low SNR in the pons that represent a barrier to more granular investigations of its functional topography. For instance, while there may be important information within the other gradients, the potential influence of noise and prominent laterality effects led us to focus our interpretation on the first gradient, which we are confident reflects a robust and meaningful pattern. The brainstem represents a worst-case scenario for functional MRI: it is compact in size with densely packed and functionally distinct nuclei, there is poor differentiation of gray matter nuclei from surrounding white matter at the resolutions possible with in-vivo MRI, and there is both elevated physiological noise and magnetic susceptibility artefacts (Sclocco et al., 2018). Group registration may also lead to misalignment of smaller brainstem nuclei, which serves to further reduce the effective resolution of fMRI (Sclocco et al., 2018). Some of these limitations are partially mitigated by averaging a large number of high qualities rsfMRI datasets, as was also done in the present study. However, this approach may also affect the level of granularity with which we can investigate the pons.

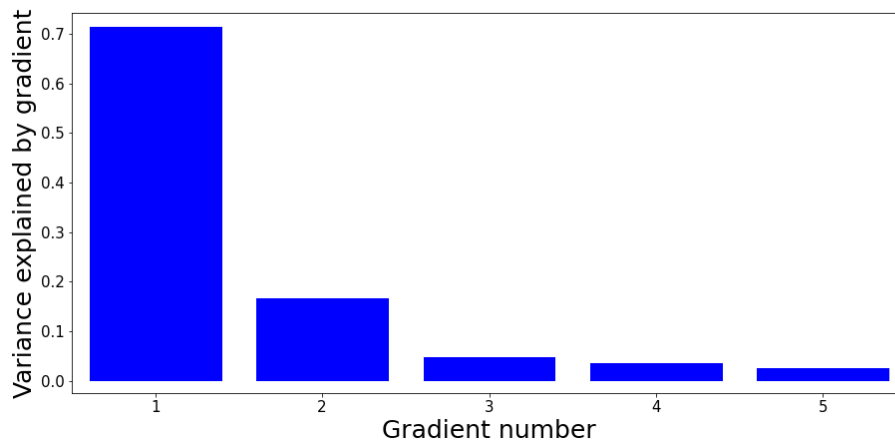
Future fMRI studies of the pons would benefit from the collection of physiological recordings, use of models of the hemodynamic response function tailored to the brainstem,

probabilistic atlases of brainstem nuclei that can improve inter-subject registration, and the use of high-field (e.g., 7T) MRI data (Groot et al., 2024; Sclocco et al., 2018). Mohamed and colleagues (2024), for instance, developed a specific protocol for brainstem fMRI with 3T and demonstrated significant improvements in SNR compared to an HCP style protocol. Improved brainstem data quality will lend itself to understanding the spatial distribution of pontine activation in task-based paradigms (e.g., similar to the approach of King et al. (2019) for the cerebellum) and allow for the further delineation of pontine resting connectivity gradients.

