Purkinje cells converge non-randomly on neurons in the cerebellar nuclei

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For my father, Alan W. Gruver, who
would have been very proud.
(1952 - 2016)

Contents

Abstract

Form fits function, and the structure of connections between cells influences how a circuit functions. The cerebellar circuit consists of two major components: a cerebellar cortex dominated by Purkinje cells, and a core set of nuclei containing the output neurons of the cerebellum. The general schema underlying connectivity between the cerebellar cortex and nuclei is known: Purkinje cells are the only cells that project outside of the cerebellar cortex, converging on neurons in the cerebellar nuclei. Purkinje cells integrate information that enters the cerebellum, while cerebellar nuclear neurons predominantly integrate Purkinje cell input and transmit this processing to many areas of the brain. Thus, the Purkinje cell – cerebellar nuclear neuron synapse represents a key node in the cerebellar circuit. However, many properties of Purkinje cell - cerebellar nuclear connectivity remain unknown. In this thesis, I explore the spatial relationships between Purkinje cells and cerebellar nuclear neurons. Chapter 1 describes the rationale for the research described in the thesis. Chapter 2 provides a literature review of the cerebellar microcircuit, focusing on cerebellar topography and heterogeneity. It also describes the relationship between Purkinje cells and cerebellar nuclear neurons in the fastigial nucleus. In Chapter 3, I present our study optimizing the methodology for optogenetic circuit mapping to explore Purkinje cell – cerebellar nuclear neuron connectivity. Chapter 4 applies our methodology from Chapter 3, uncovering structured connectivity that underlies Purkinje cell – cerebellar nuclear neuron synaptic connections. We support our electrophysiological findings with structural labeling of Purkinje cell axons. Finally, Chapter 5 offers interpretations on how structured Purkinje cell - cerebellar nuclear connectivity may influence cerebellar function and behavior. In addition to characterizing novel properties of the cerebellar circuit, this thesis highlights the benefits of meticulous experimental design when addressing challenging research questions. Overall, I demonstrate that the Purkinje cell – cerebellar nuclear neuron synapse is an overlooked yet important site of integration in the cerebellum. The diversity of connectivity patterns between Purkinje cells and cerebellar nuclear neurons adds a computational complexity that enables the cerebellum to participate in a wide variety of functions and behaviors.

Résumé

La forme et la fonction sont étroitement liées, et la structure des connexions entre les cellules influence le fonctionnement d'un circuit. Le circuit cérébelleux se compose de deux composants majeurs: un cortex cérébelleux dominé par les cellules de Purkinje et un ensemble central de noyaux contenant les neurones de sortie du cervelet. Le schéma général de connectivité entre le cortex cérébelleux et les noyaux est connu: les cellules de Purkinje sont les seules à projeter en dehors du cortex cérébelleux, convergant vers les neurones des noyaux cérébelleux. Les cellules de Purkinje intègrent les informations qui entrent dans le cervelet, tandis que les neurones des noyaux cérébelleux intègrent principalement les entrées des cellules de Purkinje et transmettent ce traitement à de nombreuses régions du cerveau. Ainsi, la synapse entre les cellules de Purkinje et les neurones des noyaux cérébelleux représente un nœud important dans le circuit cérébelleux. Cependant, de nombreuses propriétés de la connectivité entre les cellules de Purkinje et les noyaux cérébelleux restent inconnues. Dans cette thèse, j'explore les relations spatiales entre les cellules de Purkinje et les neurones des noyaux cérébelleux. Le chapitre 1 décrit la justification de la recherche présentée dans cette thèse. Le chapitre 2 propose une revue de la littérature sur le microcircuit cérébelleux, en mettant l'accent sur la topographie cérébelleuse et l'hétérogénéité. Il décrit également la relation entre les cellules de Purkinje et les neurones des noyaux cérébelleux dans le noyau fastigial. Dans le chapitre 3, je présente notre étude visant à optimiser la méthodologie de cartographie du circuit par optogénétique pour explorer la connectivité entre les cellules de Purkinje et les neurones des noyaux cérébelleux. Le chapitre 4 applique notre méthodologie du chapitre 3 et révèle une connectivité structurée qui sous-tend les connexions synaptiques entre les cellules de Purkinje et les neurones des noyaux cérébelleux. Nous appuyons nos résultats électrophysiologiques avec un marquage structural des axones des cellules de Purkinje. Enfin, le chapitre 5 propose des interprétations sur la façon dont la connectivité structurée entre les cellules de Purkinje et les noyaux cérébelleux peut influencer la fonction et le comportement cérébelleux. En plus de caractériser de nouvelles propriétés du circuit cérébelleux, cette thèse met en évidence les avantages d'une conception expérimentale méticuleuse lors de l’abordage de questions de recherche complexes. Dans l’ensemble, je démontre que la synapse entre les cellules de Purkinje et les neurones des noyaux cérébelleux est un site d’intégration négligé mais important dans le cervelet. La diversité des schémas de connectivité entre les cellules de Purkinje et les neurones des noyaux cérébelleux ajoute une complexité computationnelle qui permet au cervelet de participer à une grande variété de fonctions et de comportements.

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Contribution to original knowledge

The manuscripts presented in this doctoral thesis describe my original research and contribute knowledge to the field of neuroscience in the following ways:

1. The first manuscript included in this thesis is described in Chapter 3 and was published in a peer-reviewed journal in November 2019 (Gruver and Watt, 2019). It provides an investigation of the use of optogenetics at the Purkinje cell – cerebellar nuclear neuron synapse. While optogenetics is a powerful and versatile tool, its use is not well-suited to every synapse (Jackman et al., 2014). Therefore, careful technical optimization is required to appropriately use optogenetics to answer specific scientific questions. Here we show that Purkinje cell axons require different stimulation parameters than Purkinje cell somata when using light to induce single action potentials, which has implications for optogenetic circuit mapping in the brain. We also demonstrate that axons are significantly more vulnerable to suboptimal conditions such as ambient light exposure than are Purkinje cell somata. Lastly, we identified the photostimulus conditions required to elicit temporally precise responses from postsynaptic cerebellar nuclear neurons. Our findings have been cited in a Neuromethods book series (Holder and Prigge, 2023), highlighting the differences in stimulating cell bodies versus axons with light. Our study will likely be informative for others who wish to use optogenetics for long-range circuit mapping that necessitates the stimulation of axons rather than somata.

2. The second manuscript in this thesis was recently released as a preprint on BioRxiv. In this manuscript, presented in Chapter 4, we describe the functional convergence patterns underlying Purkinje cell – cerebellar nuclear neuron connectivity in the fastigial nucleus, the most diverse of the three cerebellar nuclei (Kebschull et al., 2020). Purkinje cell – cerebellar nuclear neuron connectivity has been characterized along the mediolateral axis of the cerebellum predominantly through anatomical labeling experiments.

Cerebellar nuclear neurons in the fastigial nucleus are thought to generally receive input from Purkinje cells throughout the mediolateral axis of the cerebellar vermis (Voogd and Ruigrok, 2004). We wanted to investigate whether specific patterns of convergence underlie connectivity at this synapse at the level of cerebellar lobules and transverse zones which are organized along the rostrocaudal axis. This rostrocaudal axis runs orthogonal to the mediolateral axis.

We explored Purkinje cell - cerebellar nuclear neuron connectivity by using whole-cell patch clamp recordings paired with optogenetic circuit mapping to assess functional synapses between these cells. We observed that Purkinje cells from different areas of the cerebellum converge on cerebellar nuclear neurons in diverse combinations with varying synaptic strengths. Our findings highlight the complexity of information transfer at this particular synapse in the cerebellum. We suggest that the cerebellar nuclei are an important yet often overlooked locus of cerebellar encoding of information. Our original approach to assess this feature of cerebellar physiology complements the existing body of cerebellar circuit literature, expanding our knowledge of information transfer at this critical synapse in the brain.

Contribution of authors

As the sole first author of the manuscripts presented in this doctoral thesis, I designed and performed the various experiments described in each manuscript, with guidance from my supervisor, Dr. Alanna J. Watt, who conceived of the original project. These experiments involved mastery of techniques in electrophysiology, immunohistochemistry, two-photon and confocal imaging, and unsupervised learning models.

Additionally, I spearheaded the data analysis and manuscript writing that appear in Chapters 3 and 4, with assistance from several co-authors (described below).

Chapter 3

- Alanna J. Watt: experimental design, two-photon image acquisition, data interpretation, and manuscript writing

Chapter 4

- Jenny W.Y. Jiao: collected and analyzed data, and wrote code for Figures 4.3-4.4, Supplementary Figures 4.10-4.10, and 4.13
- Eviatar Fields: designed and performed experiments for Figure 4.5
- Sen Song: analyzed data for Figure 4.2
- P. Jesper Sjöström: wrote custom code for data acquisition and analysis, analyzed data for Figures 4.2 and 4.4
- Alanna J. Watt: experimental design, data interpretation, and manuscript writing

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List of Abbreviations

A cell	"Anterior" zone cell
AAV	Adeno-associated virus
ACPN cell	"Anterior, Central, Posterior, Nodular" zone cell
ACSF	Artificial cerebrospinal fluid
ALM	Anterior lateral medial cortex
ARSACS	Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay
ASD	Autism Spectrum Disorder
BSA	Bovine serum albumin
C cell	"Central" zone cell
CD	Caudodorsal
ChR2	Channelrhodopsin-2
ChR2/H134R	Modified ChR2 with point mutation at position H134
CN	Cerebellar nuclei (see also "DCN")
CN neuron	Cerebellar nuclear neuron
CRACM	Channelrhodopsin-2 Assisted Circuit Mapping
CV	Caudoventral
D	Dorsal
DCN	Deep cerebellar nuclei (see also "CN")
DN	Dentate nucleus
E#	Embryonic day #
EYFP	Enhanced yellow fluorescent protein
Fig.	Figure
fcMRI	Functional connectivity magnetic resonance imaging
fMRI	Functional magnetic resonance imaging
FN	Fastigial nucleus
g	Gram
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
HSP25	Heat shock protein 25

Hz	Hertz
IPN	Interposed nuclei
IPSC	Inhibitory postsynaptic current
ISI	Inter-spike interval
LED	Light-emitting diode
LSM	Laser scanning microscope
M	Medial
M1	Primary motor cortex
mL	Milliliter
MLI	Molecular layer interneuron
mm	Millimeter
mM	Millimolar
mOsm	Milliosmole
MRI	Magnetic resonance imaging
ms	Millisecond
mV	Millivolt
mW	Milliwatt
N	Number of animals
n	Number of cells, lobules, or zones
N cell	"Nodular" zone cell
NeuN	Neuronal nuclear marker
nL	Nanoliter
nm	Nanometer
Ns	Not significant
nZone	Number of zones
P	Probability
P#	Postnatal day #
PBS	Phosphate-buffered saline
P cell	"Posterior" zone cell
pA	Picoamperes
Pcp2	Purkinje cell protein 2
PFA	Paraformaldehyde
Plcb3	Phospholipase Cβ3
Plcb4	Phospholipase Cβ4
R	Rostral
$\mathbf{R}^2$	Coefficient of determination
RD	Rostrodorsal
$\mathbf{R}_{\mathbf{in}}$	Input resistance

RV	Rostroventral
s	Second
SCA2	Spinocerebellar ataxia type 2
SCA6	Spinocerebellar ataxia type 6
SCA7	Spinocerebellar ataxia type 7
SD	Standard deviation
SEM	Standard error of the mean
YFP	Yellow fluorescent protein
μ**g**	Microgram
μ**m**	Micron

Chapter 1

Introduction

1.1 Rationale

The cerebellum is a relatively simple structure that contributes to a surprisingly diverse set of behaviors and functions. The cerebellum has historically been considered a motor region of the brain, but has more recently been implicated in cognitive, affective, and autonomic processes. Disruptions in typical cerebellar function have been shown to play a role in complex disorders such as Autism Spectrum Disorder and schizophrenia (Schmahmann, 2000). The cerebellum is well situated to contribute to diverse functions: the cerebellum receives input from many sources including the cerebral cortex via the pontine nuclei and basal ganglia (Bostan, Dum, and Strick, 2013), and from the spinal cord (Sathyamurthy et al., 2020), sending its output to areas like the thalamus, cortex, brainstem, and spinal cord (Gornati et al., 2018; Kebschull et al., 2023).

While the inputs and outputs of the cerebellum have been thoroughly described, the internal wiring within the cerebellar circuit is less clear. Two major cell populations are responsible for integrating input to the cerebellum and conveying output from the cerebellum, respectively. These populations are the Purkinje cells in the cerebellar cortex, and the neurons of the cerebellar nuclei. Precisely how Purkinje cells functionally connect to their targets in the cerebellar nuclei is unclear despite the key role these cell populations play in processing extensive information and contributing to a range of functions. Anatomical studies have identified that a general mediolateral connectivity gradient governs Purkinje cell - cerebellar nuclei connectivity and is linked with particular compartments in the inferior olive (Voogd and Ruigrok, 2004). However, Purkinje cell - cerebellar nuclear neuron connectivity has not been described using a comprehensive functional circuit mapping approach to evaluate synaptic connections between these cells. Further, how Purkinje cells from multiple areas of the cerebellum converge on individual cerebellar nuclear neurons, and the strength of these connections, has not been characterized. Identifying the spatial relationships that underlie Purkinje cell - cerebellar nuclear neuron connectivity is essential to understand cerebellar function as a whole.

1.2 Objectives

The overarching objective of my research has been to identify patterns in the functional connections between Purkinje cells and cerebellar nuclear neurons. Since Purkinje cells exhibit a topographic organization in the cerebellar cortex (Apps and Hawkes, 2009) and are more numerous than the neurons in the cerebellar nuclei (Person and Raman, 2012a), we hypothesized that Purkinje cells would likely converge on individual cerebellar nuclear neurons in non-random patterns. We combined optogenetics with electrophysiology to explore this hypothesis. The following doctoral thesis includes two first-author manuscripts that describe our work and findings.

Chapter 3

Our goals for the manuscript described in Chapter 3 were as follows: 1. evaluate whether we could pair optogenetics with electrophysiological recordings to reliably identify connections between Purkinje cells and cerebellar nuclear neurons; and 2. optimize the photostimulation parameters necessary to assess connectivity at this particular synapse. Optogenetic photostimulation grants us the ability to rapidly and focally stimulate Purkinje cells with relative ease. We determined the experimental conditions required to elicit single action potentials from Purkinje cell axons and evoke responses from functionally-connected cerebellar nuclear neurons.

Chapter 4

In Chapter 4, we used the optimized Purkinje cell optogenetic photostimulation paradigm described in Chapter 3 and performed spatial circuit mapping of Purkinje cell connectivity to cerebellar nuclear neurons in the fastigial nucleus. Electrophysiological recordings from cerebellar nuclear neurons provided rich information about functional properties of Purkinje cell input to these cells. We supported our electrophysiological findings with data from anatomical labeling experiments, situating our study within the larger body of anatomical convergence literature of the cerebellum.

Chapter 2

Review of the literature

2.1 Cerebellum overview

2.1.1 Evolutionarily conserved structure involved in a variety of functions

The cerebellum is a highly conserved brain structure located in the hindbrain of all vertebrates (Kebschull et al., 2020; Rueda-Alaña and García-Moreno, 2022). The cerebellum is best known for its role in motor coordination, though research as early as the first half of the 20th century began describing the cerebellum's additional contributions to processing autonomic and sensory information (Chambers Jr., 1947; Nisimaru and Watanabe, 1985). Within the last decade, studies have continued to point to the cerebellum's direct involvement in cognitive and affective processes (Badura et al., 2018; King et al., 2019). Described by Eccles in 1967 (Eccles, Ito, and Szentágothai, 1967) as consisting of a highly stereotyped and simple organization, the cerebellum has proven to be quite complex in spite of this simplicity.

The "simple" organization of the archetypal, or "true," cerebellum is that of a multi-layered structure consisting of a cortical sheet that is foliated around a central set of nuclei (Kebschull et al., 2020; Van Essen, Donahue, and Glasser, 2018). This "true" cerebellum is a feature of amniote brains (Kebschull et al., 2023), and is marked in part by a multi-layered cerebellar cortex dominated by γ-aminobutyric acid-mediated (GABAergic) Purkinje cells and set of nuclei that provide the major output of the cerebellum (Kebschull et al., 2023; Montgomery, Bodznick, and Yopak, 2012). The cerebellar nuclei have been hypothesized to serve as "relay stations" for communicating cerebellar output (Hohman, 2010). In mammals, the cerebellum is divided into medial and lateral compartments (Montgomery and Bodznick, 2016): along the midline of the cerebellum is the vermis, then the paravermis sits on each side of the vermis, and finally at the most lateral regions are the hemispheres (Marzban et al., 2015).

2.1.2 The cerebellum receives input from many sources

The structure of inputs to the cerebellum is consistent with this simple/complex dichotomy. The cerebellum receives two types of input: from "mossy fibers" and from "climbing fibers." Mossy fibers received their name from Ramón y Cajal due to the "mossy" appearance of their terminals (Delvendahl et al., 2013; Palay and Chan-Palay, 1974a). Mossy fiber input arises from the brainstem, spinal cord, and pontine nuclei (Kostadinov and Häusser, 2022; Palay and Chan-Palay, 1974a). Although connections between the cerebral cortex and cerebellum are thought to be largely mediated through the pontine nuclei (Biswas et al., 2019; Pijpers et al., 2006), direct connections from the motor cortex to the cerebellum have been observed (Schaefer, C. B. et al., 2022; Wild and Williams, 2000). Mossy fibers synapse on neurons in both compartments of the cerebellum: the cerebellar cortex and the cerebellar nuclei. In the cerebellar cortex, mossy fibers synapse on granule cells (further described in section 2.2.1). In the cerebellar nuclei, mossy fibers synapse on cerebellar nuclear neurons, the output neurons of the cerebellum (Fig. 2.1A).

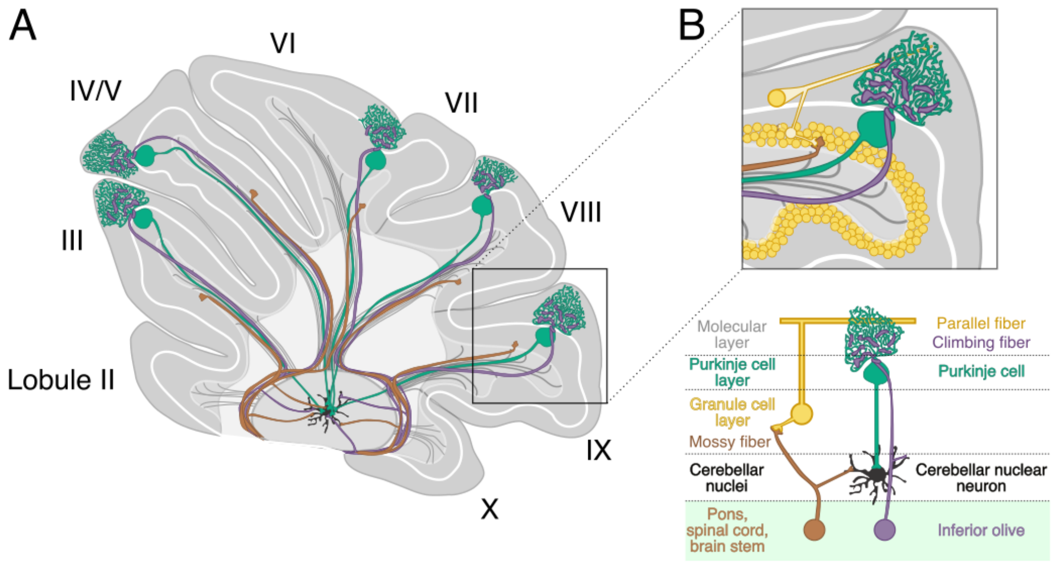

Figure 2.1: **Overview of cerebellar connections.** **A**, Schematic of a sagittal cerebellar slice showing Purkinje cells, cerebellar nuclear neurons, and mossy fiber and climbing fiber inputs to the cerebellum. **B**, top, Inset is an expansion of the cerebellar cortex in **A**, with granule cells in yellow. Bottom, diagram of major cell types in the cerebellum and layers of the cerebellar cortex (excluding interneurons). Cerebellar lobules are denoted in roman numerals. Green box shows inputs to the cerebellar cortex and nuclei that arise from outside of the cerebellum.