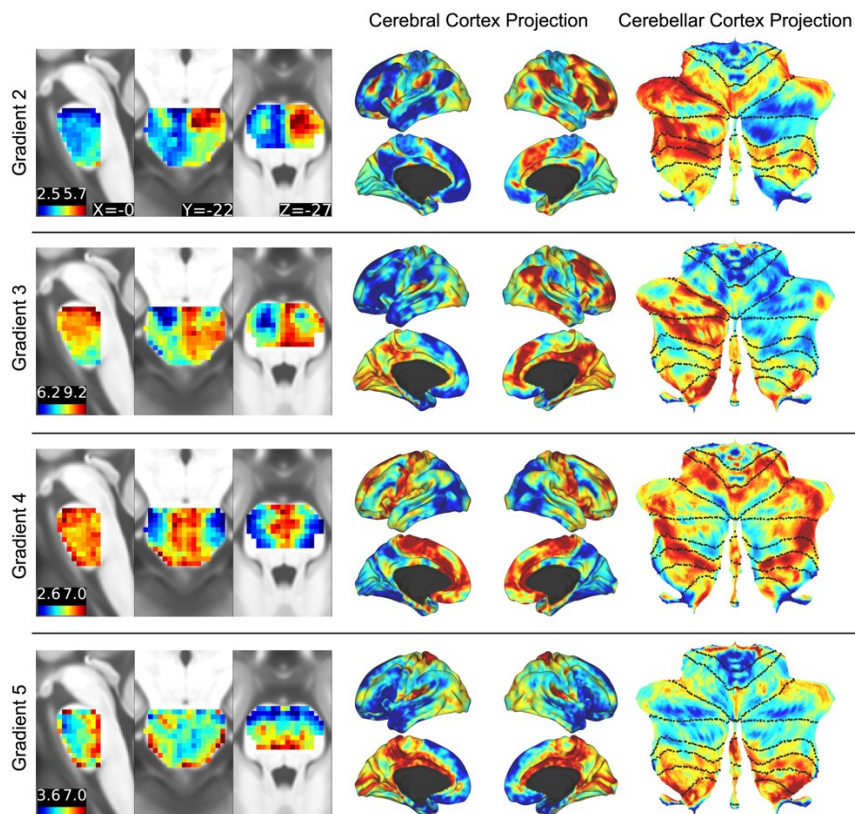
## **4.6 Conclusion**

We reconstructed the primary functional gradient in the pons based on its connectivity with cerebral and cerebellar cortices. Our analysis demonstrated that the primary gradient is organized in a rostrocaudal and mediolateral fashion that is consistent with non-human primate tract tracing and dMRI studies in humans (Rousseau et al., 2022, 2025; Schmahmann et al., 2004; Schmahmann & Pandya, 1997b). When projected to the cerebral and cerebellar cortex, the pontine-derived gradient aligns closely with the primary gradients observed within each of these separately (Guell et al., 2018; Margulies et al., 2016) but with important regional differences pointing to a level of reorganization of cerebrocortical information in the pons. Our findings provide initial in-vivo human evidence for functional integration in the pons that suggests that the pons may bind functionally relevant inputs from different cerebrocortical areas. Together our results highlight the pons as a dynamic and integrative structure that plays a complex role in the cortico-ponto-cerebellar pathway. These findings add impetus to further investigation into the nature of its contributions to information integration and transformation along this pathway.

## 4.7 Supplementary Information



**Supplementary Figure 4.1.** Proportion variance accounted for by the five reconstructed gradients in the pons. Gradient 1 accounts for approximately 70% of the variance in the connectivity data.



**Supplementary Figure 4.2.** Pontine gradients 2-5 are depicted on the left. The cerebral cortex projection for each of the gradients is depicted in the centre, and the cerebellar cortical projection (displayed on a cerebellar cortical flat map) is depicted on the right.

## Chapter 5. Discussion

### 5.1 The Organization of Human Cortico-Ponto-Cerebellar Connectivity

#### 5.1.1 Recapitulation of Key Findings

The work described in this dissertation aimed to advance our understanding of cerebellar connectivity using non-invasive neuroimaging methods. Key findings of the three studies are presented in Figure 5.1. In our first study (Chapter 2) we reconstructed the pontocerebellar segment, performing dMRI tractography between the pons and individual cerebellar lobules in a large adult cohort. We mapped the spatial distribution of lobule-specific projections and identified a topographic organization: the rostral pons was preferentially connected to cerebellar areas implicated in non-motor function, whereas caudal pons was more strongly connected to cerebellar motor areas (Figure 5.1a). Connections traversing the middle cerebral peduncle were organized in a medial-lateral configuration, with connections to cerebellar motor areas situated medially and non-motor connections situated more laterally. These findings aligned with both the non-human primate tract-tracing work and older dissection work in humans (Brodal, 1982; Jamieson, 1910). On the basis of these tractography results, we segmented the middle cerebral peduncle, and the pons based their connectivity profiles with individual cerebellar lobules. These white matter segmentations represent a tool for future researchers seeking to investigate regional changes in white matter microstructure of the pons and MCP. To our knowledge, this work represents the most detailed effort to precisely map pontocerebellar connectivity in humans and contributes to our understanding of the topographic organization of this pathway. At the same time, this study highlighted the challenges of performing tractography in this region and underscored the role of emerging methods (discussed in the Future Directions section) for achieving more precise white matter reconstructions.