In contrast to the various sources of input that send mossy fibers to the cerebellum, climbing fibers exclusively arise from the inferior olive. Climbing fibers also bypass other cells in the cerebellar cortex and synapse directly on Purkinje cells, where they wrap around Purkinje cells, “climbing” up Purkinje cells’ dendritic arbors (Cajal, 1909–1911). This extensive encircling of Purkinje cells allows climbing fibers to exert a powerful effect on both immediate Purkinje cell output (De Gruijl et al., 2013; Han et al., 2020) as well as modulate of cerebellar circuit plasticity (Rowan et al., 2018; Schmolesky, De Zeeuw, and Hansel, 2005). Like mossy fibers, climbing fibers also synapse on cerebellar nuclear neurons via collateral branches (De Zeeuw et al., 1997; Sugihara, Wu, and Shinoda, 2001). In all, input from such diverse sources, via mossy and climbing fibers, places the cerebellum in a position to serve as a modulator of motor, sensory, and autonomic processing, a role which the cerebellum has been shown to perform (Romano et al., 2020).

2.1.3 Downstream targets of the cerebellum highlight its widespread contribution to behavior

In addition to receiving input from a wide variety of sources, the cerebellum also outputs to many brain regions primarily via the cerebellar nuclear neurons. Canonically, these monosynaptic connections from cerebellar nuclear neurons target different areas of the thalamus (Asanuma, Thach, and Jones, 1983; Fujita, Kodama, and Du Lac, 2020; Gornati et al., 2018), inferior olive (Najac and Raman, 2015; Ruigrok and Voogd, 1990), brainstem (Fujita, Kodama, and Du Lac, 2020), and spinal cord (Butler, Horne, and Hawkins, 1992; Sathyamurthy et al., 2020), highlighting the cerebellum’s contribution to motor and sensory functions. More recent evidence has also demonstrated that cerebellar nuclear neurons project monosynaptically to the ventral tegmental area (Carta et al., 2019), the periaqueductal gray (Vaaga, Brown, and Raman, 2020), and the hypothalamus (Çavdar et al., 2001; Zhu et al., 2006), areas associated with reward, defense, and autonomic functions, respectively. These eclectic targets of direct cerebellar output suggest that the cerebellum is a versatile structure that contributes to a variety of important processes.

Disynaptic connections to the cerebral cortex from the cerebellum have also been observed (Gao et al., 2018; Guo et al., 2017). These disynaptic connections are mediated largely by a layover in the thalamus (Çavdar et al., 2001; Hoover and Strick, 1999). Notably, the cerebellum’s cortical targets are not restricted solely to the motor cortex. Studies performed in mice (Kelly et al., 2020), nonhuman primates (Kelly and Strick, 2003; Middleton and Strick, 2001), and humans (Krienen and Buckner, 2009) have also demonstrated a polysynaptic connection or association between the cerebellar nuclei and the prefrontal cortex, an area that is important for planning and goal-directed behavior (Kostadinov and Häusser, 2022).

The cerebellum's role as a modulator or "supervisor" or motor and non-motor learning (Hull, 2020; Raymond and Medina, 2018) is likely made possible by the "loops" it makes with many areas of the brain. The disynaptic connections between the cerebellum and the cerebral cortex loop back to the cerebellum via the pontine-cerebello-thalamic pathway (Gao et al., 2018; Guo et al., 2021). Through this interconnectivity, the cerebellum can exert feedforward control of behavioral states by comparing predicted outcomes with actual outcomes (Bastian, 2006; Giovannucci et al., 2017; Romano et al., 2020). When predicted and actual outcomes do not match up, the cerebellum is thought to update its internal models (Bastian, 2006; Ebner and Pasalar, 2008). These discrepancies are communicated via climbing fibers from the inferior olive, which is involved in a closed loop with the cerebellum (Chaumont et al., 2013; Uusisaari and De Schutter, 2011). Thus, cerebellar loops with the rest of the brain likely underlie the cerebellar contribution to complex behaviors.

2.2 Organization of the cerebellar cortex

2.2.1 Overview of the neurons in the cerebellar cortex

The largest component of the cerebellum is the cerebellar cortex, which is foliated into 10 lobules (I-X) in mice (Larsell, 1952), in addition to the expanded hemispheric lobules VI (lobulus simplex) and VII (crus I and crus II) (Apps and Hawkes, 2009; Habas, 2021). In each of these lobules are the three layers of the cerebellar cortex (Fig. 2.1B). The most predominant is the Purkinje cell layer, named for the principal cells of the cerebellar cortex. Purkinje cells are large neurons which exhibit some of the most striking structural characteristics within the brain: each cell features an elaborate dendritic arbor and a long, thin axon that projects through the cerebellar cortex to synapse on neurons in the cerebellar nuclei. The Purkinje cell dendritic arbor is a relatively flat structure (Ing-Esteves et al., 2018) such that Purkinje cells are organized in thin, parallel layers organized in the parasagittal plane.

Various interneurons populate the area immediately below the Purkinje cell layer (Hull and Regehr, 2022). These interneurons include Lugaro cells (Lainé and Axelrad, 1998), globular cells (Hirono et al., 2012), and candelabrum cells (Osorno et al., 2022), which receive input from and inhibit a variety of other cerebellar interneurons (Hull and Regehr, 2022).

Another layer of the cerebellar cortex is the molecular layer, which is populated by GABAergic molecular layer interneurons (MLIs). MLIs are made up of stellate cells and basket cells, so named by Cajal due to their morphological characteristics (Sotelo, 2015). In the adult cerebellum, the molecular layer is external to the Purkinje cell layer, where MLIs are nestled within Purkinje cell dendrites or pericellular to the Purkinje cell soma. MLIs directly inhibit Purkinje cells and have been shown to be important for shaping firing activity of Purkinje cells (Brown et al., 2019).

The third layer of the cerebellum is the granule cell layer, named for the prolific granule cells which populate it. Though small in size, granule cells are thought to account for half of the neurons in the brain (Giovannucci et al., 2017) and over 99% in the cerebellum (Consalez et al., 2021). Granule cells process input to the cerebellum from mossy fibers. Mossy fibers can carry both motor and sensory information to the same granule cell (Huang et al., 2013; Ishikawa, Shimuta, and Häusser, 2015) that originate from multiple precerebellar nuclei, suggesting that granule cells perform multimodal integration of precerebellar input.

Granule cells provide excitatory input to Purkinje cells. Granule cell axons form parallel fibers that ascend through the granule and Purkinje cell layers and bifurcate in the molecular layer, targeting Purkinje cell dendrites. There, these parallel fibers run orthogonal to the parasagittal bands of Purkinje cells (Fig. 2.1B). Parallel fibers can extend to lengths of 5 mm in the rat cerebellum, making contact with hundreds of Purkinje cells (Isope and Barbour, 2002), thus having a modulatory effect on a large population of Purkinje cells.

The granule cell layer is also home to interneuron populations: Golgi cells and unipolar brush cells (Hull and Regehr, 2022). Golgi cells are large, GABAergic interneurons that can inhibit unipolar brush cells as well as thousands of granule cells (Schilling et al., 2008). Since Golgi cells receive input from both mossy fibers and granule cell parallel fibers, they are thought to play a role in both feedforward and feedback inhibition in the cerebellar cortex (Hull and Regehr, 2022). Unipolar brush cells are also innervated by mossy fiber input. However, unipolar brush cells are glutamatergic and can exert an excitatory effect on hundreds of granule cells and other unipolar brush cells (Hull and Regehr, 2022).

2.2.2 Purkinje cells: the sole output of the cerebellar cortex

All the information processing that takes place in the cerebellar cortex, from glutamatergic climbing fibers snaking through Purkinje cell dendrites, to excitatory mossy fibers synapsing on the vast number of granule cells, to the various interneurons across the cortex inhibiting Purkinje cells or each other, culminates as the output of Purkinje cells. An estimated 220,000 Purkinje cells in the mouse cerebellum (Harvey and Napper, 1991; Person and Raman, 2012a) communicate the computations of the cerebellar cortex to its downstream targets in the cerebellar nuclei, in addition to some direct Purkinje cell-extracerebellar targets in the neighboring vestibular and parabrachial nuclei (De Zeeuw and Berrebi, 1995; Hashimoto et al., 2018; Sekirnjak et al., 2003).

Purkinje cells are spontaneously active, capable of firing at high frequencies (Lang-Ouellette et al., 2021), and can exhibit a wide array of firing patterns (Arancillo et al., 2015; Zhou et al., 2014). Since Purkinje cells are GABAergic, they are often presumed to exert a net-inhibitory effect on their target neurons (Person and Raman, 2012a). However, Purkinje cells can become synchronized with one another, leading to increased firing rates in their target cerebellar nuclear neurons (Brown and Raman, 2018; Person and Raman, 2012a). Additionally, the loss of Purkinje cell GABAergic neurotransmission results in aberrant activity in cerebellar nuclear neurons, which does not always correspond to an increase in the firing rates of these neurons (White et al., 2014).

2.2.3 Topographic organization of heterogeneous Purkinje cells

While the cerebellar cortex has a homogeneous cytoarchitecture (Eccles, Ito, and Szentágothai, 1967), Purkinje cells, the principal cells of the cerebellar cortex, are now understood to be a heterogeneous population (Apps and Hawkes, 2009; Sotelo and Wassef, 1991; Viet et al., 2022; Zhou et al., 2014). Purkinje cells are heterogeneous in their molecular profiles (Ozol et al., 1999), physiological properties (Zhou et al., 2014), morphology (Beekhof et al., 2021), connectivity patterns (Sugihara and Shinoda, 2004), and vulnerability to disease (Larivière et al., 2015; Toscano Márquez et al., 2021). Notably, Purkinje cell heterogeneity is associated with different areas of the cerebellar cortex (Apps and Hawkes, 2009; Rodriques et al., 2019).

The most apparent divisions of Purkinje cells within the cerebellar cortex are those of the 10 anatomical lobules that are most pronounced in the medial region of the cerebellum. Foliation and lobulation of the cerebellum take place during embryonic and early postnatal life in mice and occur under tight genetic control (Airey, Lu, and Williams, 2001; Sillitoe et al., 2008; Sudarov and Joyner, 2007). The conservation of lobules across species suggests that these genetic mechanisms are important evolutionary features of cerebellar morphogenesis (Sillitoe and Joyner, 2007). Purkinje cells from lobules in different areas of the cerebellum have been observed to be physiologically and morphologically different (Beekhof et al., 2021; Beekhof and Schonewille, 2023; Nguyen-Minh et al., 2019). However, each individual lobule does not appear uniquely distinct from the others, and lobules are not considered to act as discrete computational units (Apps and Hawkes, 2009; King et al., 2019). Instead, topographic Purkinje cell heterogeneity has been explored outside of the anatomical lobule framework.

2.2.3.1 Stripes, longitudinal zones, and modules in the mediolateral axis of the cerebellum

Differences in Purkinje cell patterning have been observed by labeling with a variety of molecular markers. Such markers capable of distinguishing between Purkinje cells include zebrin II (Brochu, Maler, and Hawkes, 1990), heat shock protein HSP25 (Armstrong et al., 2000; Duffin et al., 2010), phospholipase Cβ3 and Cβ4 (Plcb3 and Plcb4) (Sarna et al., 2006), and parvalbumin (Brandenburg et al., 2021). The patterning revealed from labeling the cerebellum with many of these markers, particularly zebrin II, has led to the subdivision of Purkinje cells into ~20 parasagittal bands known as "stripes" in the mediolateral axis of the cerebellum, running from the vermis through the hemispheres (Apps and Hawkes, 2009, Fig. 2.2). These stripes are reproducible across individual animals (Hawkes and Herrup, 1995). In mice, Purkinje cells are born between embryonic days E10 through E13 (Beckinghausen and Sillitoe, 2019), and their stripe fate is determined by their embryonic birthdates. Purkinje cells that share the same birthdate are fated to the same cluster that generally form distinct stripe compartments later in development (Hashimoto and Mikoshiba, 2003; Vibulyaseck et al., 2017). A study that followed the developmental trajectories of embryonic Purkinje cell clusters from E17.5 found that during development, these clusters flattened and spread into cerebellar stripes via various transformational configurations by postnatal day P6 in mice (Fujita et al., 2012).

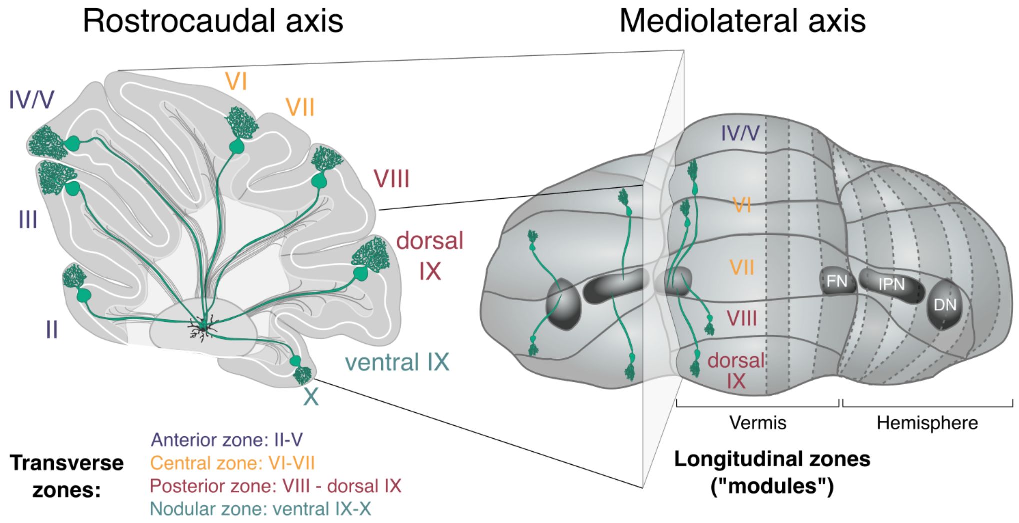

Figure 2.2: **Axes of the cerebellum.**, Left, schematic of sagittal cerebellar slice rostrocaudal axis. Lobules and their associated transverse zone are denoted by roman numeral and color. Right, mediolateral axis of the cerebellum depicting longitudinal zones (simplified for demonstration purposes, demarcations do not represent actual longitudinal zones or stripes of the cerebellum). Purkinje cells (green) shown converging on cerebellar nuclei (grey). Vermis and hemispheres are visible in the coronal plane. FN: fastigial nucleus, IPN: interposed nuclei, DN: dentate nucleus.

Purkinje cells in zebrin II-negative stripes exhibit higher firing frequencies than

Purkinje cells in zebrin II-positive stripes (Ady et al., 2018; Zhou et al., 2014). An RNA sequencing study found that Purkinje cells in zebrin II-positive and -negative bands exhibit different patterns of gene expression, including genes associated with ATP and potassium channels that are important for the high rates of spontaneous firing in Purkinje cells. These expression patterns may help explain the physiological differences between Purkinje cells in zebrin II-positive and -negative areas (Rodriques et al., 2019). Additionally, expression of Plcb4, which is involved in metabotropic glutamatergic signaling, in Purkinje cells largely overlaps with zebrin II-negative stripes (Sarna et al., 2006), and Plbc4-positive Purkinje cells undergo transcriptional plasticity in response to motor activity, while zebrin II-positive Plbc4-negative Purkinje cells do not (Chen et al., 2022). At the most extreme, Purkinje cells in zebrin II-negative stripes of the anterior region of the cerebellum have been shown to be more vulnerable to cell death than Purkinje cells in zebrin II-positive stripes (Toscano Márquez et al., 2021) in an animal model of the neurodegenerative disease Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay (ARSACS) (Larivière et al., 2015).

Like the patterning observed with Purkinje cell stripes, Purkinje cells have also been subdivided into parasagittal “longitudinal zones” (or “modules," Fig. 2.2) that are aligned with their target areas in cerebellar and vestibular nuclei (Courville et al., 1980; Voogd and Ruigrok, 1997) and share a climbing fiber termination area (Garwicz, Ekerot, and Jörntell, 1998; Trott, Apps, and Armstrong, 1998a). (Incidentally, the term “module” was first used by Voogd and Bigaré (Courville et al., 1980) at a meeting in Montréal (Apps et al., 2018), adding a local flair to the history of cerebellar research.) These longitudinal zones largely overlap with zebrin-II stripe boundaries (Sugihara and Shinoda, 2004; Voogd and Ruigrok, 1997; Voogd and Ruigrok, 2004), suggesting a connection between the input/output circuits of the cerebellum and the molecular properties of Purkinje cell (Apps and Hawkes, 2009). This will be described further in section 2.4.3.

2.2.3.2 Transverse zones in the rostrocaudal axis of the cerebellum

Orthogonal to the stripes and longitudinal zones are the four transverse zones, which are organized along the rostrocaudal axis and make up the broadest subdivisions of the cerebellum (Fig. 2.2). The transverse zones are thought to arise earlier than stripes during cerebellar development (Apps et al., 2018; Ozol et al., 1999). The transverse zones subdivide the 10 cerebellar lobules in the vermis into four areas based on molecular marker expression both during development (calbindin and lacZ) and in adulthood (zebrin II and lacZ) (Ozol et al., 1999). These transverse zones include the anterior zone (lobules I-V), central zone (lobules VI-VII), posterior zone (lobules VIII and dorsal IX), and nodular zone (ventral IX and lobule X) (Ozol et al., 1999; Reeber et al., 2012).

Notably, the edges of transverse zones overlap, such that these boundaries exhibit interdigitation (Apps and Hawkes, 2009).

The transverse zones generally map onto the principal fissures of the cerebellar cortex (Sudarov and Joyner, 2007). A study performing fate mapping of granule cells in the first two postnatal weeks in mice showed that granule cell proliferation is crucial to the anatomy of lobule development (Legué, Riedel, and Joyner, 2015), and that lobules within the same transverse zone exhibit more similar timing of granule cell proliferation than lobules belonging to different transverse zones (Legué et al., 2016). These studies highlight how transverse zones are conserved features that arise during cerebellar development.

After development, the rostrocaudal axis that underlies transverse zones remains important into adulthood. Physiologically, Purkinje cells from a lobule in the anterior (furthest rostral) zone of the cerebellum have been shown to be physiologically and morphologically different from Purkinje cells from a lobule in the nodular (furthest caudal) zone (Beekhof et al., 2021; Beekhof and Schonewille, 2023). Zebrin II expression alone does not appear to account for this heterogeneity, but rather an interplay between transverse zone and zebrin II expression. Purkinje cells in the anterior zone are predominantly zebrin II-negative, aside from narrow bands of zebrin II-positive cells, while Purkinje cells in the central zone are almost exclusively zebrin II-positive (Ozol et al., 1999). Posterior zone Purkinje cells are predominantly zebrin II-positive, and nodular zone Purkinje cells are all thought to be zebrin II-positive (Ozol et al., 1999). While it was previously stated that Purkinje cells in zebrin II-negative areas exhibit higher firing frequencies than those in zebrin II-positive areas (Zhou et al., 2014), this effect is also dependent on the rostrocaudal position of Purkinje cells: Purkinje cells in zebrin II-positive Purkinje cells in the anterior zone exhibit lower firing frequencies than those in the nodular zone (Zhou et al., 2014).

2.2.3.3 Localized cerebellar deficits in disease and their effects on behavior

Purkinje cells along this rostrocaudal axis have also been shown to be differentially susceptible in disease, where Purkinje cell degeneration is often associated with motor and/or other behavioral impairments in both mouse models and human patients (Jayabal et al., 2015; Kansal et al., 2017). A common theme is that of Purkinje cells located in more rostral lobules undergoing degeneration and cell death. This has been seen both in a variety of diseases like ARSACS (Larivière et al., 2015), spinocerebellar ataxia type 6 (SCA6) (Unno et al., 2012), and Niemann-Pick type C disease (Sarna et al., 2003), as well as in spontaneous mouse models of cerebellar ataxia (e.g. the Leaner mouse; Herrup and Wilczynski, 1982). In these cases, Purkinje cells in the more caudal regions of the cerebellum, such as in the posterior and nodular zones, preferentially survive. In the case of the Lurcher mouse, Purkinje cell death begins in the anterior zone before proceeding to the central and posterior zones, and lastly is delayed but inevitable in the nodular zone (Duffin et al., 2010).

What properties differ between rostrally- and caudally-localized Purkinje cells leading to this difference in vulnerability remains an important question. The roles of zebrin II and HSP25 have been considered, since Purkinje cells expressing either or both markers appear to be more resilient than those in zebrin II- and HSP25-negative regions in several disease models (Chung et al., 2016; Duffin et al., 2010; Toscano Márquez et al., 2021). However, while rostral degeneration and caudal survival of Purkinje cells is common in cerebellar neurodegenerative diseases, the inverse has also been observed (Cerminara et al., 2015) in diseases like SCA2 and SCA7 (Hernandez-Castillo et al., 2018), as well as in the spontaneous mutants Purkinje cell degeneration (pcd) (Mullen, Eicher, and Sidman, 1976) and Nervous mice (Wassef et al., 1987). Regardless of the locus of Purkinje cell vulnerability, there appear to be biologically relevant distinctions between Purkinje cells in different transverse zones.

Identifying how mice with patterned Purkinje cell degeneration behave can offer insight into the contributions of Purkinje cells from different areas of the cerebellum. Disorders such as ARSACS, SCA6, and Niemann-Pick type C that predominantly affect the rostral areas of the cerebellum (e.g., Purkinje cells in the anterior zone) result most obviously in motor impairments (Cendelin, 2014). A magnetic resonance imaging (MRI) study that involved 90 human subjects exhibiting a variety of cerebellar diagnoses found that damage to lobules in the anterior zone were correlated with higher ataxia scores (Schoch et al., 2006). However, disorders like SCA2 and SCA7 that preferentially target the caudal areas of the cerebellum also result in motor impairments, but these impairments often manifest as oculomotor deficits, such as slow saccadic eye movements (La Spada, 1993; Pulst, 1993). Likewise, the pcd mouse mutant similarly exhibits general ataxia, in addition to visuomotor and spatial orientation deficits (Lalonde and Strazielle, 2022), though these deficits may not be exclusively caused by Purkinje cell degeneration but also by retinal degeneration (Fernandez-Gonzalez et al., 2002).

The disruptive impact of Purkinje cell dysfunction can also be demonstrated through the study of non-ataxic disorders. A mouse model of autism spectrum disorder (ASD) PC-Tsc1 results from a Purkinje cell-specific deletion of Tuberculosis Sclerosis Complex 1 and recapitulates various autistic-like behaviors such as impaired social interactions, repetitive behaviors, and abnormal communication (Tsai et al., 2012). PC-Tsc1 mice exhibit deficits in the cerebellar vermis, hemispheres, and nuclei. Stimulation of Purkinje cells in the caudal regions (posterior and nodular zones) is sufficient to alleviate certain repetitive behaviors (Kelly et al., 2020).

Interestingly, a study exploring the relationships between lobule volumes and major symptoms of ASD in autistic children showed reduced gray matter in the central zone lobules VII and hemispheric VII (crus I/II), an area highly implicated in ASD (D'Mello et al., 2015). The authors also found that autistic subjects displayed larger posterior zone lobule VIII compared to the typically-developing subjects. Smaller anterior zone lobules I-V and central zone lobule VI were correlated with poorer social interaction and communication outcomes, while larger central zone lobule VII and posterior zone lobule VIII were associated with increased repetitive behaviors (D'Mello et al., 2015). This observation of increased repetitive behaviors associated with the posterior zone of the cerebellum is consistent with the previous findings in mice from Kelly et al., 2020. Overall, these findings highlight a topographic basis of Purkinje cell heterogeneity, where Purkinje cell dysfunction or degeneration in certain zones of the cerebellum disrupts different kinds of behaviors.

2.2.4 Activity in the cerebellar cortex

2.2.4.1 Localized Purkinje cell activity and its effects on behavior

Studies that explore the effects of cerebellar dysfunction and disease on behavior can shed light on how damage to different areas of the cerebellum disrupts normal function. However, teasing apart the localized Purkinje cell contributions to those disrupted functions from disease models can be challenging, especially for diseases that affect multiple areas of the brain. Experiments performed in healthy brains can help clarify localized cerebellar contributions to behavior.

Purkinje cells in the rostral part of the cerebellum, such as in the anterior zone, have long been associated with sensorimotor activity. More recently Purkinje cells in the caudal regions have been shown to contribute to more complex functions like cognition (Stoodley and Schmahmann, 2010; Witter and De Zeeuw, 2015). A recent study showed that Plcb4-positive (zebrin II-negative) Purkinje cells, which are enriched in the rostral cerebellum, undergo transcriptomic plasticity following motor training tasks and that these Purkinje cells facilitate motor learning (Chen et al., 2022). However, zebrin II-positive Purkinje cells, which are enriched in the caudal cerebellum, were neither associated with transcriptomic plasticity nor motor learning.

At the synaptic level, timing of inputs affects Purkinje cells from different areas in different ways. Purkinje cells from the anterior zone lobule V and central zone lobule VI have been shown to exhibit synaptic plasticity following climbing fiber activity on different timescales, ranging from windows of 0 to 150 milliseconds (Suvrathan, Payne, and Raymond, 2016). This variation in timescales may allow for sufficient flexibility in the cerebellum's contributions to a variety of tasks where feedback may occur along different timescales. The authors also found that the flocculus region of the cerebellum, which contributes specifically to oculomotor learning, exhibits synaptic plasticity at a fixed window of 120 ms, which supports the hypothesis that the flocculus does not contribute to a variety of behaviors.

However, the contribution of other areas of the cerebellum to a variety of behaviors has been explored. A transsynaptic tracing study performed in monkeys showed reciprocal connections between Purkinje cells in anterior lobules III and IV/V, central zone lobules VI and hemispheric VIII, and the primary motor cortex area M1, suggesting the involvement of these Purkinje cells in motor activity (Kelly and Strick, 2003). Interestingly, Kelly and Strick (2003) also observed reciprocal connections between Purkinje cells in the central zone lobule VII, nodular zone lobule X, and hemispheric lobule crus II with area 46 of the dorsolateral prefrontal cortex, suggesting a non-motor role for Purkinje cells in these areas.

Studies performed in awake behaving animals can be especially useful in elucidating the role of localized Purkinje cell contribution to motor and non-motor behavior. Lobules IX and X in the nodular zone encode vestibular information such as adaptive balance (Barmack and Pettorossi, 2021). Recordings from Purkinje cells in lobules IX and X in monkeys highlight their contribution to inertial motion in response to head movement (Yakusheva et al., 2007). Activity in Purkinje cells from the central zone lobules VI and VII, termed the “oculomotor vermis” (Herzfeld et al., 2015), has been shown to be involved in producing rapid eye movements, or saccades, both in mice (Herzfeld et al., 2015) and macaques (Fujikado and Noda, 1987). Purkinje cells in these lobules have also been associated with eyeblink conditioning in mice (Badura et al., 2018), while silencing Purkinje cells in paravermis lobules IV/V (anterior zone) and VI (central zone) resulted in non-ocular motor behaviors such as mouth opening and whisker movements (Heiney et al., 2014).

Another study explored the effects of altering cerebellar activity through chemogenetic inhibition of developmental molecular layer interneurons, thus disrupting Purkinje cell activity (Badura et al., 2018). The authors observed that developmental chemogenetic perturbation in central zone lobule VI resulted in disrupted eyeblink conditioning, while chemogenetic perturbation in lobule VII disrupted a variety of complex behaviors, including behavioral choice reversal, self-grooming, and exploratory behavior impairments. Additionally, mice exhibited altered social preference following chemogenetic perturbation of hemispheric lobule crus II, in line with what has been observed in ASD mouse models (Kelly et al., 2020).

Other surprising behaviors have been identified when modulating Purkinje cell activity with *in vivo* mouse models. A recent calcium imaging study exploring the relationship between Purkinje cell activity and reward prediction showed that in lobules V, VI, and the hemispheric lobule simplex, climbing fiber input to Purkinje cells can provide both predictive and error correction signals in the context of reward expectation (Kostadinov et al., 2019). In a different study, optogenetic stimulation of Purkinje cells in central zone lobule VII was shown to reduce aggression in a resident-intruder assay in mice, while optogenetic inhibition in the same region increased aggression (Jackman et al., 2020). However, existing research does not concur on whether a cerebellar contribution to cognition or affect can be reliably identified using mice. In a study that used four mouse models of cerebellar dysfunction and assayed a variety of cognitive and emotional tests, mice rarely demonstrated marked cognitive or emotional deficits (Galliano et al., 2013). As such, turning toward human-centered studies to explore how the cerebellum might contribute to complex behaviors like cognition may provide more insight into the role of the cerebellum in non-motor behaviors.

2.2.4.2 Localized cerebellar activity from human neuroimaging studies

A functional topography that underlies cerebellar activity has been observed in humans (Stoodley and Schmahmann, 2018). A study using functional connectivity magnetic resonance imaging (fcMRI) identified four distinct cerebro-cerebellar circuits in humans. These included associations between the motor cortex and anterior zone lobule V and posterior zone lobule VIII, between the dorsolateral prefrontal cortex and central zone lobule VII and hemispheric lobule crus II, between the medial prefrontal cortex and posterior lobule IX and hemispheric lobule crus I, and between the anterior prefrontal cortex and central zone lobules VI and VII and hemispheric lobule crus I (Krienen and Buckner, 2009). The latter three cortical regions are associated with non-motor higher order cognitive processing (Collette et al., 2005). Another study performed by the same authors set out to map a complete topography of the cerebellum using fcMRI (Buckner et al., 2011). The authors identified multiple somatomotor representations, such as representations of the tongue, hand, and foot, making up a small proportion of the cerebellum in the rostral (e.g., anterior zone) and most caudal and ventral regions (e.g., posterior and nodular zones) of the cerebellum. The majority of the cerebellum was linked to a range of cerebral association networks, which include those associated with cognitive control (King et al., 2019) as well as the default mode network, which has also been connected to the cerebellum in other studies (Eryurek et al., 2022).

Task-based neuroimaging methods have been used to identify not only passive connectivity associations between the cerebellum and other parts of the brain, but also correlations between cerebellar activity and active behavior. Lobules in the human anterior zone have been associated with leg, foot, and hand movements, while those in the central zone have been associated with orofacial and eye movements and more complex tasks involving motor planning (Stoodley and Schmahmann, 2018). In the human posterior zones, visuospatial, executive function, and language tasks have been associated with central zone lobules, while working memory has been associated both with central and posterior zone lobules, and hemispheric lobule crus I and posterior zone lobule IX have been engaged during abstract mentalizing (Stoodley and Schmahmann, 2018).

It was previously mentioned that neighboring transverse zones overlap (Ozol et al., 1999) rather than represent sharp separations (e.g., there is some overlap of the anterior and central zones between lobules V and VI) (Apps and Hawkes, 2009). Consistent with this observation, a study based on resting-state functional MRI (fMRI) and task-based data from a large cohort (>1000 subjects) identified continuous gradients rather than sharp divisions between functional areas of the cerebellum. These gradients particularly displayed a gradual organization of regions processing motor, default mode network, motor, and finally non-motor information (Guell et al., 2018). In particular, the authors identified motor (lobules I-VI and VIII) and non-motor (lobules VI, hemispheric VII/crus II, and IX-X) regions of the cerebellum. Despite this large cohort dataset, others have taken issue with the small number of tasks (fewer than 7) performed to map out the relationship between the cerebellum's contributions to different behaviors and the spatial organization of the cerebellum. In particular, it has been suggested that a task-free (or "default mode"-like) parcellation of the cerebellum exhibits more overlapping edges between regions, while a more robust task-based testing regime results in strong functional boundaries between regions (King et al., 2019).

To determine if different areas of the cerebellum transition between one another as gradients or as more discrete boundaries, a recent study used a large battery of 26 tasks to comprehensively assay the cerebellar contribution to a wide set of behaviors (King et al., 2019). Specifically, the authors explored the relationship between the lobule and functional boundaries within the cerebellum based on their large task battery, which included, among other tasks, motor sequences, the Stroop task (Hershman, Beckmann, and Henik, 2022), emotional movie scenes (e.g., the love story from Pixar's "Up"), word prediction, and mental spatial navigation assays (King et al., 2019). This allowed the authors to sample cerebellar activity across a range of motor, cognitive, and affective tasks. They found that lobule boundaries only weakly correlate with functional boundaries identified during the task battery, which is in line with observations from molecular labeling and tracing studies (Ozol et al., 1999; Sugihara and Shinoda, 2004).

The authors did, however, observe stronger lobular boundary segregations that appear to segregate in part along the axis of transverse zones. These stronger boundaries were identified between lobules V and VI (anterior/central zone boundary), VI and VII (within the central zone), and lobules VIII and IX (near the posterior/nodular zone boundary) (King et al., 2019), suggesting groups of lobules belonging to the same transverse zone tend to exhibit more similar patterns of activity than those across zones. The authors also found that the cerebellar cortex can be subdivided along discrete boundaries separating regions that perform different types of processing. These areas broadly feature motor regions that align with the anterior zone involved in primary motor action, as well as a posterior motor region near lobule VIII involved with action planning. Other behaviors such as oculomotor activity in lobule VI in the vermis, as well as a wide variety of higher-order processing such as working memory, language processing, attention, and emotional processing that were associated with the caudal and hemispheric regions of the cerebellum (King et al., 2019). It is important to note, however, that the signal measured by fMRI corresponds to hemodynamic changes, used as a proxy for neuronal activity (Bandettini, 2014), and that fMRI activity largely correlates with climbing fiber and parallel fiber activity, and not with Purkinje cell firing (Lauritzen, 2001).

2.3 Organization of the cerebellar nuclei

2.3.1 The three cerebellar nuclei contain the output cells of the cerebellum

Downstream of Purkinje cells are the cerebellar nuclei, which lie at the core of the cerebellum. The cerebellar nuclei contain the output cells of this brain region which project to a wide range of targets across the brain. These nuclei are made up of three bilateral masses of neurons on either side of the cerebellar vermis, encased by extensive white matter made up of Purkinje cell axons, climbing fibers, and mossy fibers.

Moving from medial to lateral, the three pairs of cerebellar nuclei include the fastigial nucleus (also known as the medial nucleus), the interposed nuclei (subdivided into the anterior interposed and posterior interposed nuclei), and the dentate nucleus (also known as the lateral nucleus). Evolutionarily, the fastigial nucleus is the oldest of the three nuclei, while the dentate nucleus is the youngest (Kebschull et al., 2020). Like cells in the cerebellar cortex, neurons in the cerebellar nuclei receive input from inferior olive climbing fibers and multi-source mossy fibers, though their predominant input comes from Purkinje cell axons whose puncta engulf cerebellar nuclear somata as well as synapse on their dendrites (Hirono et al., 2018; Kebschull et al., 2023). More than 70 percent of synaptic boutons that target the cerebellar nuclei arise from Purkinje cells, highlighting the powerful control Purkinje cells exert onto cerebellar nuclear neurons (De Zeeuw and Berrebi, 1995). Unlike the cerebellar cortex, the cerebellar nuclei exhibit a less stereotyped organization.

The cerebellar nuclei are thought to have evolved from a single pair of nuclei, a feature that is still apparent in certain species of fish and amphibians, while reptiles and birds have two pairs of nuclei, and mammals boast three pairs (Kebschull et al., 2020). The size and shape of each nucleus varies across different animals, and these differences appear to correlate with differing cognitive abilities across species, suggesting that the cerebellar nuclei evolved independently to support new or more complex functions (Kebschull et al., 2020). Most evident among these differences is the increased size of the dentate (lateral) nucleus in primates including humans, where its growth is correlated with expansion of the prefrontal cortex (Bostan, Dum, and Strick, 2013) and increased size of hemispheric lobules crus I and crus II (Balsters et al., 2010), which may account for the increased cognitive abilities in these species.

2.3.2 Cell types and projection targets of cerebellar nuclear neurons

Each of the three cerebellar nuclei contains excitatory glutamatergic neurons, inhibitory GABAergic neurons, and inhibitory glycinergic neurons, most of which are spontaneously active (Bagnall et al., 2009; Uusisaari, Obata, and Knöpfel, 2007). Projection neurons from the cerebellar nuclei are made up of glutamatergic (Fujita, Kodama, and Du Lac, 2020) and GABAergic (De Zeeuw et al., 1997) neurons, while an additional subset of large glycinergic projection neurons are located exclusively in the rostrolateral portion of the fastigial nucleus (Bagnall et al., 2009). Small GABAergic and glycinergic inhibitory cells make up putative interneurons within the nuclei (Kebschull et al., 2023; Uusisaari and Knöpfel, 2008).

During development, cerebellar nuclear neurons arise from different proliferative zones depending on their cell lineage: future glutamatergic neurons, and potentially glycinergic neurons (Kebschull et al., 2020), arise from the rhombic lip beginning around embryonic day E9.5 in mice (Kebschull et al., 2023; Wang, Rose, and Zoghbi, 2005), while future GABAergic cells arise from the ventricular zone around E10.5 (Hoshino et al., 2005; Kebschull et al., 2023). Neurogenesis in the cerebellar nuclei continues into the early postnatal days, though embryonic-born neurons are thought to represent the projection neurons of the cerebellar nuclei, while the postnatal-born cells are more likely to represent local interneurons (Kebschull et al., 2023). How cerebellar nuclear neurons migrate and organize into separate nuclei is poorly understood, though preliminary findings suggest that neurons belonging to the dentate nucleus are born before those in the fastigial nucleus, with earlier-born cells potentially being laterally displaced by later-born medially-fated cerebellar nuclear neurons (Kebschull et al., 2023).

In adulthood, glutamatergic and glycinergic nuclear neurons exhibit similar firing properties (Bagnall et al., 2009), while GABAergic cells are on average smaller and fire at lower frequencies than non-GABAergic cells (Uusisaari, Obata, and Knöpfel, 2007).

A recent single nucleus-RNA sequencing study demonstrated that the GABAergic neurons could be divided into five transcriptomic cell types of inhibitory neurons that were present in each of the three nuclei (Kebschull et al., 2020). The largest class of GABAergic neurons are thought to represent the inhibitory cerebellar nuclear projection neurons that target the inferior olive, forming closed loops between the cerebellar nuclei, inferior olive, and cerebellar cortex (De Zeeuw et al., 1997). Meanwhile, the glutamatergic neurons are more complex: excitatory cerebellar nuclear neurons are made up of 15 transcriptomic cell types that are somewhat preferentially localized to a specific nucleus (Kebschull et al., 2020). These excitatory glutamatergic cell types in the fastigial nucleus were also shown to be more distinctive than the cell types in the interposed and dentate nuclei, which exhibit more overlap.

Projection neurons of the cerebellar nuclei are commonly assumed to be made up predominantly of glutamatergic neurons (Fujita, Kodama, and Du Lac, 2020), with a subset of GABAergic neurons projecting to the inferior olive. A recent viral tracing study in the interposed nuclei demonstrated that, in addition to the inferior olive-projecting cells, GABAergic neurons also synapse on widespread targets in the midbrain and brainstem (Judd, Lewis, and Person, 2021). In general, neurons from each of the three nuclei target many (> 50) areas of the central nervous system, often projecting to the same gross regions such as the thalamus, midbrain, and hindbrain. However, these neurons synapse on adjacent regions rather than on completely overlapping targets (Kebschull et al., 2020; Teune et al., 2000). Labeling experiments performed in each nucleus demonstrated that the fastigial and interposed nuclei also project to the spinal cord (Sathyamurthy et al., 2020), while the dentate nucleus does not (Kebschull et al., 2020). The fastigial nucleus projects to more distinct regions when compared to the interposed and dentate nuclei, consistent with the observation of more distinctive cell types within the fastigial nucleus, while the interposed and dentate nuclei are more similar in their projection patterns (Kebschull et al., 2020).

2.3.3 The fastigial nucleus is distinct among the cerebellar nuclei

As was described in the previous section, the fastigial nucleus has both the most distinct projection patterns and cell types of all three cerebellar nuclei. While evidence from disease and neuroimaging studies often highlights the role of the cerebellar hemispheres and the dentate nucleus in higher-order cognitive processing (Badura et al., 2018; King et al., 2019), the fastigial nucleus has also been implicated in a wide variety of functions. These include both motor and non-motor behaviors, such as respiration and orofacial behaviors (Lu et al., 2013), visual saccades (Bourrelly, Quinet, and Goffart, 2018), freezing behavior (Vaaga, Brown, and Raman, 2020), fear extinction (Frontera et al., 2023), repetitive behavior (Kelly et al., 2020), and blood pressure control

(Miura and Reis, 1969; Rector, Richard, and Harper, 2006). While six different excitatory glutamatergic cell types have been identified in the fastigial nucleus in mice in a single nucleus RNA sequencing study (Kebschull et al., 2020), five major types of excitatory cell types were identified by their gene expression patterns, connectivity patterns, and morphology in a contemporaneous study by another research group (Fujita, Kodama, and Du Lac, 2020). These five cell types were localized to specific areas of the fastigial nucleus which generally overlap with an earlier topographic subdivision of the fastigial nucleus into molecular domains (Chung, Marzban, and Hawkes, 2009). The topography of these five cell types was also associated with distinct output circuits. Moving from rostral to caudal, these groups of cells were associated with posturomotor, oromotor, autonomic, self-protection/vigilance, and self-orienting behaviors (Fujita, Kodama, and Du Lac, 2020). These findings highlight the modular nature of the fastigial nucleus, suggesting that it is not homogeneous but rather consists of subregions that underpin a particular set of functions and behaviors.