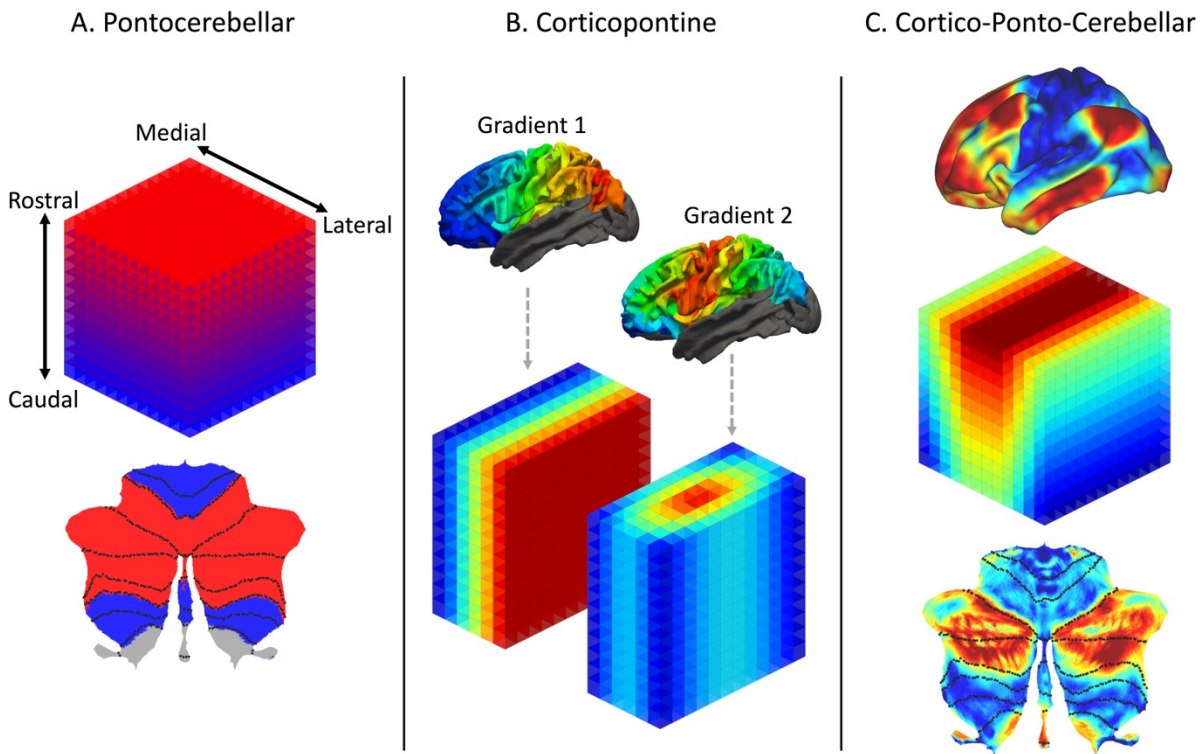
In our second study (Chapter 3), we sought to characterize the organization of the corticopontine segment with dMRI tractography. In our first study we were limited to identifying organizational patterns along the rostro-caudal and dorsal-ventral axes of the pons, due to the inability to resolve terminations of streamlines in the pontine nuclei. By contrast, the corticopontine approach allowed us to capture medial-lateral organization in the pons (but not rostro-caudal), complementing our initial findings and providing a more complete characterization

of its topographic organization. Corticopontine tractography represented a much larger methodological challenge compared to the pontocerebellar segment, as tractography is performed over a much longer distance, with massive convergence of the much larger cerebrocortical area onto the pons. This required careful calibration of tractography parameters and the implementation of anatomical constraints to maximize the validity of reconstructed pathways. While our first study used cerebellar lobules as discrete anatomical units for performing tractography and generating segmentations of corticopontine connections, here we applied a more data-driven approach: utilizing gradients to capture overlapping patterns of connectivity within the data. As noted previously, the lobules are useful anatomical landmarks, but do not closely correspond to functional boundaries (King et al., 2019). The gradient method allowed us to forgo pre-defined anatomical parcellations and instead capture continuous transitions in corticopontine connectivity to tease apart overlapping organizational patterns in this pathway. In our first study, we observed a differential pattern of connectivity between the pons and motor and non-motor areas of the cerebellum. There was, however, a considerable degree of overlap between these connections in the pons and there was no clear boundary between motor and non-motor connections. The majority vote segmentation approach, while practical for generating lobule specific white matter maps, introduced boundaries that undercut the more continuous nature of connectivity transitions in this region. The use of connectivity gradients in the second study offered a means of extracting and visualizing continuous transitions in corticopontine connectivity. We performed this analysis in a subset of participants from our first study and validated our findings in a high-resolution postmortem sample of the human brainstem. Our primary gradient, present at the individual level and across both datasets, reflected a medial-to-lateral organization within the pons that corresponded to a rostral-caudal organization in the cerebral cortex (Figure 5.1b). Our secondary gradient, a core-shell organization in the pons, corresponded to a central to distal organization in the cerebral cortex that was centered on sensorimotor cortex. Our results recapitulated core findings in the non-human primate tracing work, demonstrating frontal areas projecting to the most mesial parts of the pons, parietal areas to lateral pons, and sensorimotor areas to central pons (Schmahmann et al., 2004b; Schmahmann & Pandya, 1989, 1995). The superimposed organizational patterns of corticopontine connectivity – detectable only through the gradient approach – also extended our understanding of the pathway by suggesting a more complex mapping of cerebrocortical inputs onto the pons. In other words, the connections between the

cortex and the pons appear to follow two organizational or transformational principles, that when considered together suggest a mixture of preserved cortical topography with a degree of reorganization. This complex arrangement of connectivity aligns with the foundational tracing work (e.g., Brodal, 1978) showing inputs from disparate cortical sites being brought into close proximity, but without overlapping, in the pons. In our view, this arrangement may underlie the integration of functionally diverse cerebrocortical information at the level of the pons.

The configuration of corticopontine anatomical connections revealed by connectivity gradients – namely the apparent preservation and reconfiguration of cerebrocortical organization – suggested that the pons could serve an integrative role in the downstream pathway. Investigating this more directly necessitated a change of methodology. Our dMRI studies are useful in demonstrating macroscale patterns of anatomical connections and suggest an arrangement that may support integration; however, they do not directly inform our understanding of how information may be dynamically integrated in the pons. In our third study (Chapter 4) we therefore shifted from dMRI tractography analyses to a rsfMRI functional connectivity analysis, applying a gradient based approach to characterize the functional organization of the pons based on its connectivity with the cerebral and cerebellar cortices. Here we found that the functional connectivity of the pons was organized along a rostrocaudal and mediolateral gradient (Figure 5.1c). One end of the gradient, strongest within the rostro-medial pons, mapped onto default mode and frontoparietal control regions of the cerebral cortex, and cerebellar regions functionally connected to these. On the opposite end of the gradient, the caudal pons was preferentially connected to sensorimotor and dorsal and ventral attention network cerebrocortical areas, and corresponding cerebellar areas. These findings recapitulated the unimodal to transmodal organization previously seen in cerebrocortical and cerebellar connectivity gradients (Guell et al., 2018; Margulies et al., 2016). The pattern of organization in the pons was also broadly compatible with the combined findings of our first two studies, which separately demonstrated rostrocaudal and mediolateral organization of pontocerebellar and corticopontine structural connectivity. The functional findings extended our structural connectivity work in demonstrating convergence of functionally distinct cerebrocortical networks within distinct and overlapping areas of the pons. This supports the conceptualization of the pons as a functionally dynamic structure that may be involved in coordinating information from different cerebrocortical areas, depending on the specific context (Schwarz & Thier, 1999). These findings raise important questions regarding the

exact mechanisms underlying this hypothesized integration. In particular, there is a need to understand local pontine circuitry and the manner in which feedback projections from the deep cerebellar nuclei to the pons modulate or shape pontine activity. These lines of inquiry will inform our understanding of how the pons reconfigures cerebrocortical inputs before they are relayed to the cerebellum.



**Figure 5.1.** Schematic of the key findings from the three studies presented in this dissertation. To facilitate comparisons, the pons is represented as an array of cubes colour-coded according to organizational principle/s observed in each of the studies.

**A.** Mon-motor versus motor organization of pontocerebellar structural connectivity. Rostral pons (red) is more highly connected to cerebellar non-motor lobules (depicted in red in the cerebellar cortical flatmap). Caudal pons regions are more connected to motor lobules (depicted in blue on the cerebellar cortical flatmap).

**B.** Corticopontine structural connectivity gradients organized in medial-lateral and core-belt fashion, represented in the left pons, along with their respective cerebrocortical projections.

**C.** Functional connectivity gradient of the pons based its connectivity with the cerebral and cerebellar cortices and the corresponding gradient projections.

To summarize, our primary aim across this series of studies was to uncover the organizational principles of corticocerebellar connectivity – specifically focusing on the feedforward limb linking the cerebral cortex to the cerebellar cortex via the pons. Collectively, the

three studies in this thesis provide evidence for a complex pathway whose organization is characterized by both segregation and integration. That is, while aspects of the anatomical and functional organization of the cerebral cortex are maintained across the different levels of the pathway, there is also evidence for convergence or the mixing of different cerebrocortical channels. The first study (Chapter 2) highlighted the spatial segregation of pontocerebellar motor and non-motor connections in a manner compatible with the non-human primate work (e.g., Brodal, 1979, 1982). The second study (Chapter 3) demonstrated smooth transitions in corticopontine connectivity, and transformational principles for the mapping of cerebrocortical inputs on to the pons. Like our first study, this highlighted a degree of segregation in demonstrating that different cerebrocortical areas map to different zones of the pons. The use of a gradient approach, however, allowed us to infer the presence of superimposed corticopontine organizational principles, suggesting a more complex arrangement that we hypothesize allows for the integration of different sources of information at the level of the pons. The transition to a functional approach in the final study (Chapter 4) allowed us to investigate this question of integration within the pons more directly. We found a pattern of both convergence and differentiation of distinct cerebrocortical networks within specific zones of the pons in a manner that appeared to be functionally meaningful and broadly compatible with our structural findings.

### 5.1.2 Summary of Main Contributions to the Cerebellar Connectivity Literature

Our studies can be considered as a part of an effort to understand the function and contributions of the cerebellum. Together, the first two studies (Chapters 2 and 3) are bridges to the foundational anatomical tracing studies in non-human primates. While these studies may not introduce entirely novel discoveries regarding the pathways in question, they provide strong evidence that organizational principles identified in non-human primates are applicable to humans. As discussed further in subsequent sections, these studies and the reconstructions of corticocerebellar white matter that they produced, represent invaluable tools for future cerebellar research. While these studies were important in extending the non-human primate work to humans, and generating tools for future researchers, the final study (Chapter 4) represents an entirely novel contribution to our understanding of the role of the pons within corticocerebellar circuits in humans. It provides insights regarding the functional organization of this structure that go beyond direct extensions of the animal work. It also opens multiple avenues for future investigation and

brings to the forefront the idea that pons is a functionally significant structure deserving of greater scientific attention.