Inhibitory projection neurons in the fastigial nucleus also contribute to a variety of behaviors. GABAergic projection neurons from the fastigial nucleus target multiple areas in the inferior olive (Sugihara and Shinoda, 2004) and contribute to the closed nucleo-olivary-cortical loops (De Zeeuw et al., 1997). These nucleo-olivary GABAergic neurons are thought to contribute to a feedback system to control: 1. associative learning, 2. activity in the nucleo-olivary-cortical loop, possibly including spontaneous Purkinje cell activity, and 3. electrotonic coupling in inferior olive neurons (Bengtsson and Hesslow, 2013). GABAergic fastigial nucleus projection neurons also target regions outside of the inferior olive, such as the ventromedial hypothalamic nucleus where they may contribute to feeding behavior (Li et al., 2017). A subset of mostly GABAergic neurons in the fastigial nucleus have also been documented crossing the cerebellum along the roof of the fourth ventricle to synapse on neurons in the contralateral fastigial nucleus (Gómez-González and Martínez-Torres, 2021), which suggests further complexity in the role of GABAergic fastigial nuclear neurons modulating cerebellar output.

While excitatory glutamatergic neurons project contralaterally, the large inhibitory glycinergic neurons of the fastigial nucleus have been shown to project to areas in the ipsilateral brainstem such as the vestibular and reticular nuclei, as well as the spinal cord (Bagnall et al., 2009). These glycinergic projection neurons are unique to the fastigial nucleus and exhibit physiological properties that are similar to the glutamatergic projection neurons of the cerebellar nuclei (Bagnall et al., 2009; Kebschull et al., 2023). Ipsilateral-projecting inhibitory glycinergic neurons may be well suited to perform fast modulation of behavior, complementing the contribution of contralateral-projection excitatory glutamatergic neurons (Bagnall et al., 2009).

2.3.4 Fastigial nucleus activity and its effects on behavior

Recordings from fastigial nucleus neurons in awake, behaving animals can help support the conclusions made about the fastigial nucleus contribution to behavior from tracing studies (Fujita, Kodama, and Du Lac, 2020). Activity in the rostral part of the fastigial nucleus in monkeys has been associated with encoding movement of the body through space (Brooks and Cullen, 2009; Shaikh, Meng, and Angelaki, 2004). Studies focusing on the caudal part of the fastigial nucleus describe these neurons as being associated with changes in visual saccade adaptation following eye injury (Scudder and McGee, 2003) as well as general saccadic activity and in responsiveness to optokinetic stimuli (Gruart and Delgado-García, 1994; Kawamura et al., 1990; Noda et al., 1988).

Recent studies have shown the contribution of the fastigial nucleus to more complex behaviors. Lesioning and optogenetic perturbation of fastigial nuclear neurons results in disrupted preparatory behavior mediated by neurons in the anterior lateral motor cortex (ALM; Gao et al., 2018). Optogenetic inhibition of the fastigial nucleus during the "delay period" before movement in a behavioral choice test disrupted movement initiation and correct response selection, with minimal effect on movement execution. Optogenetic inhibition of fastigial nuclear neurons altered ALM activity, and inhibition of ALM neurons altered fastigial nucleus activity, highlighting the fastigial nucleus-ALM circuit as a cerebellum-cerebral cortex loop involved in motor planning and preparatory behavior (Gao et al., 2018).

Fastigial nuclear neurons have also been linked with defensive behaviors (Vaaga, Brown, and Raman, 2020). Glutamatergic projection neurons from the fastigial nucleus can target cells in the ventrolateral periaqueductal gray, a structure associated with responding to aversive stimuli (Mokhtar and Singh, 2023). Stimulation of cells in the ventrolateral periaqueductal gray that receive input from the fastigial nucleus results in freezing behavior in mice (Vaaga, Brown, and Raman, 2020). Robust connectivity between the fastigial nucleus and the periaqueductal gray has also been observed in humans (Cacciola et al., 2019). These findings highlight the wide range of the fastigial nucleus contribution to behavior.

Studies reporting the effects of lesions in the fastigial nucleus are also helpful for identifying its contribution to behavior. Bilateral lesions in the fastigial nuclei of juvenile rats produced long-lasting deficits in motor coordination and social interactions (Al-Afif et al., 2019), as well as deficits in spatial and operant learning (Helgers et al., 2020). These observations suggest a role of the fastigial nucleus in motor, social, and cognitive behaviors. Human patients with fastigial nucleus lesions regularly experience postural and gait abnormalities (Ilg et al., 2008; Schoch et al., 2006), saccadic impairments (Helmchen et al., 2022), and cerebellar mutism (Albazron et al., 2019). It has also been suggested that damage to the fastigial nucleus in humans results in neuropsychiatric symptoms such as disrupted affect (Heath, Cox, and Lustick, 1974; Schmahmann, 2004; Schmahmann, 2000; Schmahmann et al., 2019), though evidence for this claim is currently scarce.

Activity in the fastigial nucleus has been linked to surprising functions. Stimulation of the fastigial nucleus can be used as a neuroprotective mechanism in the brain against cerebral ischemia (Ma et al., 2022). Comparatively, stimulation of the dentate nucleus does not provide such neuroprotection following ischemia (Wang et al., 2014). Fastigial nucleus stimulation can improve post-ischemic outcomes through a variety of mechanisms, such as reduction in the inflammatory response and apoptotic processes (Wang et al., 2014). This neuroprotective effect may be due to the fastigial nucleus' widespread projections in the brain, as well as its contribution to autonomic processes like blood pressure control (Miura and Reis, 1969; Wang et al., 2014). A recent study performed in mice showed that activation, but not inhibition, of the fastigial nucleus successfully attenuated hippocampal seizures (Streng and Krook-Magnuson, 2020). The link between fastigial nucleus activity and neuroprotection in multiple species is surprising but may fit with the notion of the cerebellum's contributions to widespread function.

Reports from human fMRI studies on fastigial nucleus activity are rare. Some human fMRI studies have reported fastigial nucleus activation in response to pain (Dimitrova et al., 2003), thirst (Parsons et al., 2000), and autonomic function (Henderson et al., 2002). However, a robust characterization of fastigial nucleus activity from human neuroimaging studies is lacking. This is largely due to technical limitations, such as isolating fastigial nucleus activity from activity in the interposed and dentate nuclei (Küper et al., 2012), as well as imaging signal loss from the high iron content of the cerebellar nuclei (Diedrichsen et al., 2011). Additionally, distinguishing between fMRI activity from the cerebellar cortex and from the cerebellar nuclei is a non-trivial task since the fMRI signal largely reflects mossy fiber activity, and mossy fibers target both the cerebellar cortex and nuclei (Küper et al., 2012). As such, animal models may be better suited to providing a more comprehensive picture of fastigial nucleus function.

2.4 Connectivity between the cerebellar cortex and cerebellar nuclei

Identifying the connections between Purkinje cells and cerebellar nuclear neurons is critical for understanding cerebellar function: Purkinje cells transmit cerebellar cortical computation, while cerebellar nuclear neurons transmit the results of cerebellar processing to the rest of the brain. Purkinje cell - cerebellar nuclear neuron connections have commonly been referred to as "corticonuclear" projections or "corticonuclear" connectivity. These terms will be used interchangeably in the following sections.

2.4.1 Corticonuclear connectivity during embryonic development

Since Purkinje cells constitute the sole output of the cerebellar cortex and project to cerebellar nuclear neurons, the output cells of the cerebellum, identifying how and when the connections between these two populations form is important for understanding the development of the cerebellar circuit. Purkinje cells (born embryonic day E10-13) and cerebellar nuclear neurons (born E9.5 for glutamatergic cells from the rhombic lip, E10.5 for GABAergic projection neuron from the ventricular zone) (Butts, Green, and Wingate, 2014; Elsen et al., 2019; Hoshino et al., 2005; Kebschull et al., 2023) represent two of the earliest-born neuronal populations of the cerebellum. Connections between Purkinje cells and cerebellar nuclear neurons are formed very early in development (Rahimi-Balaei et al., 2018), though the exact mechanism by which Purkinje cells contact individual cerebellar nuclear neurons remains unclear (Sotelo and Rossi, 2022). In mice, Purkinje cell axonogenesis begins around E12.5 and their axons start to fasciculate and target the cerebellar nuclei by E14.5, which occurs at the same time as Purkinje cells begin forming the multi-layered Purkinje plate (Miyata et al., 2010). Nuclear neurons from each lineage migrate from their prospective proliferative zones to the nuclear transitory zone via Netrin and Slit signaling (Rahimi-Balaei et al., 2018) before beginning to form the cerebellar nuclei at E14.5 (Elsen et al., 2019; Laumonnerie and Solecki, 2020). Thus, Purkinje cell – cerebellar nuclei contact begins while both Purkinje cells and cerebellar nuclear neurons are still migrating. By E18.5, many Purkinje cell axons can already be found in each of the three cerebellar nuclei (Sillitoe, Gopal, and Joyner, 2009).

While Purkinje cell axons reach the cerebellar nuclei early, the exact mechanisms by which they form synaptic contacts with individual cerebellar nuclear neurons is unknown (Kebschull et al., 2023). Since Purkinje cell axons are already present in the cerebellar nuclei shortly after their final cell division, short-range cues between individual Purkinje cell axons and cerebellar nuclear neurons, rather than long-range axon guidance cues, are thought to govern Purkinje cell – cerebellar nuclear neuron connectivity (Sotelo and Rossi, 2022). Potential short-range mechanisms may include: cadherins and protocadherins (Sarpong et al., 2018), where Purkinje cells and nuclear neurons that express the same cadherin subtype are connected (Neudert and Redies, 2008; Redies, Neudert, and Lin, 2011); netrin and its repulsive receptor Unc5c, which is strongly expressed by both Purkinje cell and nuclear neuron progenitors (Kim and Ackerman, 2011; Sotelo and Rossi, 2022); and Anosmin-1, a branch-inducing factor affecting both terminal arbors and collateral branches of Purkinje cell axons (Gianola, Castro, and Rossi, 2009; Sotelo and Rossi, 2022). However, the causal mechanisms of Purkinje cell-cerebellar nuclear neuron contact remain speculative at the present time.

2.4.2 Corticonuclear connectivity during postnatal development

Purkinje cell – nuclear neuron synapses become functional in the first postnatal week of life (Garin and Escher, 2001; Van Welie, Smith, and Watt, 2011). Purkinje cells exhibit features of presynaptic development starting around postnatal day P4, while expression of postsynaptic $GABA_A$ receptors in cerebellar nuclear neurons remains low until P8. Surprisingly, excitatory and inhibitory postsynaptic potentials can be evoked from cerebellar nuclear neurons before seemingly functional $GABA_A$ receptors are found in the cerebellar nuclei (at P0 and P2, respectively; Gardette et al., 1985). These findings suggest that synapses between these cells become functional in early postnatal life, despite Purkinje cell axons' proximity to cerebellar nuclear neurons before birth.

Maturation of Purkinje cell synapses on cerebellar nuclear neurons occurs with the development of perineuronal nets in the first few weeks of life (Hirono, 2021). Perineuronal nets are made up of extracellular matrix molecules that enrobe cell bodies, pre- and postsynaptic structures, and glia, helping to fortify the tripartite (or 'tetrapartite') synapses (Hirono, 2021). In the cerebellum, perineuronal nets preferentially develop around large glutamatergic cerebellar nuclear neurons starting from P7 in rats (Carulli et al., 2006; Carulli, Rhodes, and Fawcett, 2007), and are fully formed by P19 (Bekku et al., 2012; Hirono et al., 2018). The formation of perineuronal nets is associated with the closing of critical periods in development (Carulli, Rhodes, and Fawcett, 2007), as these structures stabilize synapses and thus mark the end of developmental synaptic refinement (Bekku et al., 2012; Hirono, 2021).

Acute chemical depletion of perineuronal nets results in increased GABAergic transmission between Purkinje cells and cerebellar nuclear neurons through increased probability of GABA release from Purkinje cells (Hirono et al., 2018; O'Dell et al., 2021). This induced change in perineuronal net dynamics impacts motor learning. Motor learning itself has been shown to alter perineuronal nets in adult mice: eyeblink conditioning was sufficient to temporarily reduce perineuronal net density around cerebellar nuclear neurons, which was restored after memory consolidation (Carulli et al., 2020). Perineuronal net depletion has been shown to enhance memory formation through its effect on synaptic plasticity (Hirono, 2021). These results suggest that, while developmental perineuronal net formation stabilizes synaptic contacts between Purkinje cells and cerebellar nuclear neurons, perineuronal nets, and thus Purkinje cell – cerebellar nuclear neuron synapses, can remain dynamic into adulthood.

Additionally, myelination of Purkinje cell axons has been shown to be necessary for proper development of Purkinje cell presynaptic terminals and communication between Purkinje cells and cerebellar nuclear neurons (Barron et al., 2018). Myelination of Purkinje cell axons occurs differentially across the cerebellar cortex: while many areas had differentiated oligodendrocytes in the rat P10 cerebellum, other areas still lacked myelin at this timepoint (Reynolds and Wilkin, 1988). Purkinje cell axons generally appear to be myelinated by P15 in rats, following a similar time course as perineuronal net stabilization of cerebellar nuclear neuron synaptic contacts. These results highlight that multiple developmental phenomena likely contribute to the formation and maturation of Purkinje cell – cerebellar nuclear contacts.

2.4.3 Corticonuclear connectivity at the macro-scale level

2.4.3.1 Mediolateral corticonuclear connectivity

Efforts to uncover the topographic organization of Purkinje cell – cerebellar nuclear connections began in earnest in the first half of the 20th century (Haines and Manto, 2011; Haines and Manto, 2010; Jansen and Brodal, 1940). Several scientists including R.H. Clarke and V. Horsley (1905), L.B. Hohman (1929), and J. Jansen and A. Brodal (1940), explored the organization of corticonuclear projections (Haines and Manto, 2011; Haines and Manto, 2010; Hohman, 2010; Jansen and Brodal, 1940). These researchers described a generally ipsilateral and mediolateral organization to the corticonuclear projections.

Scientists in the second half of the 20th century expanded upon these early findings from lesion and labeling studies, confirming the ipsilateral and mediolateral organization (Apps and Hawkes, 2009; Groenewegen and Voogd, 1977; Groenewegen, Voogd, and Freedman, 1979), often with the use of more complex techniques such as electrophysiology (Armstrong and Schild, 1978a; Buisseret-Delmas, 1988; Deura, 1969; Duffin et al., 2010; Yu, Ebner, and Bloedel, 1985). They observed that Purkinje cells in the vermis target the fastigial nucleus while Purkinje cells located more laterally in the hemispheres typically target the dentate nucleus (Armstrong and Schild, 1978a; Armstrong and Schild, 1978b). This mediolateral corticonuclear organization has also been observed in non-mammals, such as in lizards (Bangma and Donkelaar, 1984) and chickens (Arndt et al., 1998). Exceptions to these rules have been noted, where the fastigial nucleus also receives some hemispheric input from crus I and II Purkinje cells (Armstrong and Schild, 1978b; Fujita, Kodama, and Du Lac, 2020).

The work of Jan Voogd continued expanding upon this mediolateral organization. Voogd identified longitudinal zones of Purkinje cells in the sagittal plane that topographically target particular areas within the cerebellar nuclei (see section 2.2.3.1)

(Haines and Manto, 2011; Voogd, 2011). Voogd's original longitudinal zones were expanded to describe several broad parasagittal cerebellar cortical zones (A, X, B, C, and D, each with their own subdivisions) that project to distinct areas in each cerebellar nuclei (Apps and Hawkes, 2009; Voogd and Ruigrok, 2004). The concept of longitudinal zones also came to incorporate evidence of reciprocal connections between the cerebellum and the inferior olive. Purkinje cells located in longitudinal zones (also called "modules") target cerebellar nuclear neurons that then target the inferior olivary cells which connect back to the same Purkinje cells, forming an "olivo-cortico-nuclear loop" (Gravel et al., 1987; Ito, 2006; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004). Longitudinal zones incorporate non-contiguous but molecularly-similar stripes of Purkinje cells, where Purkinje cells from two zebrin II-positive stripes are considered linked stripes belonging to the same longitudinal zone, despite being separated a zebrin II-negative stripe (Sugihara, 2021).

The modular organization of olivo-cortico-nuclear connectivity has been uncovered by both microinjection labeling (Groenewegen, Voogd, and Freedman, 1979; Teune et al., 2000; Voogd et al., 2003) and immunostaining studies (Sugihara and Quy, 2007; Sugihara and Shinoda, 2004; Sugihara and Shinoda, 2007; Voogd et al., 2003). The longitudinal modules have also been corroborated with electrophysiological experiments combined with anatomical tracing (Apps and Garwicz, 2000; Campbell and Armstrong, 1985; Garwicz, Apps, and Trott, 1996; Trott and Armstrong, 1987; Trott, Apps, and Armstrong, 1998a; Trott, Apps, and Armstrong, 1998b; Voogd et al., 2003). Further, these modules generally colocalize with the parasagittal zebrin II patterning of the cerebellar cortex (Apps and Hawkes, 2009; Cerminara et al., 2013; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004), though there are differences between rostrally- and caudally-located Purkinje cells since zebrin II stripes are not continuous across transverse zones (Ozol et al., 1999). This suggests that, while a mediolateral organization underlies Purkinje cell connections to the cerebellar nuclei, there is likely to be variability in these connections along the rostrocaudal axis.

From the cerebellar nuclear perspective, the fastigial nucleus has been historically associated with the "A" module, which extends through all of the lobules along the rostrocaudal axis (Ruigrok, Sillitoe, and Voogd, 2015). The "A" module targets different areas within the caudal medial accessory olive, a region within the greater inferior olive (Apps and Hawkes, 2009; Sugihara and Shinoda, 2007; Voogd and Ruigrok, 2004). This inferior olive-cerebellar module compartmentalization is also consistent with the recent circuit compartmentalization found for glutamatergic fastigial nuclear neurons (Fujita, Kodama, and Du Lac, 2020). This suggests that, rather than simply reflecting a topography for GABAergic fastigial neurons which directly contact the inferior olive, glutamatergic fastigial nuclear neurons also exhibit a similar compartmentalization.

Thus, a general fastigial nucleus topography of cell types and connectivity to downstream areas of the brain appears to be a feature of a larger cerebellar topographic organization.

2.4.3.2 Rostrocaudal corticonuclear connectivity in the fastigial nucleus

Corticonuclear connectivity has been mostly explored in the mediolateral plane, whereas such connectivity along the perpendicular rostrocaudal axis, which is delineated by the cerebellar lobules, has received less attention (Sugihara, 2021). A recent review of cerebellar connectivity has described the olivo-cortico-nuclear system as predominantly exhibiting a zonal rather than a lobular organization (Voogd et al., 2013). Nonetheless, several studies have explored the relationship between cerebellar lobules and corticonuclear connectivity, particularly using anatomical tracing and lesion methods.

Purkinje cells from different lobules tend to project to the closest region of the fastigial nucleus (Courville and Diakiw, 1976; Haines and Koletar, 1979). The authors found that rostrally-situated lobules (e.g. lobules I, II, and IV/V) tend to terminate in rostral areas of the fastigial nucleus, caudally-situated lobules (e.g. lobule IX) terminate in caudal areas, and lobules in the middle (e.g. lobule VI) terminate in medial areas. This topography was also observed by others in the rat (Armstrong and Schild, 1978a; Hawkes and Leclerc, 1986; Umetani, Tabuchi, and Ichimura, 1986), cat (Courville and Diakiw, 1976; Dietrichs, 1983), pigeon (Arends and Zeigler, 1991), and primate cerebellum (Haines, 1976; Vachananda, 1959).

A study that combined histological and electrophysiological techniques in cats found similar results, where corticonuclear projections follow both a mediolateral and rostrocaudal organization (Fanardzhyan, Oganesyan, and Melik-Mus'yan, 1973). These authors presented examples of Purkinje cell convergence from across zones on the same area of a cerebellar nucleus, as well as divergence from Purkinje cells in the same zone on different areas of a nucleus. To better understand these complex Purkinje projection patterns, the discussion will now turn to evaluating corticonuclear projection patterns at the level of individual Purkinje cell axons.

2.4.4 Corticonuclear connectivity at the level of individual cells

Corticonuclear connectivity has also been elucidated through laborious but extremely informative Purkinje cell axonal reconstructions. Early studies featuring reconstructed Purkinje cell axons were performed in cats (Bishop et al., 1979), rabbits (De Zeeuw et al., 1994; Wylie et al., 1994), and rats (Sugihara, Ebata, and Shinoda, 2004). These studies demonstrated properties of corticonuclear connectivity at the level of individual cells, compared corticonuclear connectivity between areas of the cerebellar cortex, and demonstrated that an individual Purkinje cell can contact multiple nuclei.

A subsequent study systematically explored properties of Purkinje cell axonal trajectories across many areas of the cerebellar cortex by anterogradely labeling entire Purkinje cell axons from their position in the cerebellar cortex to their terminal fields in the cerebellar nuclei (Sugihara et al., 2009). Purkinje cell axonal projections generally corresponded with the previously described olivo-cortico-nuclear loops: mediolateral convergence typically occurs for Purkinje cells in the same zebrin II-positive or -negative compartments onto a small area in each of the three cerebellar nuclei. In this study, the authors also observed some cross targeting: in 30 percent of reconstructed Purkinje cells, puncta that targeted both zebrin II-positive and -negative compartments were identified in the fastigial and interposed nuclei. However, "opposite"-zebrin II targeting Purkinje cell axonal terminals (e.g., a zebrin II-positive Purkinje cell with axonal terminals in a zebrin-II negative area of the cerebellar nuclei) occurred 5 percent of the time, suggesting that zebrin II cross targeting is typically an exception to the olivo-cortico-nuclear rule.

Interestingly, while Purkinje cell axons that projected to the interposed and dentate nuclei had tightly compact termination fields within a zebrin II-positive or -negative region, the authors observed different termination patterns for the fastigial-projecting Purkinje cell axons. Fastigial-projecting Purkinje cell axons often had much wider terminal arbors. In some cases, these fastigial-projecting Purkinje cells from the vermis also synapsed on extracerebellar targets like the vestibular nucleus and the parabrachial nucleus. Only Purkinje cells from the vermis targeted both the cerebellar nuclei and nuclei outside the cerebellum (Sugihara et al., 2009). These results add further support to the hypothesis that neurons from the fastigial nucleus may be involved in more diverse processes than those from the interposed or dentate nuclei (Kebschull et al., 2020).

Reconstruction of individual Purkinje cell axons also showed that Purkinje cells from multiple lobules within the same mediolateral plane can converge on the same area in the cerebellar nuclei where their terminal axons overlapped significantly. Individual Purkinje cell axons were found to target multiple nuclei, corroborating earlier findings (De Zeeuw et al., 1994). The authors additionally performed retrograde labeling of cerebellar nuclear neurons to identify how Purkinje cells converge onto a particular area in the nuclei using fluorescent latex microspheres to label Purkinje cell convergence. Unfortunately, this method did not offer the same level of resolution necessary to trace individual corticonuclear projections. Tracing the entire trajectories of Purkinje cell axons from an individual cerebellar nuclear neuron would be necessary to identify how all Purkinje cells that converge onto that individual neuron. Rather than describing convergence at the level of individual cerebellar nuclear neurons, the retrograde labeling demonstrated that Purkinje cells from many lobules in the vermis can converge onto a small patch in the fastigial nucleus. To better understand corticonuclear connectivity at a high resolution, the discussion will now focus on properties of the Purkinje cell – cerebellar nuclear synapse.

2.4.5 Corticonuclear synaptic transmission

In addition to providing evidence for corticonuclear connections as mentioned in section 2.4.3.1, electrophysiological recordings can be used to elucidate the synaptic properties of the corticonuclear synapse. For instance, GABAergic Purkinje cells synapse on the wide variety of cerebellar nuclear neurons. Whole-cell electrophysiological recordings in cerebellar nuclear neurons have shown that Purkinje cell synaptic transmission elicits fast postsynaptic inhibitory events in cerebellar nuclear neurons via ionotropic $\mathrm{GABA_A}$ receptor signaling (Person and Raman, 2012a; Pugh and Raman, 2005). Blocking $\mathrm{GABA_A}$ receptors with the antagonist bicuculline abolishes spontaneous currents from cerebellar nuclear neurons (Anchisi, Scelfo, and Tempia, 2001). While cerebellar nuclear neurons also contain metabotropic $\mathrm{GABA_B}$ receptors, these receptors do not appear to be involved in fast Purkinje cell signaling (Morishita and Sastry, 1995; Sastry et al., 1997). Presynaptic $\mathrm{GABA_B}$ receptors in Purkinje cells, however, may modulate the size of inhibitory postsynaptic potential (IPSP) amplitudes from cerebellar nuclear neurons (Mouginot and Gähwiler, 1996).

Different types of cerebellar nuclear neurons respond differently to Purkinje cell firing. For example, when compared to inferior olive-projecting small GABAergic cerebellar nuclear neurons, the large putatively glutamatergic cerebellar nuclear neurons exhibit faster kinetics, higher firing rates, and larger inhibitory postsynaptic currents (Najac and Raman, 2015; Uusisaari and Knöpfel, 2008). Purkinje cell axons appear to preferentially terminate on the dendrites of small GABAergic cells in the cerebellar nuclei, rather than on the somata as they do on large cerebellar nuclear neurons (Najac and Raman, 2015; Uusisaari and Knöpfel, 2008). The differences in kinetics between putative glutamatergic and GABAergic cerebellar nuclear neurons may also be due to differences in $\mathrm{GABA_A}$ receptor subunit compositions between these cell types. Putative glutamatergic cerebellar nuclear neuron $\mathrm{GABA_A}$ receptors consist of only the $\alpha 1$ subunit, but GABAergic nuclear neuron $\mathrm{GABA_A}$ receptors have both the faster $\alpha 1$ and slower $\alpha 3$ subunits (Uusisaari and Knöpfel, 2008). In all, these differences may allow different classes of cerebellar nuclear neurons to integrate different types of information (Najac and Raman, 2015).

Purkinje cells and cerebellar nuclear neurons are both spontaneously active and capable of firing at high frequencies. This puzzling phenomenon has led to investigations of how cerebellar nuclear neurons are able to sustain regular spiking despite the barrage of inhibitory input they receive from Purkinje cells (Gauck and Jaeger, 2000; Person and Raman, 2012a). While cerebellar nuclear neurons do receive excitatory input from climbing fibers and mossy fibers, their predominant (∼70 percent) source of input comes from inhibitory Purkinje cells that consist of dense, multi-site axonal terminals on each cerebellar nuclear neuron (De Zeeuw and Berrebi, 1995; Sugihara et al., 2009). Consistent with this observation, inhibitory input from Purkinje cells has been shown to exert greater influence on cerebellar nuclear neuron firing than excitatory input from climbing and mossy fibers in a slice preparation (Gauck and Jaeger, 2003).

Since cerebellar nuclear neuron firing rates do not always vary inversely with Purkinje cell firing rates (Person and Raman, 2012b), many have asked what factors mediate cerebellar nuclear neuron output. Different hypotheses have been put forward to explain how cerebellar nuclear neurons fire spontaneously under a deluge of GABAergic neurotransmission. While their intrinsic properties allow for spontaneous firing (Mouginot and Gähwiler, 1995; Person and Raman, 2012b), cerebellar nuclear neurons may also maintain regular firing through rebound depolarization. During rebound depolarization, these neurons experience a burst of calcium-mediated action potentials after hyperpolarization, typically during a pause in Purkinje cell firing (Aizenman and Linden, 1999). Rebound depolarization can be evoked by injecting a hyperpolarizing current into a cerebellar nuclear neuron to simulate Purkinje cell inhibition (Aizenman and Linden, 1999). Spontaneous rebound depolarization has been observed from cerebellar nuclear neurons following direct suppression of Purkinje cell activity (Heiney et al., 2014; Witter et al., 2013) as well as after putative climbing fiber activity (Hoebeek et al., 2010). Synchronous Purkinje cell activity that occurs in response to induced climbing fiber activity yields robust rebound depolarization in the cerebellar nuclei (Bengtsson, Ekerot, and Jörntell, 2011). However, hyperpolarization events that are strong enough to induce rebound depolarization in nuclear neurons may be rare *in vivo* (Alviña et al., 2008). Further, unlike large glutamatergic cerebellar nuclear neurons, small GABAergic nuclear neurons do not exhibit prolonged rebound depolarization (Najac and Raman, 2015), thus indicating that rebound depolarization does not fully explain the firing properties of all cerebellar nuclear neurons.

Action potential synchrony between Purkinje cells has been suggested as a mechanism by which cerebellar nuclear neurons fire regularly (Person and Raman, 2012b). Mimicking synchronous and asynchronous convergent Purkinje cell activity while recording from a cerebellar nuclear neuron yields either regular or inhibited spontaneous cerebellar nuclear firing, respectively (Person and Raman, 2012a). Purkinje cell synchrony may result in consistent spiking pauses that release cerebellar nuclear neurons from tonic inhibition, thus enabling rebound depolarization (Person and Raman, 2012b). Climbing fiber stimulation of a Purkinje cell has been shown to synchronize the suppression of activity in neighboring Purkinje cells through ephaptic coupling, and this may result in increased firing rates in the cerebellar nuclei (Han et al., 2020). Purkinje cell synchrony has been observed during behavior, where bands of Purkinje cells were synchronized *in vivo* during a learned motor task (Heck, Thach, and Keating, 2007). This suggests that Purkinje cell synchrony may be important for downstream neural activity and subsequent behavioral output.

2.4.6 Modes of information transfer between Purkinje cells and cerebellar nuclear neurons

What is the relationship between Purkinje cell firing and cerebellar nuclear firing, and how does that enable successful behavioral outcomes? This remains a dynamic and contested question in the field of cerebellar neuroscience. Two dominant theories set out to address the complex question of cerebellar computation at the Purkinje cell – cerebellar nuclear neuron synapse (Heck et al., 2013). These theories describe the roles of rate coding and temporal coding of Purkinje cell firing in mediating cerebellar output (Cook, Fields, and Watt, 2020; De Zeeuw et al., 2011).

Rate coding in the cerebellum

The "rate code" theory postulates that Purkinje cell firing frequency is the main mechanism of neural computation in the cerebellum and the subsequent driver of behavior, irrespective of regularity (Payne et al., 2019). The rate code is hypothesized by some to dominate cerebellar computation where there is a linear relationship between Purkinje cell firing rate and behavioral output (Payne et al., 2019; Walter and and Khodakhah, 2009). Linear computation of firing rate has been observed at other synapses in the cerebellum (Walter and Khodakhah, 2006), and is suggested to offer a mathematically simple framework to explain information encoding at the Purkinje cell – cerebellar nuclear neuron synapse (Walter and and Khodakhah, 2009). Biophysical modeling studies of this synapse suggest that a rate code is sufficient to account for cerebellar nuclear neuron output (Abbasi et al., 2017; Gilbert, 2022; Zang, Hong, and De Schutter, 2020). *In vitro* studies have demonstrated that properties at the Purkinje cell – cerebellar nuclear neuron synapse are specialized to overcome synaptic depression, enabling linear encoding of Purkinje cell firing rate by cerebellar nuclear neurons (Pedroarena, 2020).

Behavioral studies exploring the rate code hypothesis are useful for identifying its validity in living animals. *In vivo* recordings from Purkinje cells in the flocculus, an area important for oculomotor performance (Suvrathan, Payne, and Raymond, 2016), show a high correlation between Purkinje cell firing rate and eye movements (Chaisanguanthum et al., 2014; Medina and Lisberger, 2007). Floccular-mediated eye movements have been shown to be insensitive to fluctuations in Purkinje cell regularity (Payne et al., 2019). Similar findings were observed from Purkinje cells located in the oculomotor vermis (Herzfeld et al., 2015). A linear rate code between Purkinje cell firing and behavior has also been implicated during voluntary whisking in mice (Chen, Augustine, and Chadderton, 2016). However, while a linear relationship appears to exist between Purkinje cell firing rate and certain behaviors like eye movements, evidence of Purkinje cell rate coding in other behaviors like limb movements is less compelling (Ebner, Hewitt, and Popa, 2011).

Temporal coding in the cerebellum

The "temporal code" theory encompasses two major observations related to Purkinje cell firing. First, the regularity (pacemaker firing) or irregularity of Purkinje cell spikes within a cell affects cerebellar output (De Zeeuw et al., 2011). Purkinje cell irregularity has been observed in many diseases (Achilly et al., 2021; Alviña and Khodakhah, 2010; Peter et al., 2016), where rescuing Purkinje cell firing regularity relieves motor deficits (Jayabal et al., 2016). This observation has led to the hypothesis that the timing of individual Purkinje cell spikes within a cell is important for normal cerebellar function. In healthy mice, Purkinje cell firing rates have been shown to shift during the initiation phase of different behaviors (Brown and Raman, 2018; Gaffield, Sauerbrei, and Christie, 2022; Sarnaik and Raman, 2018; Tsutsumi et al., 2020), often resulting in brief periods of increased irregularity or changes in the inter-spike intervals. These results suggest that the temporal dynamics of Purkinje cell firing are important for normal behavior.

The second component of the temporal code hypothesis is that of Purkinje cell synchrony (Person and Raman, 2012b), which was described previously in section 2.4.5. In addition to allowing cerebellar nuclear neurons to fire consistently, synchronous firing of Purkinje cells enables cerebellar nuclear neurons to faithfully encode and transmit these firing rates *in vivo* (Person and Raman, 2012a; De Solages et al., 2008). Purkinje cell synchrony has been observed during different behaviors (Heck, Thach, and Keating, 2007; Sedaghat-Nejad et al., 2022), and appears to contribute to the initiation and cessation of movement (Sedaghat-Nejad et al., 2022). Models of temporal coding of Purkinje cell firing describe an interplay between Purkinje cell synchrony and regularity, in which the firing output of cerebellar nuclear neurons is dependent upon both factors (Luthman et al., 2011).

Multiple codes driving cerebellar output

Still others have suggested that a combination of neural computation strategies may be required for flexible, adaptive, and time-sensitive behavioral output (De Zeeuw et al., 2011; Gauck and Jaeger, 2000; Gilbert, 2022). Purkinje cells are capable of both rate and temporal coding (Person and Raman, 2012b; Steuber and Jaeger, 2013). Purkinje cells may alternate between rate and temporal coding in a context-dependent manner rather than use both coincidentally (Stahl et al., 2022). Modeling studies have demonstrated that each coding strategy is important for cerebellar output (De Schutter and Steuber, 2009; Gauck and Jaeger, 2000; Sudhakar, Torben-Nielsen, and De Schutter, 2015; Tang et al., 2021). A recent study set out to directly assess the contribution of rate and temporal coding in controlling eye movements in monkeys and found that Purkinje cells use both forms of coding during behavior (Hong et al., 2016). The authors suggest that synchrony may complement the rate code as a "multiplexed code" to represent a variety of firing frequencies at the population level, thus coordinating sensorimotor integration and output (Hong et al., 2016). Finally, recent evidence highlights that specific cerebellar nuclear neuron types are tuned to encode Purkinje cell firing via a particular coding strategy (Özcan et al., 2020). Large glutamatergic projection neurons in the fastigial nucleus can respond to both rate and temporal coding, while small GABAergic cerebellar nuclear neurons respond to the population average Purkinje cell firing rate (Özcan et al., 2020). These results indicate that the cerebellum likely encodes and transfers information using a variety of mechanisms, which may contribute to the versatility of this brain region.

2.5 Circuit mapping in the cerebellum with optogenetics

Electrophysiology can be used to assess functional connections between putative pre- and postsynaptic cells (Campagnola et al., 2022). By electrically stimulating a putative presynaptic cell and recording from a putative postsynaptic cell, complex patterns of connectivity can be identified within a circuit (Song et al., 2005). The ability to precisely control the extent of electrical stimulation is, however, limited when using a fully electrophysiological approach (Linders et al., 2022). The development of genetically-encoded light-based actuators of neurons such as Channelrhodopsin-2 (ChR2) (Boyden et al., 2005) rapidly changed the landscape of experimental neuroscience. Actuators like ChR2 offer the precision and speed lacking from traditional electrical stimulation methods. These benefits have made optogenetics, or the genetically-mediated optical control of cell activity, an incredibly useful tool for exploring temporally- and spatially-sensitive experimental questions (Zeng and Madisen, 2012).

2.5.1 The origins of optogenetics

In the early 2000s, research on light-gated ion channels (Nagel et al., 2002; Nagel et al., 2003) offered a glimpse into the possibility of controlling the activity of cells with light (Boyden, 2011). ChR2, a light-gated non-selective cation membrane channel from the green alga Chlamydomonas reinhardtii, was described in 2003 (Nagel et al., 2003). By 2005, it was demonstrated that ChR2 could be expressed in mammalian neurons through viral transfection (Boyden et al., 2005). The activity of neurons transfected with ChR2 could be controlled via light, with kinetics on a sub-millisecond timescale (Boyden et al., 2005). The precision of ChR2, which works on the timescale of synaptic events (Graziane and Dong, 2016), made optogenetics a very appealing technique in modern neuroscience research (Häusser, 2014).

Shortly after the dawn of optogenetics, concerns emerged regarding ChR2's ability to act as a reliable actuator of neuronal activity (Wang et al., 2007). The original ChR2 was effective at driving action potentials in neurons up to but not above 30 Hz due to rapid inactivation of the channel and its slow recovery from the inactivated state (Lin et al., 2009). Additionally, the low conductance of ChR2 made it difficult to adequately depolarize cells (Berndt et al., 2011). To address this, different variants of the original ChR2 were engineered to enhance the kinetics and photocurrent conductance of ChR2, including ChR2(H134) (Nagel et al., 2005). Compared to the original ChR2, the ChR2(H134) variant has slightly slower kinetics, but generates a significantly larger photocurrent (Lin et al., 2009).

Additional issues arose when optogenetics began to be used widely in neuroscience. For example, it became apparent that optogenetic activation was not equally suitable for every circuit. At particular synapses like the CA3 – CA1 synapse in the hippocampus, optogenetic activation resulted in greater depression of synaptic currents than electrical stimulation (Jackman et al., 2014), which may confound data interpretation. However, the efficacy of some ChR2 variants, such as ChR2(H134), has been assessed in the cerebellum (Jackman et al., 2014).

2.5.2 Achieving cell-type specificity with optogenetics

To complement the spatial and temporal specificity of ChR2 and its variants, pairing optogenetics with genetic recombinant tools allows for an added dimension of precision. The possibility of cell-type specific ChR2 expression enhances its versatility and potential reach. Recombinase systems like the Cre/loxP system facilitate conditional expression or silencing of genetic sequences (Nagy, 2000). Cre can be expressed under a specific promoter to allow for cell-type specific expression of ChR2 or its variants (Madisen et al., 2012). In the mouse cerebellum, Purkinje cell-specific expression of ChR2 can be driven by the Purkinje cell protein 2 (Pcp2/L7) promoter (Zhang et al., 2004) when crossed with a ChR2 reporter mouse (Madisen et al., 2012). In Pcp2-Cre/ChR2(H134) mice, photostimulation of Purkinje cell axons reliably elicits responses from cerebellar nuclear neurons (Jackman et al., 2014). These light-induced postsynaptic responses in cerebellar nuclear neurons are comparable to those elicited following Purkinje cell electrical stimulation.

2.5.3 Functional optogenetic mapping of circuits

Lastly, due to its potential for spatially- and temporally-restricted activation of cells, optogenetics is a powerful tool for circuit mapping in the 21st century, overcoming many technical limitations of more traditional techniques (Petreanu et al., 2007). ChR2-assisted circuit mapping (CRACM) was first described in mice in 2007 (Petreanu et al., 2007), shortly after the advent of optogenetics. When paired with whole-cell recordings in putative postsynaptic cells, CRACM was effective at identifying functional connections in long-range circuits with intact axons, even if the soma was severed in the slice preparation. This set CRACM apart from other circuit mapping techniques, such as dual electrophysiological recordings in the pre- and postsynaptic cells and glutamate uncaging, which are best suited for mapping local circuits where pre- and postsynaptic cells are present in the slice (Petreanu et al., 2007). Glutamate uncaging also requires an intact soma to induce activity (Katz and Dalva, 1994), so it cannot be used for assessing long-range circuits that preclude the preservation of both pre- and postsynaptic somata. While electrical stimulation can be used to stimulate an axon of a presynaptic cell in the absence of its soma, the spatial extent and cell specificity of its activation cannot be precisely controlled (Linders et al., 2022; Petreanu et al., 2007). Thus, CRACM offers greater flexibility and precision to explore the functional properties of circuits in the brain.