## **5.2 Future Directions**

### **5.2.1 Mesoscale White Matter Reconstruction using Novel Imaging Techniques**

Our first two studies represent the most comprehensive efforts to map corticopontine and pontocerebellar structural connectivity in humans. We chose to reconstruct these two segments of the feedforward limb, because of the novelty and the feasibility of tractography in these regions. The prospect of accurately reconstructing the entirety of the corticocerebellar loop with dMRI tractography, while appealing, is unrealistic with in-vivo methods. Connections between the cerebellar cortex and the dentate nucleus have been mapped by a previous study by Steele et al. (2017) but the rest of the feedback limb has not yet been systematically characterized. This represents a considerable challenge from a methodological perspective. The nucleothalamic segment is narrow, travels along complex trajectory and has a diffuse and complex pattern of termination within the thalamus (Schmahmann & Pandya, 1997b). Accurate reconstruction of the thalamocortical segment presents an even more significant challenge, due to the difficulty in identifying precise tracking origins in the thalamus, and the complex intermingling of corticothalamic and passing corticopontine fibres in this area. We focused heavily on corticocerebellar loops in this dissertation, but there are a number of other related fibre systems that remain to be explored. Notably: the olivocerebellar pathway that contributes the climbing fibre input to the cerebellar cortex (Azizi, 2007). There are also reciprocal connections between the basal ganglia, the pons, and cerebellar nuclei (Bostan & Strick, 2018). In our view these smaller pathways also are also infeasible to accurately reconstruct with current dMRI tractography, yet these are critical to our understanding of cerebellar connectivity and function.

While dMRI continues to improve, novel methods of reconstructing white matter connections at levels of spatial resolution between neuronal tract-tracing and dMRI represent a promising future approach. These techniques may allow for more precise delineations of previously reconstructed connections (i.e., corticopontine and pontocerebellar) and open the door to mapping additional cerebellar connections, such as the nucleothalamic pathway. Two related microscopy techniques that can be used on postmortem brain tissue are Polarized Light Imaging (PLI) and polarization-sensitive Optical Coherence Tomography (psOCT) (Bullock et al., 2022).

These are sometimes collectively referred to as label-free techniques, because they do not require the use of stains and instead leverage the optic properties inherent to white matter (Bullock et al., 2022). PLI, uses mounted serial sections of brain tissue and exploits the birefringent properties of myelinated axons to extrapolate fibre orientations (Axer et al., 2011a; Liu et al., 2023). psOCT similarly exploits the birefringence of white matter, this time acquiring images from a block face of brain tissue and using polarized light to generate depth-resolved images of axonal orientation (Wang et al., 2018). While PLI has been used for longer, it suffers the limitation of needing to slice and mount tissues before images are acquired (Axer et al., 2011a) . This is labour intensive, and slicing introduces distortions in the tissue (Wang et al., 2018). psOCT lends itself to automation through an approach referred to as automatic serial sectioning psOCT (as-psOCT). Here a depth resolved volume is acquired on a block face of a tissue sample, the tissue is automatically sectioned, and the process is repeated to obtain multiple three-dimensional volumes representing each slice of the sample (Wang et al., 2018). Individual slices in the case of PLI, or volumes in the case of psOCT are stitched together using registration methods. Tractography is performed on the basis of local fibre orientations, in a way which is analogous to dMRI tractography (Wang et al., 2018). Compared to dMRI, however, both techniques allow for levels of spatial resolution in the range of 1-15 micrometres (Marchant et al., 2024). The level of resolution may be insufficient to visualize individual axons but is orders of magnitude superior to that of the best dMRI acquisitions (Wang et al., 2018). In our second study, for example, we used a high-resolution postmortem diffusion dataset with 200 micrometre isotropic resolution – approaching the upper limit of what is currently achievable with dMRI (Marchant et al., 2024). These label-free techniques allow us to bridge the divide between in-vivo dMRI tractography and non-human animal tract tracing (Marchant et al., 2024). They enable more accurate reconstructions and make it possible to resolve pathways that are infeasible to reconstruct with dMRI.

A promising future direction is to use these label-free approaches in conjunction with in-vivo dMRI methods. High resolution fibre orientation information and tractography, obtained on postmortem samples with the label-free approaches, can be used to guide and refine dMRI in-vivo tractography (Blanke et al., 2023; Yendiki et al., 2022). Specifically, anatomical priors generated with these methods could be used to constrain in-vivo tractography to reduce the number of implausible connections (Rheault et al., 2020). For example, in our first study (Chapter 2) we observed unexpected behaviour of streamlines connecting the pons and the anterior lobe.