Chapter 3

Optimizing Optogenetic Activation of Purkinje Cell Axons to Investigate the Purkinje cell—DCN synapse

3.1 Preface to Chapter 3

The principal goal of my doctoral research has been to identify how Purkinje cells functionally converge on their target neurons in the cerebellar nuclei. Much of our knowledge on Purkinje cell – cerebellar nuclear connectivity comes from anatomical tracing studies. These studies offered widespread insight into general patterns of Purkinje cell convergence in the mediolateral axis, though these techniques are limited in their ability to provide functional information. Other techniques such as traditional electrical stimulation also have limitations that make it prohibitively challenging to comprehensively assess Purkinje cell convergence on individual cerebellar nuclear neurons. In light of these technical limitations, the idea of using optogenetics to activate presynaptic Purkinje cells and evaluate connections to postsynaptic cerebellar nuclear neurons was promising. However, since various concerns arose regarding the reliability of Channelrhodopsin-2 across different experimental conditions, we first evaluated its suitability to circuit-mapping at the Purkinje cell – cerebellar nuclear neuron synapse. The following manuscript details our investigation into this question.

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Optimizing Optogenetic Activation of Purkinje Cell Axons to Investigate the Purkinje cell—DCN synapse

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3.2 Abstract

Optogenetics is a state-of-the-art tool for interrogating neural circuits. In the cerebellum, Purkinje cells serve as the sole output of the cerebellar cortex where they synapse on neurons in the deep cerebellar nuclei (DCN). To investigate the properties of this synaptic connection, we sought to elicit time-locked single action potentials from Purkinje cell axons. Using optical stimulation of channelrhodopsin-2 (ChR2)-expressing Purkinje cells combined with patch-clamp recordings of Purkinje cells and DCN neurons in acute cerebellar slices, we determine the photostimulation parameters required to elicit single time-locked action potentials from Purkinje cell axons. We show that axons require longer light pulses than somata do to elicit single action potentials and that Purkinje cell axons are also more susceptible to light perturbations. We then demonstrate that these empirically-determined photostimulation parameters elicit time-locked synaptic currents from postsynaptic cells in the DCN. Our results highlight the importance of optimizing optogenetic stimulation conditions to interrogate synaptic connections.

3.3 Introduction

Optogenetics is a powerful tool that has transformed the investigation of neural circuits. The ability to genetically target and optically activate distinct cell populations of presynaptic neurons allows for functional circuit mapping which has refined our understanding of the brain (Cruikshank et al., 2010; Huber et al., 2008; Pfeffer et al., 2013). Genetically-targeted opsins distribute throughout cell membranes and can be detected in all cellular compartments, including the soma, dendrites, and axons (Boyden et al., 2005; Lewis et al., 2009). Light pulses can thus be focused onto subcellular compartments to elicit neuronal activity that originates locally (Jackman et al., 2014; Petreanu et al., 2007). For example, targeting axons with focal optical stimulation can be an effective means by which to probe connectivity, especially in acute slices where presynaptic axons are preserved even if their soma is lesioned. However, this approach raises the question of whether focal stimulation of a neuron's axon requires different conditions than focal stimulation of its soma. This is important to address given that there are several recent reports showing that focal axonal stimulation with inhibitory optogenetic tools paradoxically produces excitation rather than inhibition (Mahn et al., 2016; Messier et al., 2018). These studies highlight the importance of empirically testing conditions for optogenetic experiments.

Cerebellar Purkinje cells carry information from the cerebellar cortex via synapses made onto neurons in the deep cerebellar nuclei (DCN) (Palay and Chan-Palay, 1974b; Person and Raman, 2012a). Previous studies have demonstrated that this connection can be investigated with Channelrhodopsin-2 (ChR2), since the synaptic responses elicited optogenetically resemble those elicited from extracellular electrical stimulation (Jackman et al., 2014). However, the parameters to elicit action potentials optogenetically can differ with different equipment, for example with a LED versus a laser. To study the synaptic properties of the Purkinje cell – DCN neuron connection optogenetically, we first need to understand how to elicit well-timed single action potentials reliably from Purkinje cell axonal stimulation. Here we determine the experimental conditions necessary to reliably activate Purkinje cells using a patterned illuminator with a LED light source. We find that focal illumination of Purkinje cell axons requires longer light pulses than somata, and that axons are more susceptible to perturbations from ambient light. Finally, we show that these empirically determined conditions enable us to elicit well-timed synaptic responses in DCN neurons.

3.4 Materials and methods

Animals.

Transgenic mice hemizygous for Purkinje cell-specific Cre (strain B6.Cg-Tg(Pcp2-cre)3555Jdhu/J; stock number: 010536; PCP2-Cre) and mice homozygous for Channelrhodopsin-2/H134R fused with enhanced YFP (strain: B6;129S-Gt(ROSA)26Sortm32 (CAG-COP4* H134R/EYFP)Hze/J; stock number 012569; Ai32), or ChR2(H134R)-EYFP, were acquired from The Jackson Laboratory (Bar Harbor, ME, USA) and bred to produce hemizygous PCP2-Cre/Ai32 mice expressing modified ChR2 in Purkinje cells (Jackman et al., 2014). All animal procedures were approved by the McGill Animal

Care Committee, in accordance with guidelines established by the Canadian Council on Animal Care.

Acute slice preparation.

Slices were prepared as described previously (Ady et al., 2018; Jayabal, Ljungberg, and Watt, 2017). Male and female mice (P20 to P31) were deeply anesthetized with isoflurane and decapitated. Brains were removed and immediately placed in ice-cold artificial cerebrospinal fluid (ACSF; in mM: 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 MgCl2, 1.25 NaH_2PO_4, 25 $NaHCO_3$, and 25 glucose, bubbled with 95% O2–5% CO_2 to maintain pH at 7.3; osmolality $\sim$317 mOsm) for Purkinje cell experiments, or partial sucrose replacement slicing solution (in mM: 50 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 10 $MgCl_2$, 1.25 NaH_2PO_4, 25 NaHCO3, 25 glucose, and 111 sucrose bubbled with 95% O2–5% CO2 to maintain pH at 7.3; osmolality $\sim$317 mOsm) for DCN experiments. Chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) and/or Fisher-Scientific (for $CaCl_2$ and $MgCl_2$; Toronto, ON, Canada). Parasagittal slices of cerebellar vermis and paravermis were cut using a Leica VT 1000S vibrating blade microtome at a thickness of 200 μm. All slices were then incubated in ACSF at 37ºC for 30-45 minutes, and subsequently stored at room temperature for up to 6 hours. Slices were typically stored in a chamber that minimized light exposure. However, for ambient light experiments, slices were stored in ACSF in a clear glass chamber in a laboratory with bright overhead lights, and were illuminated with a halogen lamp to visualize Purkinje cells in acute slices. In the “ambient light” condition, slices were exposed to a continuous spectrum of white background light.

Imaging.

Slices were imaged with a custom two-photon microscope equipped with a Ti:Sapphire laser (MaiTai; Spectra Physics, Santa Clara, CA, USA) tuned to 890 nm and image stacks (1 μm z-step) were acquired with ScanImage running in Matlab (Mathworks, Natick, MA, USA) (Pologruto, Sabatini, and Svoboda, 2003). Maximal intensity projections of image stacks were generated in ImageJ (US National Institutes of Health, https://imagej.nih.gov/ij/).

Electrophysiology.

Borosilicate patch pipettes (2 – 9 MΩ) were pulled with a P-1000 puller (Sutter Instruments, Novato, CA, USA). For current-clamp experiments in Purkinje cells, the internal solution contained (in mM): 130 potassium gluconate, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, 0.4 Na-GTP, 10 NaCl, 10 KCl, with 286 mOsm and pH 7.3 (adjusted with KOH). For voltage-clamp experiments in deep cerebellar nuclei neurons, the internal solution contained (in mM): 150 potassium gluconate, 3 KCl, 10 HEPES, 0.5 EGTA, 3 Mg-ATP, 0.5 GTP tris salt, 5 phosphocreatine-(di)tris, with 297 mOsm and pH 7.2 (adjusted with KOH). Recordings were acquired with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) on a SliceScope Pro 3000 microscope (Scientifica, Uckfield, UK) from neurons in slices maintained at a temperature of 34ºC ± 1ºC and bathed with oxygenated ACSF. Purkinje cells whose resting membrane potential was >-40 mV were excluded from analysis. For voltage-clamp recordings in DCN neurons, cells were voltage-clamped to -60 mV, and Rin and resting membrane potential were monitored. Recordings in which the Rin changed more than 25% were excluded from analysis. Data acquisition and analyses were performed using custom IGOR Pro acquisition and data analysis software (Sjöström, Turrigiano, and Nelson, 2001) (Wavemetrics, Portland, OR, USA).

Optical stimulation.

Slices expressing ChR2 were optically stimulated using a Polygon400E patterned spatial illuminator with a 470 nm LED light source (Mightex, Toronto, ON, Canada), through a 60X water-immersion objective (Olympus LUMPLFLN60XW, Tokyo, Japan). Visually-identified regions of interest for photostimulation were delineated using PolyScan2 software (Mightex). Photostimulation was induced while patch-clamping the soma of either Purkinje cells or DCN neurons. We used a 40 X 40 μm blue square light pulse with an estimated focal plane power density of 100 mW/mm^2 for both axonal and somatic photostimulation, or in some cases, circular light pulses ($\sim$20 μm diameter) were used for somatic stimulation. For axonal photostimulation during Purkinje cell experiments, the area illuminated was 120-200 μm from the Purkinje cell soma. This distance varied due to variation in the thickness of the granule cell layer, but was always in the white matter close to the recorded Purkinje cell. For axonal photostimulation during DCN experiments, the area illuminated was $\sim$200 μm from the DCN neuron soma, in the white matter adjacent to the DCN. Interstimulus intervals were 15 s for evoking action potentials from Purkinje cell somata or axons and 20 s for eliciting postsynaptic responses in the DCN.

Data analysis.

All electrophysiological data were analyzed using custom Igor Pro data analysis software (Watt et al., 2009). Action potential latency was measured as the time in ms from the onset of the light stimulus to the peak of the action potential. For inhibitory postsynaptic currents (IPSCs), the rise time was measured as the time between 20 – 80% of the peak. For Purkinje cell recordings, jitter was measured as the variability (represented as standard deviation, SD) in time from the beginning of the light pulse to the peak of the action potential. For DCN recordings, jitter in the onset of the postsynaptic response was measured as the variability (SD) in time to reach 20% of the peak IPSC. Statistics Mann Whitney U tests were performed using JMP software (SAS, Carey, NC, USA) with the level of significance (α) set at $P < 0.05$. Data are reported as mean ± SEM. For all data, n = number of cells and N = number of mice.

3.5 Results

We wondered whether focal photostimulation of Purkinje cells would result in differential effects depending on the targeted subcellular compartment. To address this, we first confirmed that ChR2 is expressed in Purkinje cell axons of ChR2(H134R)-EYFP mice. Consistent with what has been previously reported (Jackman et al., 2014), we observed robust ChR2 expression in Purkinje cell axons located in the white matter of the cerebellum (Figure 3.1A).

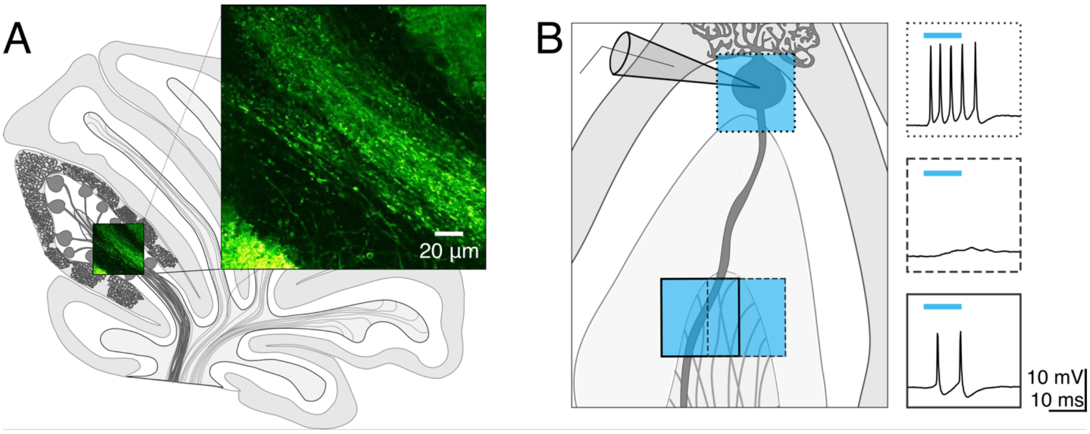

Figure 3.1: **ChR2 expression in Purkinje cell axons.** **(A)** Schematic of sagittal cerebellar slice. Inset is a maximal intensity projection of a two-photon stack showing ChR2(H134R)-EYFP expression (green) in axons in the white matter. **(B)** Left, schematic showing multiple photostimulation regions (blue squares) and somatic recording electrode. Right, representative current-clamp traces show optically-evoked action potentials following somatic (box with dotted line outline) and white matter (dashed and solid line outline on boxes) stimulation locations. Shifting the photostimulation location $\sim$30 μm in the white matter of the cerebellum produced action potentials. Blue bars above trace indicate onset and duration of light pulse.

We next sought to test whether spatially-targeted photostimulation of Purkinje cells can be reliably elicited in axons. While this has been demonstrated by others using short light pulses from a laser (Jackman et al., 2014), to our knowledge this has not been characterized from a LED light source. We made whole-cell current-clamp recordings from Purkinje cell somata and injected negative current until we hyperpolarized the cell to silence spontaneous action potentials. Using a spatial illuminator delivering 470 nm light from a LED, we applied a 40 X 40 μm square light pulse either to the soma or to the cerebellar white matter to stimulate axons and recorded antidromic action potentials. To elicit action potentials in the axon, we photostimulated in the white matter while monitoring the somatic recording for the presence of an elicited action potential(s). If no action potential was evoked in one location, we would then parametrically shift our photostimulation location (in 30-40 μm steps) until action potentials were evoked (Figure 3.1B). If we were unable to elicit action potentials after illuminating multiple stimulation locations, we concluded that the axon of the Purkinje cell was likely cut.

Once we had identified a white matter photostimulation location from which we could elicit action potentials (Figure 3.2A), we tested photostimulus pulses of different durations to explore the conditions required to elicit single action potentials when light was delivered to the soma (Figure 3.2B, left; 3.2C) and axon (Figure 3.2B, right; 3.2D). We found that there was variability in the numbers of action potentials elicited at a given light duration across cells (Figure 3.2D).

Since our aim was to identify light stimulation conditions that reliably elicit single action potentials across cells, we wanted to avoid eliciting multiple action potentials, although in most cases we were unable to accomplish this without occasional action potential failures (and used a failure cut-off of <33.3%). We found that 1 ms light stimulation reliably elicited single action potentials with somatic illumination (0.98 ± 0.12 spike for 1 ms, 1.55 ± 0.15 spike for 2 ms n = 10 cells; N = 7 mice; Figure 3.2E), but not with axonal illumination (0.08 ± 0.06 spike for 1 ms, n = 7 cells; N = 6 mice; Figure 3.2E). The optimal light stimulation duration that elicited single action potentials for axonal stimulation was typically 2 or 3 ms for individual cells (2 ms: 0.81 ± 0.26 spike; 3 ms: 1.02 ± 0.21 spike, Figure 3.2E). We sought to identify the optimal minimal light stimulation to elicit action potentials from the soma and axon for each cell, and found that the average minimal light duration necessary for axons (axon minimal light duration: 2.86 ± 0.55 ms; Figure 3.2F), was significantly longer than for somata (soma minimal light duration = 1.10 ± 0.10 ms, n = 10, N = 7; P = 0.0003; Figure 3.2F). This was also longer than what has previously been reported with a laser light source (Jackman et al., 2014). The latency from light onset to the evoked action potential was also significantly shorter for the soma than for the axon (soma: latency = 3.80 ± 1.03 ms; axon: latency = 6.07 ± 1.02 ms; P = 0.042; Figure 3.2G). However, although the latency to fire single action potentials with somatic or axonal photostimulation differed, we found no significant differences in the jitter of evoked spikes (soma: jitter = 3.81 ± 2.59 ms; axon: jitter = 2.42 ± 1.66 ms; P = 0.46; Figure 3.2H), suggesting that photostimulation results in consistently time-locked action potentials from both the axon and soma.

Since photostimulation of Purkinje cell axons requires longer light pulses to elicit

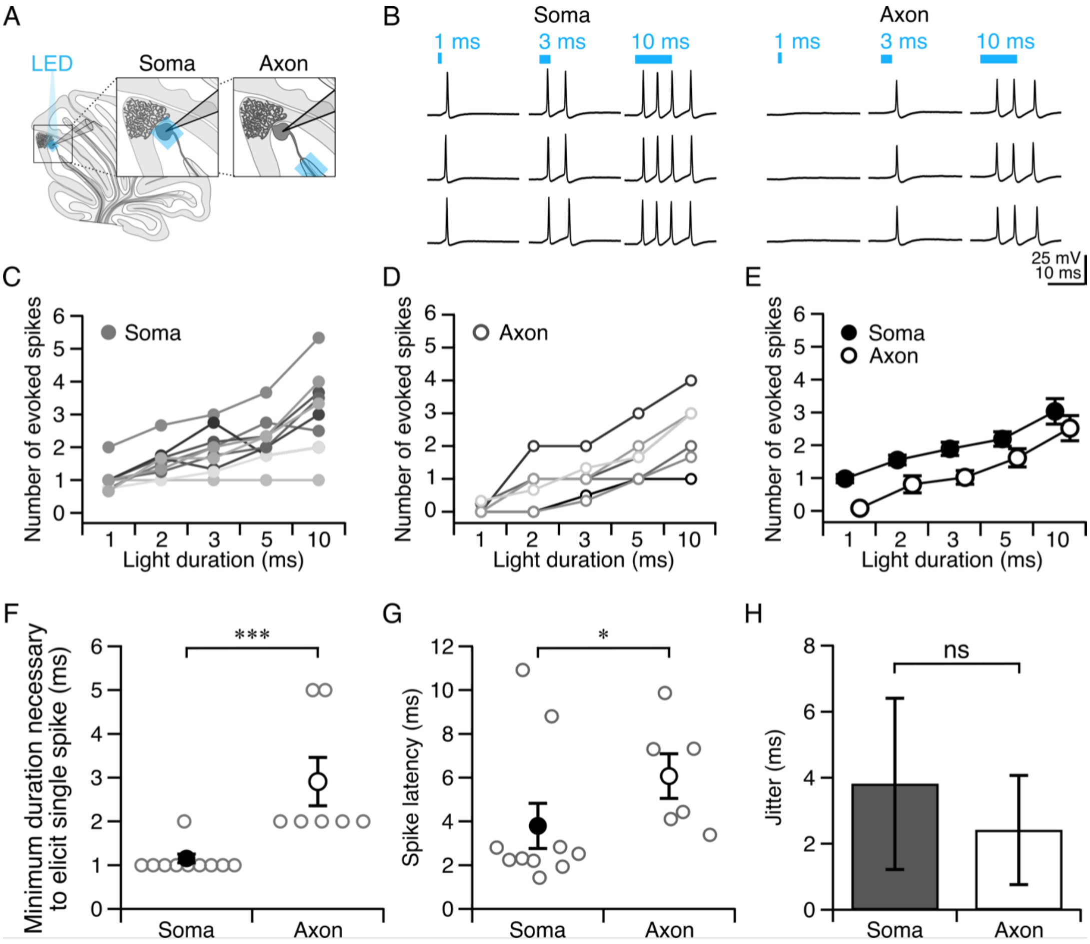

Figure 3.2: **Purkinje cell axons require longer light durations to evoke an action potential than somata. (A)** Schematic depicting the recording configuration. **(B)** Representative current-clamp traces of optically-evoked action potentials evoked following somatic (left) and axonal (right) stimulation. **(C-E)** The number of action potentials evoked for different photostimulus durations. **(C)** Soma, individual cells. **(D)** Axon, individual cells. **(E)** Averages. **(F)** Minimum duration of light stimulus required to elicit a single action potential from each subcellular locus. **(G)** Latency to the first action potential evoked by photostimulus was longer in axons than in soma. **(H)** Jitter of spike latency. Soma: $n = 10$; Axon: $n = 7$. Data represented as mean ± SEM. ns = not significant, $P > 0.05$; *$P<0.05$; ***$P < 0.001$.

an action potential than somatic stimulation, we wondered whether axons might be more susceptible to light perturbations, such as exposure to background white light that might result in inactivation of ChR2 channels (Lin, 2011). To test this, we exposed Purkinje cells to ambient light during slice incubation and recordings, and elicited action potentials as before (Figure 3.3A, see 3.4). We observed an increase in the pulse duration necessary to evoke a single action potential from axons exposed to ambient light compared to what was observed for experiments performed in low light ($P = 0.013$; Figure 3.3B). By comparison, we did not find a difference in the pulse duration necessary to reliably elicit a single action potential from the soma between ambient light and low light conditions ($P = 0.35$; Figure 3.3B). Although the spike latency showed a tendency to increase in the ambient light condition compared to the low light condition for both the soma and axon (Figure 3.3C), these differences were not significant. These results suggest that Purkinje cell axons are more susceptible to ambient light than somata are, perhaps due to the presumed lower density of ChR2 channels in axons rendering them proportionally more sensitive to photo-inactivation.