Specifically, we found two distinct bundles originating in rostral and caudal pons, that merged before entering the anterior lobe. We hypothesized that this behaviour was caused intersecting bundles in close proximity connecting to different areas cerebellum. It is unlikely that this reflected a real anatomical feature, and this anomaly underscored the complexity of the white matter architecture of this area, and the limitations of in-vivo dMRI data in fully accurately capturing some these intricacies. If we are able to reconstruct the pontocerebellar pathway on a more granular scale with one of these aforementioned techniques, we can use these as priors in an in-vivo analysis, preventing the tracking of streamlines along anatomically improbable trajectories. For the moment, these techniques are applicable to smaller brain samples (e.g., Wang et al., 2018; Zeineh et al., 2017) but there is no fundamental reason why the method cannot eventually be scaled up to the whole brain (Wang et al., 2018). Overall, multimodal approaches that combine fine-grained neuronal tracing with animal models, intermediate scale postmortem imaging in humans, and continued advances in in-vivo dMRI methods represent a promising avenue towards more precise mapping of human brain connectivity (Marchant et al., 2024; Yendiki et al., 2022). The multimodal, multiscale approach is especially pertinent to the study of cerebellar connectivity, given the numerous challenges that have been detailed in this dissertation. These include the densely packed and intersecting white matter pathways, complex fibre trajectories, and the difficulty in clearly resolving the pontine nuclei (Lundell & Steele, 2024).

### **5.2.2 Research and Clinical Applications of Corticocerebellar Tractography**

Cerebellar involvement in normal and pathological processes is well established, but there is a need for greater precision in quantifying how these processes affect cerebellar white matter. The goals of the first two studies were to reconstruct corticocerebellar connections with new levels of precision, these results are valuable both to future cerebellar researchers and clinicians. Our lobule specific parcellations of pontocerebellar white matter have been transformed to a commonly used MNI standard space. These parcellations can be used for the extraction of white matter microstructural parameters such as diffusion tensor metrics like fractional anisotropy and mean diffusivity. In terms of the corticopontine tractography results, we projected embedding values in the pons along the corticopontine tractogram. These projected embedding values can be used as the basis for segmenting this large tract into discrete segments that can be used to extract microstructural parameters. These MNI space white matter reconstructions will be made publicly available on the lab's GitHub page (<https://github.com/neuralabc>). These allow for improved

precision in tracking region-specific differences or changes in the white matter connecting to the cerebral cortex to the cerebellum. They can be used in the context of group comparisons, for instance in evaluating white matter differences in individuals with autism spectrum disorder, a condition with well-established cerebellar involvement (D’Mello & Stoodley, 2015). Microstructural parameters of different segments of these pathways can be related to different symptoms, helping to elucidate the anatomical correlates of motor and non-motor (e.g. social cognitive) symptoms of the condition. Alternatively, they can be used to track the progression of regionally specific white matter microstructural changes in degenerative conditions like the spinocerebellar ataxias. Prior studies have often used whole brain approaches (e.g., Hernandez-Castillo et al., 2016; Olivito et al., 2017) in which microstructural parameters are compared across a white matter skeleton of the entire brain. These approaches may lack sufficient statistical sensitivity when there are specific questions related to corticocerebellar connections.

Our white matter segmentations can also be used in conjunction with a tool developed in our lab – MVComp – for performing multivariate analysis of brain microstructure (Tremblay et al., 2024). Integrating our corticopontine and pontocerebellar white matter segmentations with this multivariate framework improves anatomical precision and sensitivity in studying corticocerebellar white matter microstructure. MVComp combines multiple microstructural features into a single model that can then be used for group and individual level comparisons. There are a multiplicity of different metrics based on different models of diffusion (e.g., diffusion tensor, fODF) that aim to assess white matter microstructural properties (Tremblay et al., 2024). Individual metrics, however, are not physiologically specific and do not correspond in a one-to-one fashion to white matter properties (Tardif et al., 2016; Tremblay et al., 2024). The combination of several different parameters in a multivariate framework, may lend itself to greater sensitivity in capturing the biological properties of the tissue being studied (Tremblay et al., 2024). In addition to the more standard group level analyses, our white matter parcellations, in combination with a multivariate framework like MVComp, may be used to perform comparisons and predictions at the individual level (Tremblay et al., 2024). For instance, they may be used to track the progression of white matter changes in individual patients with spinocerebellar ataxia and relate these changes to symptom progression. A related application is using our bundle reconstructions and the multivariate framework to derive biomarkers for predicting clinical outcomes. In sensorimotor stroke, and Parkinson’s disease, the cerebellum has been hypothesized play a compensatory role

(Baker et al., 2023; Schulz et al., 2017; Simioni et al., 2016; T. Wu & Hallett, 2013). By quantifying the integrity of corticocerebellar white matter in a more precise fashion, we may be better able to predict the extent to which the cerebellum can engage in this compensatory role.