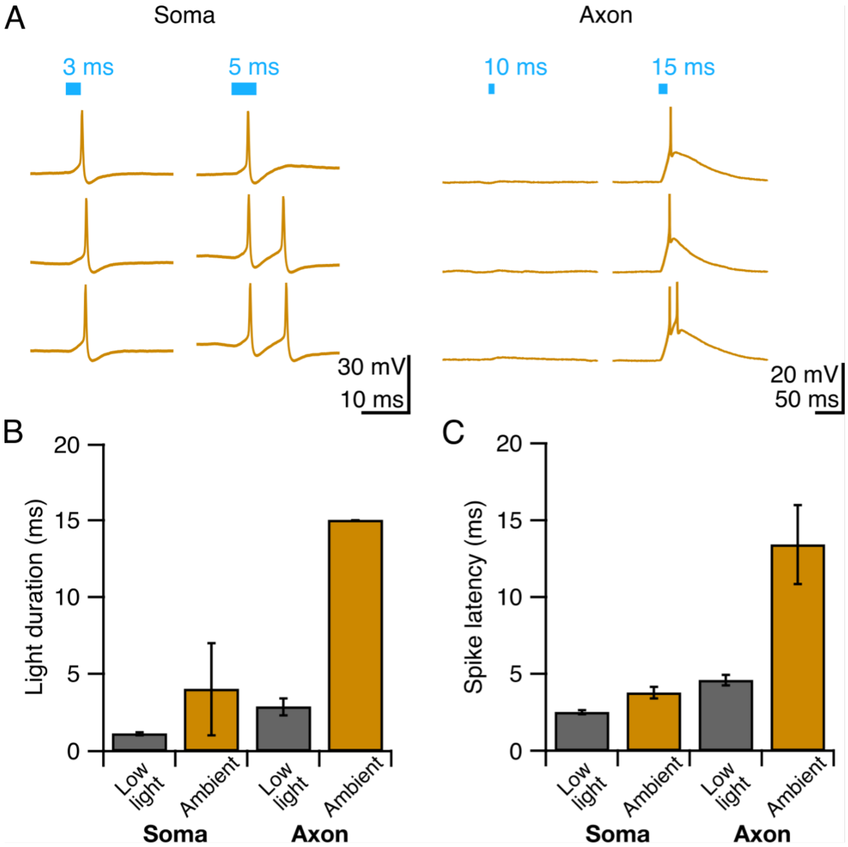

Figure 3.3: **Purkinje cell axons are more vulnerable to suboptimal experimental conditions than cell bodies. (A)** Representative current-clamp traces of optically-evoked action potentials elicited from soma (left) and Purkinje cell axons (right) when exposed to ambient light. **(B)** Axons require longer light durations to elicit single spikes in ambient light than somata. **(C)** The latency to spike after pulse onset for somata and axons exposed to ambient light. Ambient light: Soma: n =3; Axon: n = 3.

Having identified conditions that reliably elicit single well-timed action potentials in Purkinje cell axons, we then sought to determine whether this paradigm would allow us to robustly elicit well-timed postsynaptic responses in DCN neurons. After making whole-cell voltage-clamp recordings from DCN neurons (Figure 3.4A), we stimulated Purkinje cell axons with variable light durations in the white matter ∼200 μm from the patched cell, and recorded evoked IPSCs (Figure 3.4B). IPSC amplitude increased modestly with increasing photostimulus duration (Figure 3.4C), which may be due to additional action potentials elicited with longer light stimulation durations (Figure 3.4D, E), or from additional presynaptic axons being recruited by longer pulses. Rise times of evoked IPSCs were rapid (0.88 ± 0.06 ms, n = 7 cells; N = 3 mice, Figure 3.4D), with averages varying <0.2 ms across different stimulation durations, consistent with fast kinetics previously reported for this synapse (Pedroarena and Schwarz, 2003; Pugh and Raman, 2005). The jitter of the onset of postsynaptic response was low for all photostimulus durations, consistent with well-timed action potentials (Table 3.1; Figure 3.4E).

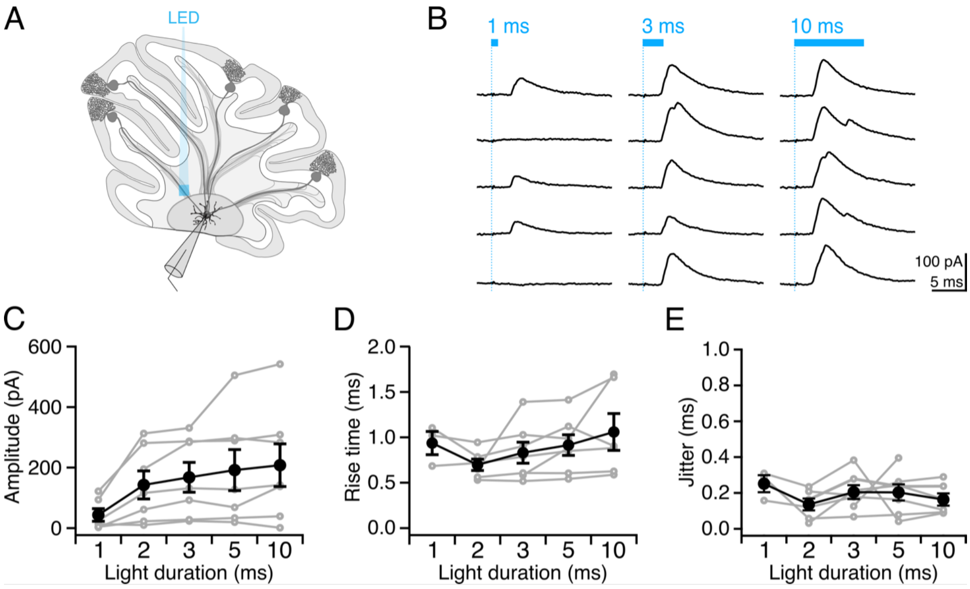

Figure 3.4: **Precisely-timed synaptic responses in DCN neuron with optogenetic Purkinje cell activation. (A)** Recording configuration. Light pulses were delivered to Purkinje cell axons while performing whole-cell voltage-clamp recordings in DCN neurons. **(B)** Representative traces of IPSCs evoked with durations of light in same location. Blue bar above traces indicates duration of light pulse and blue line indicates light onset. **(C)** Average IPSC amplitude across photostimulus durations. **(D)** Average IPSC rise time. **(E)** Jitter of onset of IPSCs. Individual cells, grey. Average, black. n = 7.

We found that with increasing light duration we saw more instances of multi-peak IPSCs (Figure 3.4B), which is in line with our observation that longer light durations elicit multiple presynaptic action potentials (Figure 3.4B), but may also reflect the recruitment of additional axons with longer pulses. Based on our empirical results above, we conclude that a 2 or 3 ms photostimulation duration is best suited to reliably elicit well-timed single presynaptic action potentials in Purkinje cell axons in order to investigate the Purkinje cell – DCN synapse.

Duration	1 ms	2 ms	3 ms	5 ms	10 ms
Amplitude (pA)	43.59 ± 20.92	142.80 ± 46.33	168.30 ± 49.18	191.80 ± 67.71	207.90 ± 70.32
Rise time (ms)	0.94 ± 0.13	0.70 ± 0.06	0.83 ± 0.12	0.91 ± 0.11	1.06 ± 0.2
Jitter of onset (ms)	0.25 ± 0.05	0.14 ± 0.03	0.21 ± 0.04	0.20 ± 0.05	0.16 ± 0.03

Table 3.1: **Synaptic properties of evoked IPSCs from DCN neurons.** Amplitude, rise time, and jitter of the onset of the postsynaptic response (time to 20% of the peak) were determined from the average IPSCs evoked for each photostimulus duration. Amplitude analysis includes failures. n = 7.

3.6 Discussion

We determined the light pulse duration from a 470 nm LED required to elicit well-timed single action potentials in Purkinje cell axons in acute sagittal slices from transgenic mice expressing ChR2 in Purkinje cells. We show that axons require longer pulse durations than somata to elicit the same number of action potentials, and that axonal photostimulation causes longer latencies to spike than somatic photostimulation. We also found that axons are more susceptible to perturbation from background light exposure. Finally, we demonstrate that the conditions we have used elicit well-timed single action potentials from Purkinje cell axonal stimulation allow us to elicit robust time-locked synaptic currents in postsynaptic neurons in the DCN. Since several recent studies using inhibitory optogenetic tools have shown that focal photostimulation of somata and axons yields different results, where stimulation of axons can result in paradoxical effects on activity(Mahn et al., 2016; Messier et al., 2018), we set out to confirm whether the conditions required for axonal photostimulation were similar to those for Purkinje cell somatic stimulation from mice transgenically expressing EYFP-fused ChR2(H134R). We found that we could elicit well-timed action potentials in both the soma and axon with focal photostimulation, although axons required longer light pulses, and displayed longer latencies. These light pulses were longer than what has been previously reported using a laser for photostimulation (Jackman et al., 2014).

Since we measured action potentials with a somatic patch pipette recording, we expected action potential latencies to be shorter when evoked from the soma than from the axon. Purkinje cell axons have been estimated to have a conduction velocity of ∼1-10 m/s (Khaliq and Raman, 2005), so given the distances between axonal stimulation location and somatic patch pipette (<200 μm separation), only a small fraction of the increased latency (up to 0.2 ms) should be attributed to the conduction latency arising from the distal site of axonal action potential initiation. Several other factors likely contribute to the increased latency of action potentials arising from axonal stimulation. Purkinje cell axons are myelinated (Ljungberg et al., 2016) and action potentials travel between nodes of Ranvier in the axon. However, given that internodal spacing ranges between 60 and 260 μm (Clark et al., 2005), the area of focal photostimulation is likely to only occasionally overlap with a node of Ranvier. In support of this, internodal spacing of nodes of Ranvier have been shown to be a limiting factor in the induction of action potentials in myelinated axons (Arlow, Foutz, and McIntyre, 2013). Since light scattering is increased in lipid-rich tissues such as myelin which is abundant in the cerebellar white matter, lower light intensities likely reach Purkinje cell axons compared to the soma (Mattis et al., 2012). However, although the latency to action potential is longer for axons, the jitter between trials is not significantly different, suggesting that action potentials can be elicited reliably and with high temporal precision following axonal photostimulation.

We observed that Purkinje cell axons required longer light pulses and showed longer latencies than somata do to elicit action potentials, so we then wondered whether they may have heightened sensitivity to light perturbations. To test this, we exposed slices to ambient light and repeated our measurements. Axons required longer light pulses in this condition compared to axons maintained in low light, while there was no significant difference in the light pulse duration necessary to elicit spikes from somata held in either low light or ambient light. These results may be due to a slow recovery from inactivation induced by exposure to ambient light: ChR2(H134R) recovers from desensitization and inactivation more slowly than other engineered ChR2 variants (Lin, 2011). Since transgenically-expressed ChR2 is not specifically clustered at nodes of Ranvier in myelinated axons (Figure 3.1; (Arlow, Foutz, and McIntyre, 2013; Grubb and Burrone, 2010), inactivation of individual ChR2 molecules in a region with an already limited availability may greatly reduce the efficacy of a photostimulus. This axonal sensitivity supports our hypothesis that Purkinje cell axons are more affected than somata are by light perturbations in optogenetic experiments and suggests that extra care should be taken when photostimulating axons to minimize unnecessary light exposure.

Finally, we confirm that the parameters which elicit well-timed single action potentials from Purkinje cell axons allow us to elicit temporally-precise synaptic responses in DCN neurons with little trial-to-trial jitter. The parameters we established to best elicit single action potentials from Purkinje cell axons matched well to conditions we observed to best elicit IPSCs in target DCN neurons when focally stimulating a population of presynaptic Purkinje cells (2 or 3 ms). Given the relatively large size of our photostimulation pulse and because Purkinje cell axons bundle together in the white matter, we do not expect to have stimulated single axons, but rather, small subpopulations of Purkinje cell axons. However, further optimization of the size and location of the photostimulation pulse might allow us to reliably photostimulate individual presynaptic axons in the future. Our findings highlight the importance of empirically determining photostimulation parameters from presynaptic neurons to optimize conditions for optogenetic experiments. We expect that some of our findings, such as that axons typically require longer light pulses for similar responses to axons and are more susceptible to background ambient light, are general features that will likely be observed across cell types and recording configuration. However, the major conclusion of this work is that it is important to determine photostimulation parameters empirically when precise temporal control of action potentials is desired for optogenetic experiments.

Conflict of Interest

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

Author Contributions

KG performed experiments and analyzed electrophysiological data. AW conceived of the project, and acquired and analyzed two-photon imaging and electrophysiological data. KG and AW designed the experiments, interpreted the data, and wrote the paper. Both authors have approved of the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Contribution to the Field

Optogenetics, or the use of light to activate neurons in a genetically-targeted manner, has opened the doors for scientists to investigate longstanding questions in neuroscience. Here we test conditions so that we can reliably activate a cell population in the cerebellum with light. We show that targeting different compartments of a cell, such as the cell body and the axon, requires different stimulation parameters to evoke similar activity. We describe how perturbations like light exposure can diminish the effect of a stimulus on Purkinje cell output, and that this differs depending on where the cell is stimulated. Purkinje cells send information via their axons to neurons of the deep cerebellar nuclei, and form synaptic connections that carry information from the cerebellar cortex. Here we show that we can reliably evoke precise synaptic responses from these cells using the conditions that we empirically determine to be best suited to stimulating Purkinje cell axons. This work can thus facilitate future investigations into how information is transferred in the cerebellum.

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Chapter 4

Structured connectivity in the output of the cerebellar cortex

4.1 Preface to Chapter 4

In Chapter 3, I presented a first-author methods paper where I demonstrated that we can use optogenetics and electrophysiology to identify functional connections between Purkinje cells and cerebellar nuclear neurons. I determined the Purkinje cell photostimulation parameters required to consistently evoke: 1. fast, 2. temporally-precise, and 3. single-peaked postsynaptic currents from cerebellar nuclear neurons that are indicative of monosynaptic connections with Purkinje cells. This paved the way for us to perform optogenetic circuit mapping between Purkinje cells and cerebellar nuclear neurons with high temporal and spatial control. The goal of the following manuscript was to identify how Purkinje cells converge from different areas of the cerebellum on individual cerebellar nuclear neurons.

In Figure 3.1B, I showed that our photostimulation configuration allows for high spatial precision. By recording antidromic action potentials from Purkinje cells following axonal photostimulation, we observed that moving our photostimulus region of interest over by 30-40 μm (the width of about two Purkinje cell somata, see ref. Ing-Esteves et al., 2018), could either evoke multiple action potentials or evoke none. This was because a Purkinje cell axon needed to be under the specific photostimulation area, which highlights the high spatial precision of our photostimulation paradigm. Our meticulous exploration of the optogenetic photostimulation parameter space described in the previous chapter set us up to evaluate the spatial properties of functional Purkinje cell convergence on the cerebellar nuclei in the current chapter. The following preprint presents our findings of Purkinje cell - cerebellar nuclear connectivity.

Preprint server information

The following chapter was published as a preprint in *BioRxiv* in March 2023, and is currently under review in a peer-reviewed journal. The DOI for the preprint is: https://doi.org/10.1101/2023.03.17.533173

Structured connectivity in the output of the cerebellar cortex

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4.2 Abstract

Circuits in the brain are built from connections between neurons, where the spatial organization and functional properties of these connections determines circuit function. In the cerebellum, Purkinje cells transmit information to neurons in the cerebellar nuclei, but how Purkinje cell - nuclear neuron connections are organized remains unclear. Here, we explored the connections between Purkinje cells and cerebellar nuclear neurons using whole-cell electrophysiology and optogenetics to produce spatial connectivity maps of cerebellar cortical output. We observed non-random connectivity between Purkinje cells and their target neurons, with inputs to cerebellar nuclear neurons clustering along cerebellar transverse zones. While many nuclear neurons received inputs from a single zone, a number of different connectivity motifs were observed. Neurons receiving inputs from all four zones were more common than predicted by a random model and showed topographic organization in the nucleus. Finally, we observed that small Purkinje cell inputs were sufficient to pause the output of nuclear neurons, suggesting that widespread Purkinje cell synchrony may not be necessary

to influence cerebellar output. These findings reveal cerebellar nuclear neurons as an important locus of multimodal cerebellar integration.

4.3 Introduction

Connections between neurons give rise to circuits that shape brain function. Identifying how these connections are organized is therefore necessary to understand how a circuit functions. The cerebellum is a highly conserved structure that receives input from many brain regions. In the cerebellar cortex, Purkinje cells are organized into lobules, with both neuroimaging studies and disease research suggesting that Purkinje cells in different regions of the cerebellar cortex process different types of information (Kelly et al., 2020; King et al., 2019; Larivière et al., 2015; Stoodley et al., 2017). In mammals, the cerebellar cortex is subdivided into four functional transverse zones (Apps and Hawkes, 2009; Reeber et al., 2012) that have been associated with different patterns of gene expression (Apps et al., 2018; Ozol et al., 1999) and physiological properties (Zhou et al., 2014; Zhou et al., 2015). These zones include the anterior (lobules II-V), central (VI-VII), posterior (VIII and dorsal IX), and nodular zones (ventral IX and X).

As the sole output of the cerebellar cortex, Purkinje cell axons target cerebellar nuclear (CN) neurons with strong inhibitory synapses. It is these CN neurons that form the vast majority of output neurons from the cerebellum. Previous studies have suggested that individual CN neurons receive input from $\sim$40 converging Purkinje cells (Person and Raman, 2012a), and anatomical studies have shown that narrow bands of neighboring Purkinje cells converge on similar areas in the CN (Fujita, Kodama, and Du Lac, 2020). Despite the critical role that the Purkinje cell - CN neuron synapse plays in cerebellar information processing, an understanding of how Purkinje cells spatially converge on CN neurons has been lacking.

In our study, we set out to explore how Purkinje cells inputs onto CN neurons are spatially organized. We focused on the cerebellar fastigial nucleus, since neurons in the fastigial nucleus receive most Purkinje cell input from the vermis (Gould, 1979). We used electrophysiology and focal optogenetic stimulation to build spatial connectivity maps of Purkinje cell inputs onto individual CN neurons. We found that Purkinje cell inputs to the CN are organized along the same four transverse zones that are defined during cerebellar development (Ozol et al., 1999). While many CN neurons receive input from a single zone, others receive Purkinje cell input from multiple zones, and connectivity between Purkinje cells and CN neurons is not random. We performed viral labeling of Purkinje cells from different zones and observed that their axons terminate in close proximity within the CN, providing anatomical support for our functional data. Finally, we performed both cell-attached and whole-cell recordings from single CN neurons to determine how Purkinje cell synaptic inputs affect CN output. We found that even small synaptic inputs could shape CN neuron output, and that the amplitude of the synaptic response influenced the length of the pause in spontaneous CN firing. Our findings support CN neurons as a locus of integration within the cerebellum that is important for cerebellar information processing.

4.4 Results

Mapping Purkinje cell input onto CN neurons

To build functional connectivity maps of Purkinje-cell convergence onto cerebellar nuclear (CN) neurons, we used transgenic mice that expressed modified Channelrhodopsin-2 in Purkinje cells, (Fig. 4.1a-c) (Jackman et al., 2014). We recorded from CN neurons in voltage-clamp configuration and used focused blue light to stimulate Purkinje cell axons at the base of individual lobules sequentially from lobule II to X (Fig. 4.1d, Supplementary Fig. 4.7) to evoke inhibitory postsynaptic currents (IPSCs) in CN neurons. Stimulation from single lobules often evoked no synaptic response (e.g. Fig. 4.1d, bottom), but occasionally IPSCs with fast rise and decay time constants and low failure rates were observed (Fig. 4.1d, bottom, Supplementary Fig. 4.7), consistent with previous descriptions of Purkinje cell - CN synapses (Jackman et al., 2014). By sequentially stimulating individual lobules with focally restricted light, we identified the location of functional connections between Purkinje cells and CN neurons which enabled us to build connectivity maps of Purkinje cell convergence (Fig. 4.1d, e). As a positive control, we shone light over the Purkinje cell axonal terminals directly surrounding the patched CN neuron soma and were able to produce large IPSCs (Fig. 4.1d, Axonal terminals, Supplementary Fig. 4.7), since approximately half of CN neurons received no synaptic input from lobule stimulation within the acute slice plane (Supplementary Fig. 4.8).