Accurate mapping of cerebellar connectivity is also important from an interventional perspective, as both invasive and non-invasive cerebellar stimulation represents promising therapeutic approaches in a range of neurological or psychiatric conditions (Benussi et al., 2023; Miterko et al., 2019; van Dun et al., 2018). There is promising preliminary work that has demonstrated that direct stimulation of the dentate nucleus in individuals having experienced sensorimotor stroke improves clinical outcomes (Baker et al., 2023). Individuals receiving stimulation showed improvement in motor function that was related to increased brain metabolism in the ipsilesional areas. Cerebellar stimulation has also been investigated in the context of refractory epilepsy, with promising findings in animals models that combined simultaneous recording in the cerebral cortex (to monitor seizure activity) and optogenetic stimulation of the cerebellum (Streng & Krook-Magnuson, 2021). In both of these examples, the success of interventions depends in part on the specificity of anatomical information. For instance, in the case of direct stimulation to the dentate nucleus in sensorimotor stroke, we need to be confident that stimulation is being applied to the precise location of the dentate with downstream projections to sensorimotor areas. This also represents an area in which a multimodal approach that integrates both structural and functional data is particularly valuable. Diffusion MRI based reconstructions inform our understanding of the organization of white matter pathways at the individual level, while functional imaging provides insights into the functional organization of gray matter areas like the cerebellar cortex or dentate nucleus. High resolution individualized dMRI tractography – guided by anatomical constraints obtained with psOCT – can be combined with functional parcellations of both the cerebellar cortex (e.g., Nettekoven et al., 2024) and cerebellar nuclei (e.g., Guell et al., 2020). The convergence of these complementary sources of information will help to optimize interventions by enabling anatomically precise, and individually tailored stimulation strategies.

### **5.2.2 What Role Do the Pontine Nuclei Play in Brain Function?**

Our final study offered evidence of functional integration at the level of the pons. Further investigation of the dynamic characteristics of this structure requires a multipronged approach with both cellular and systems level investigations. While this question can be approached through a

multitude of potential studies, we highlight a few select directions that we think are both feasible and informative. Schwarz and Thier (1999) conceive of the pontine nuclei as functioning as an adaptive filter. They actively shape which cortical inputs are conveyed to the cerebellum (i.e., what inputs from where are transmitted). Integration at the level of the pons is complex. It is well established that individual pontine neurons do not receive convergent input from multiple cortical areas (Schwarz & Thier, 1999). The presence of pontine interneurons has been demonstrated in non-human primates (Cooper & Beal, 1978; Mihailoff et al., 1992), but their extent and the nature of their interactions are largely unexplored. It also appears that pontine projection neurons do not form intrinsic collaterals within the pontine nuclei (Schwarz & Thier, 1999). In the Schwarz and Their (1999) view, the pons routes specific combinations of channels to the cerebellum. Their framework emphasizes the role of projections from the deep nuclei to the pons in modulating the excitability of specific groupings of pontine neurons. While there is research documenting these projections in non-human primates (e.g., Chan-Palay, 1977; Teune et al., 2000) and demonstrating their role in modifying excitability of pontine neurons (Xiao et al., 2023) their spatial distribution – and putative topographic organization – are not well characterized. As a concrete example, corticopontine inputs from medial prefrontal cortex and the inferior parietal lobule, terminate in distinct but potentially adjacent patches within the pons. In humans (based on resting-state functional connectivity) we have evidence that these separate channels appear to overlap in cerebellar cortex, particularly within Crus I and II. Do pontine interneurons mediate interactions between projections originating in these functionally related frontal and parietal areas? Do the feedback projections from the deep cerebellar nuclei play a role in synchronizing their channels before they are relayed to cerebellum? In our view, there are two potential directions for future anatomical investigations: First, mapping the putative topographic organization and distribution of feedback projections from the deep cerebellar nuclei to the pons with systematic tract tracing studies. There is clear evidence for topographic organization of dentate nucleus outputs to the thalamus in non-human primates (Dum & Strick, 2003). Future tracing studies can address the following questions: do connections from the dentate to the pons show a similar topography, and what is the size and spatial distribution of their terminal fields within the pons. Second, we consider better characterization of pontine interneurons to be essential. For instance, immunohistochemistry (Border & Mihailoff, 1990) and *in situ* hybridization techniques (Jin & Lloyd, 1997) can be used to map the distribution of GABAergic interneurons in the pons. Serial block-face electron

microscopy (Denk & Horstmann, 2004) can be used to obtain nanometer level resolution volumes of pontine tissue, and allow for the delineating of pontine interneuron trajectories. Anatomical insights gathered from these research directions, together with further electrophysiology and optogenetic manipulation studies of the pons, will deepen our understanding of functional integration within the pons.

Questions regarding the functional organization of the pons, and the view that it is an adaptive filter can also be approached through non-invasive functional neuroimaging. In our final study we emphasized the challenges of conducting fMRI studies in the brainstem and stressed the need for brainstem optimized acquisitions. These challenges include poor signal-to-noise ratio, and the dense packing of functionally distinct nuclei within a small anatomical area (Beissner, 2015). These challenges, and suggested optimizations, have been documented in depth by others (e.g., Beissner, 2015; Beissner et al., 2014; Brooks et al., 2013). To our knowledge, however, there have been no systematic investigations of the parameters for reliably detecting meaningful effects in these structures. For example, the seminal study King et al. (2019) demonstrated a complex functional organization of the cerebellum based on a comprehensive task battery. This study represents one of the most significant contributions to our understanding how different behaviours or cognitive processes are mapped on to specific regions of the cerebellar cortex. A similar approach to mapping the functional organization of the pons would be invaluable. However, it remains unclear what combination of acquisition parameters – including coil strength (i.e., 3T vs 7T), sample size, scan times, etc. – would be needed to achieve comparable results in the pons. In the course of our second study (Chapter 3), we ran an exploratory analysis using the task-based data of the Human Connectome Project to assess whether we could use the dataset to extract meaningful topographic organizational patterns in the pons. We conducted a principal component analysis (PCA) to identify the dominant patterns of pontine activation across a number of motor and non-motor tasks conducted during fMRI acquisition. The first two components reflected motor activation in the caudal left and right pons, but subsequent components did not reflect meaningful patterns. Even with such a large and relatively high-quality dataset, sensitivity was sufficient to detect lateralized motor-related activity localized to caudal pons, but little beyond that. This highlighted an important distinction in this dataset between the HCP's task-based and resting-state fMRI data. The resting-state data of the HCP benefits from a relatively long acquisition time (1 hour) (Van Essen et al., 2013). This combined with the averaging of a large number of participants

allowed for the delineation of meaningful resting-state patterns in the brainstem. In contrast, in the task-based data, the same total acquisition time is divided between a number of different tasks (Van Essen et al., 2013), which reduces the ability to detect reliable activation patterns for a given task in this noisy brain region. In our view, before further resources are directed towards studying functional organization of the pons, there is a need for systematic investigation of the necessary parameters to detect more nuanced activity patterns in brainstem structures.

Beyond mapping task-based activation in the pons, which should eventually be feasible with the optimization of acquisition parameters, there are approaches towards investigating the role of the pons in the downstream pathway with fMRI data. King et al. (2023) provides preliminary evidence that the pons plays a dynamic role in the corticopontine pathway. In their study, predictive models were used to estimate cerebellar activation based solely on cerebral cortical activation. While these models performed well, especially when different cortical sites were permitted to converge in the cerebellum, they did not fully account for cerebellar activation. That is to say, cerebellar activation was not entirely predictable based on cerebral cortical activation. Different areas of the cerebellum are more or less active than predicted depending on a particular task – activity in the cerebellum is not a simple mirror of that of the cerebral cortex. The authors highlight a potential role for the pontine nuclei as an intermediary that acts as an adaptive gate for inputs to cerebellar cortex. This is a fascinating possibility that is compatible with Schwarz and Thier's (1999) framework. This notion can be addressed in follow-up study that investigates whether the discrepancy between predicted and actual cerebellar activation is a function of activity in the pons. By incorporating pons activity into predictive models, we can test whether activity patterns in this structure account for some of the unexplained variance in cerebellar activity. This would suggest that the pons is indeed involved in reshaping cortical inputs before they are conveyed to the cerebellum. While I have highlighted a few select examples of future research on the question of the role of the pons, many potential avenues remain to be explored. Importantly, answering this complex question will require leveraging complementary insights from electrophysiology and cellular biology alongside non-invasive studies in humans.

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## Chapter 6. Conclusion

The series of studies presented in this dissertation aimed to advance our understanding of cerebellar connectivity in humans. We focused on the downstream pathway connecting the cerebral cortex to the cerebellar cortex. Our first two studies built upon a strong foundation of tract-tracing work in non-human primates using diffusion MRI tractography. The first study reconstructed connections between the pons and individual cerebellar lobules and demonstrated a segregation of motor and non-motor inputs to the cerebellum. In the second study, we shifted to using data-driven gradient methods, demonstrating overlapping organizational principles in the mapping of the cerebral cortex onto the pons. Together these studies represent the most comprehensive efforts to detail the anatomical organization of the downstream, corticocerebellar, pathway in humans. The reconstructed components of this pathway will also serve as a valuable tool for future researchers investigating normal or pathological variation and help guide interventional approaches. The final study built upon and extended the findings of the first two, using rsfMRI connectivity data to reconstruct gradients in the pons based on convergent functional connectivity with the cerebral and cerebellar cortices. We demonstrated a complex functional organization of the pons, characterized by both segregation and integration of particular input and output channels. The findings highlighted the pons as being a functionally dynamic structure that may serve to integrate behaviourally relevant inputs from distinct cerebrocortical areas. This final study opens the door to future investigations of the pons at both macroscopic and cellular levels. Viewed collectively, these studies advance our understanding of the connectivity of the cerebellum and offer new avenues for investigating its diverse contributions to brain function.

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