Determining whether Purkinje cells from a single lobule or multiple lobules (Fig. 4.1f) converge on an individual CN neuron has important implications for cerebellar function. We found that ∼50% of CN neurons that produced IPSCs following lobule stimulation received input from Purkinje cells located in a single lobule (Fig. 4.1g), while the remaining ∼50% of CN neurons received Purkinje cell input from between two and five lobules (Fig. 4.1h). We found no differences in IPSC amplitudes from single-lobule stimulation in CN neurons compared with those receiving multi-lobule input (Fig. 4.1i, Supplementary Fig. 4.7), suggesting that the total number of spatially-distinct inputs does not influence the strength of connections from a single lobule. Furthermore, the cumulative IPSC amplitude for the total input across lobules increased with the number

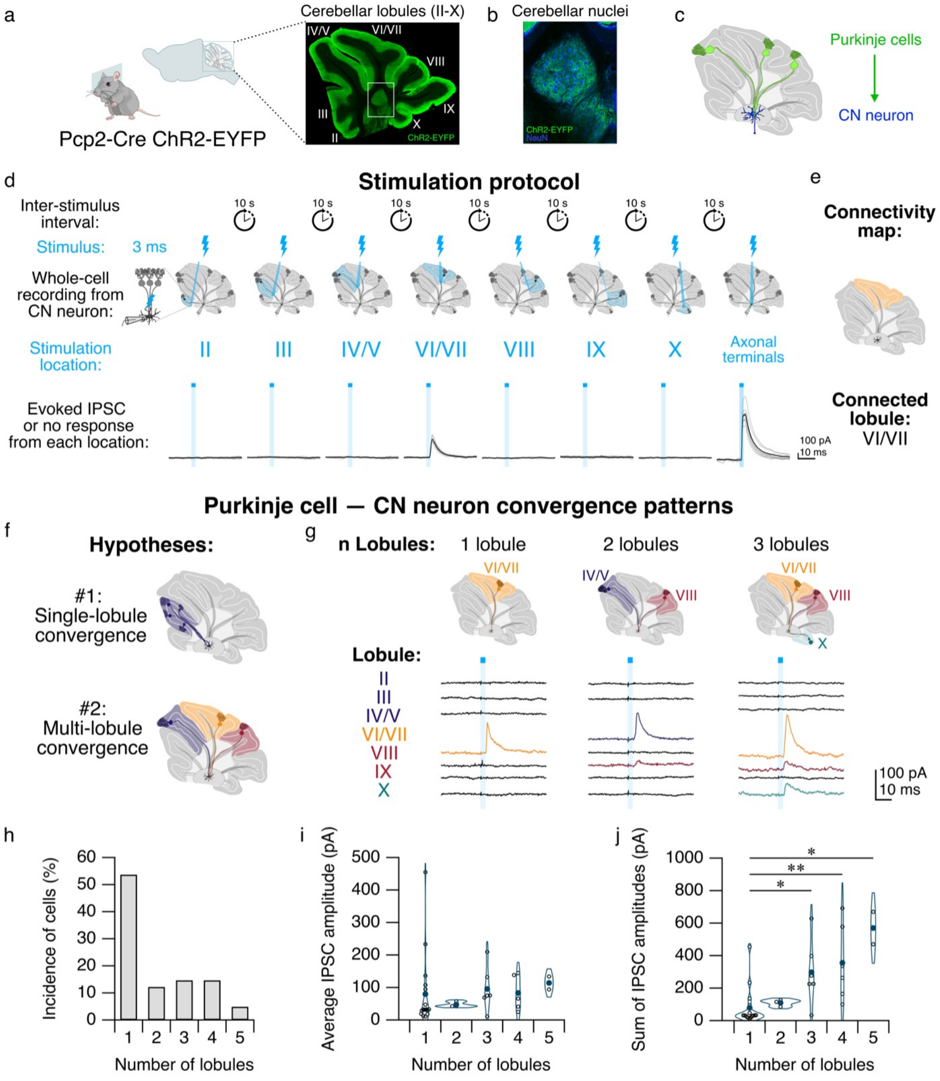

Figure 4.1: **Cerebellar nuclear (CN) neurons receive both single-lobule and multi-lobule input.** **a**, Left, cartoon of a transgenic mouse expressing Channelrhodopsin-2 (ChR2) fused with enhanced yellow fluorescent protein (EYFP) in Purkinje cells. (Legend continued on following page.)

of lobules from which a cell received input (Fig. 4.1j). To address whether there were differences in the subcellular location of Purkinje cell inputs onto CN neurons, we examined the disparity index (Hashimoto and Kano, 2003) of both IPSC amplitudes and rise times. We found no differences in the properties of IPSCs for individual CN neurons based on the number of connected lobules (Supplementary Fig. 4.7). These findings suggest that both single-lobule and multi-lobule innervation patterns are observed in Purkinje cell convergence on CN neurons.

Anatomically defined lobules of the cerebellar cortex are not discrete processing units (Apps and Hawkes, 2009; King et al., 2019). Rather, the cerebellar cortex has been subdivided into four major transverse functional zones: the anterior, central, posterior, and nodular zones (Fig. 4.2a). Since retrograde labeling experiments have shown that CN neurons receive input from narrow bands of neighboring Purkinje cells (Fujita,

Figure 4.1: (Continued) Right, ChR2-EYFP (green) in Purkinje cells, with cerebellar lobule divisions. **b**, Expansion of white box in a shows ChR2-EYFP expression is restricted to Purkinje cell puncta (green) in the CN, while CN neurons express NeuN (blue). **c**, Purkinje cells predominantly project unidirectionally to CN neurons. **d**, Stimulation protocol. Top, durations of inter-stimulus interval and stimulus. Blue lightning bolts indicate a single 3 ms flash of 470 nm blue light above stimulation location from a focal spatial illuminator via a light-emitting diode. Middle, CN neurons were patch-clamped while Purkinje cell axons in each area (left, base of individual cerebellar lobules II through X, right, axonal terminals) were stimulated in rapid succession. Blue lobule indicates stimulation of Purkinje cell axons within each lobule that are located within the acute slice plane. Bottom, the evoked IPSC was recorded following stimulation. Grey traces represent individual IPSCs, and black traces indicate average. Blue rectangles indicate onset and duration of light pulse. Lobule average IPSC: 99.85 ± 10.65 pA, axonal terminals average IPSC: 402.20 ± 55.13 pA. **e**, Connectivity map representing Purkinje cell – CN connectivity from cell shown in **d**. **f**, Representation of two hypotheses of Purkinje cell convergence on CN neurons. Top, Purkinje cells originating from a single lobule converge on a CN neuron (hypothesis #1), bottom, Purkinje cells originating from multiple lobules converge on a CN neuron (hypothesis #2). **g**, Connectivity maps (top) and average evoked IPSCs (bottom) from three CN neurons receiving input from 1 (left), 2 (middle), or 3 (right) lobules. **h**, Percentage of cells connected to n lobules. **i**, Average IPSC amplitude per cell with n connected lobules. **j**, Sum of IPSC amplitudes per cell with n connected lobules. IPSC amplitudes compared using non-parametric multiple comparison Mann-Whitney U test. *$P < 0.05$, **$P < 0.01$. Unlabeled comparisons = not significantly different. n = 41 cells with functional connections out of 75 cells.

Kodama, and Du Lac, 2020), we hypothesized that Purkinje cells in lobules belonging to the same functional zone were more likely to converge on a CN neuron than Purkinje cells in lobules from across zones. To address this, we performed unsupervised hierarchical clustering analysis of our connectivity data. In agreement with our hypothesis, we observed that Purkinje cells in lobules within the same functional zone converged more often onto the same CN neuron than those in lobules from across zones (Fig. 4.2b). This suggests that Purkinje cell output tends to respect the boundaries of the functional zones of the cerebellar cortex.

Connectivity motifs in Purkinje cell input onto CN neurons

Do Purkinje cell inputs to CN neurons preferentially arise from single zones? As we found that CN neurons can receive inputs from multiple lobules and since these lobules are grouped into functional zones, we wondered whether multi-lobule innervations arose due to convergence from Purkinje cells within the same zone. To address this, we looked at convergence properties of CN neurons and found both single-zone and multi-zone convergence (Fig. 4.2c). We observed no differences in IPSC amplitudes or other properties between CN neurons innervated by different zones or numbers of zones (Fig. 4.2d, Supplementary Fig. 4.7), suggesting that there is no zone that forms a systematically stronger input onto CN neurons. We found that multi-zonal input was not rare: over a third of CNs received multi-zone input (Fig. 4.2e, inset). Different connectivity patterns appear with different frequencies in our dataset (Supplementary Fig. 4.9), suggesting the existence of non-random connectivity motifs.

To determine whether Purkinje cells converge across zones on individual CN neurons in non-random motifs (Rieubland, Roth, and Häusser, 2014; Song et al., 2005), we used a random model that assumes Purkinje cell connectivity from each zone occurs

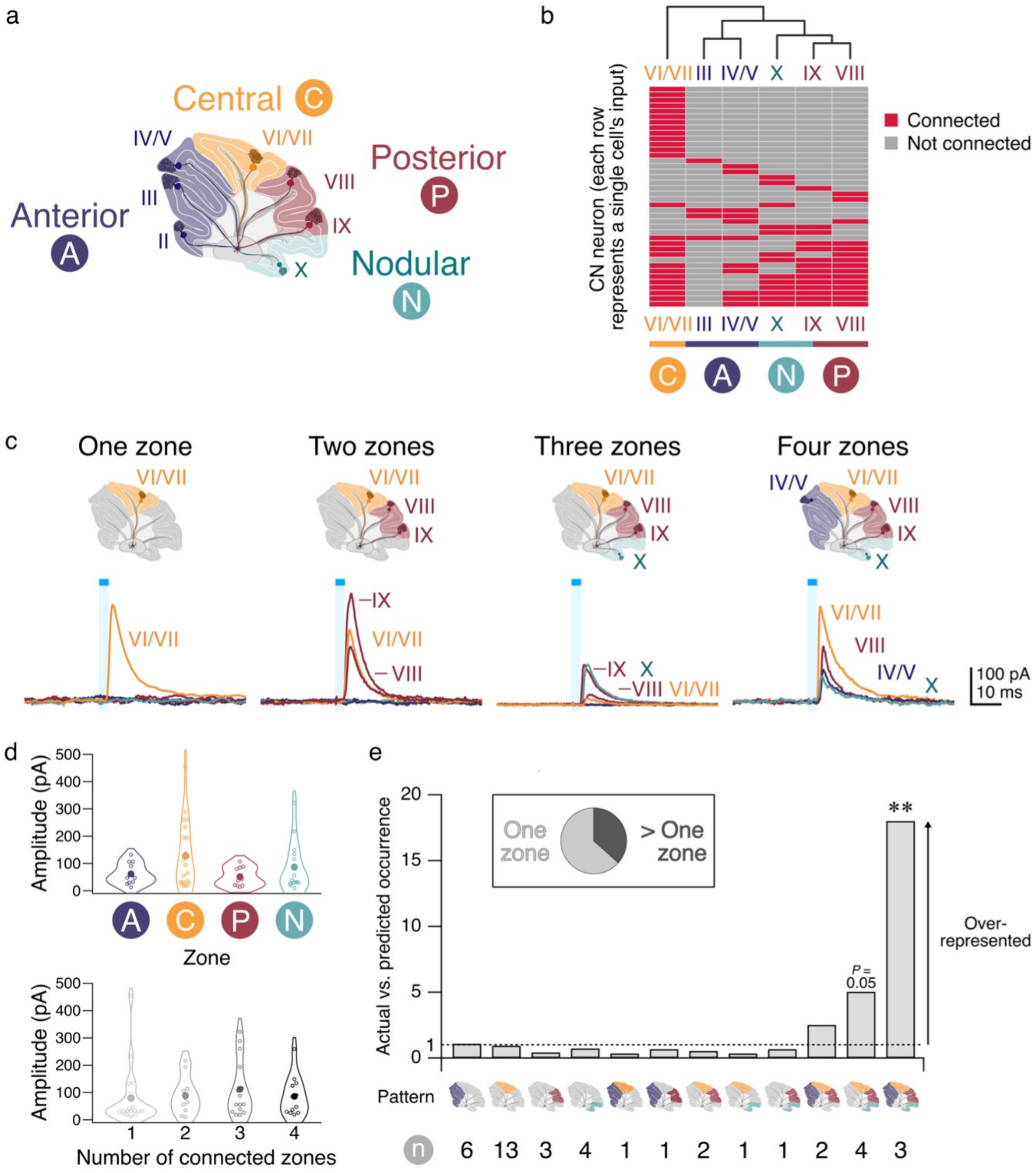

Figure 4.2: **Purkinje cell – CN neuron connectivity is not random. a**, Cartoon of cerebellar lobules (roman numerals) and zones (circles). (Legend continued on following page.)

independently of other zones and determined how well it describes our data (Fig. 4.2e). We found that four-zone convergence occurred in our dataset ∼18 times more often than predicted by the model, and three-zone connections trended toward significant over-representation as well. This over-representation suggests that a four-zone connectivity motif may be an important feature of cerebellar information transfer, and by extension, that the CN may be an important locus of information integration in the cerebellum.

Figure 4.2: (Continued) **b**, Heatmap representing lobule inputs (columns) to individual CN neurons (rows) displaying connected lobules (red) and unconnected lobules (grey). Dendrogram of lobules created with unsupervised hierarchical clustering (top) using the Hamming distances between lobules' connectivity patterns with the unweighted average linkage reveals inputs cluster along functional zones (delineated by colour, bottom). **c**, Connectivity maps (top) and evoked IPSCs (bottom) from four CN neurons receiving input from one (left), two (middle left), three (middle right), and four (right) zones. Roman numerals beside traces indicate responsive lobule. **d**, Top, average IPSC amplitude for each zone is not statistically different from other zones. Bottom, average IPSC amplitude per cell based on number of connected zones. **e**, Comparison of the actual vs. predicted occurrence of cells with each zone combination found in our dataset. Dotted line represents equal occurrence of predicted and actual combinations. P values represent the difference between the actual and predicted occurrence as determined using a binomial distribution test, with multiple hypothesis corrections performed using False Discovery Rate testing. Inset shows proportion of CN neurons receiving input from one zone or multiple zones. IPSC amplitudes compared using non-parametric multiple comparison Mann-Whitney U test. **$P < 0.01$. Unlabeled comparisons = not significantly different. $n = 41$ cells with functional connections out of 75 cells.

No morphological signature for CN neurons with different input patterns

Different classes of CN neurons have been distinguished by their morphological and physiological properties (Uusisaari, Obata, and Knöpfel, 2007). To determine whether differences in morphological properties can be used to classify CN neurons with different connectivity patterns, we filled CN neurons with AlexaFluor 594 and performed two-photon imaging of filled cells after characterizing their synaptic inputs (Fig. 4.3a). To characterize their physiological properties, we sampled their spontaneous firing in cell-attached mode prior to recording synaptic input (Fig. 4.3a, bottom). We compared the soma area, Sholl radius, branch index, number of primary dendrites, total dendrite length, and firing rate from CN neurons with different connectivity patterns and found that these properties were indistinguishable for CN neurons with different connectivity patterns (Fig. 4.3b-g) or number of input zones (Supplementary Fig. 4.10). Thus, we observe that CN neurons with different types of input could not be discerned by their morphological or physiological properties.

We next wondered whether the orientation of CN neuron dendrites was related to the location of their Purkinje cell inputs (Supplementary Fig. 4.10). We found that most cells had at least some dendrites oriented towards their Purkinje cell inputs, but that it was typically a small fraction of the dendritic tree. The amount of dendrite - zone overlap increased as the number of zones connected to a given CN neuron increased, likely due to the greater area covered by the input direction from multiple lobules, suggesting that the orientation of CN dendrites is not shaped by its input. We also did not observe a relationship between IPSC amplitudes or rise times and the degree of overlap, suggesting that dendrites are not preferentially oriented toward or away from larger lobule inputs, and that input from different lobules is not clustered on different regions of the dendritic trees.

The CN has previously been described to exhibit a topographic organization that

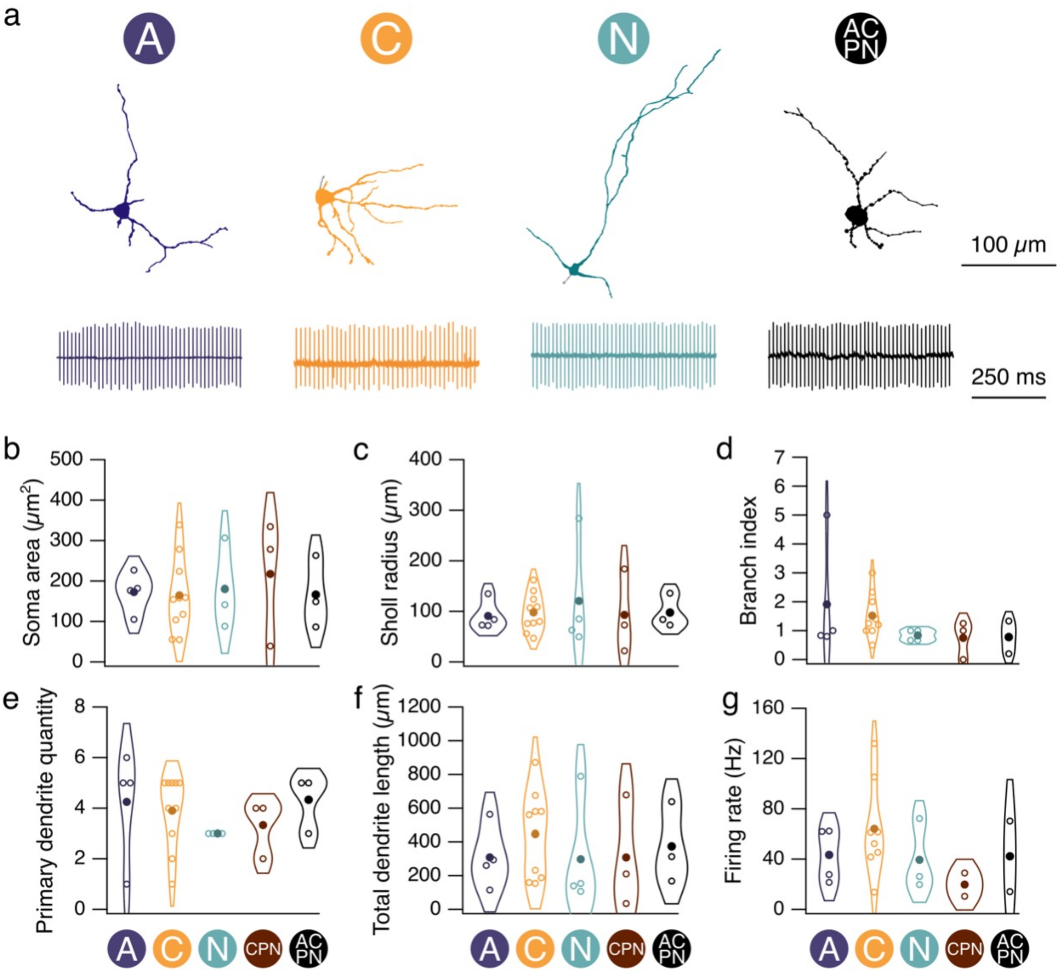

Figure 4.3: **CN neurons with different input connectivity patterns are morphologically similar. a**, Top, reconstruction of CN neurons from two-photon stack. Circle denotes cell receives input from the anterior ("A") zone. Bottom, representative traces of spontaneous firing from CN neurons receiving input from the "A," "C," "N," or "ACPN," zones, respectively, obtained before breaking through into whole-cell configuration. **b-g**, Soma area, Sholl radius, branch index, primary dendrite quantity, total dendrite length, and firing rate for cells with different connectivity patterns, groups with < 3 reconstructed cells were excluded. Morphological properties compared across connectivity patterns using non-parametric multiple comparison Mann-Whitney U test. Unlabeled comparisons = not significantly different. n = 24 cells **(b-f)**, n = 18 cells **(g)** out of 75 cells.

corresponds with the post-synaptic targets of CN neurons (Fujita, Kodama, and Du Lac, 2020; Sugihara and Shinoda, 2007; Sugihara et al., 2009). We wondered whether CN neurons also showed topographic organization according to their input connectivity patterns. Using coordinates from the Allen Mouse Brain Atlas (Wang et al., 2020), we visualized our recorded cells in 3-D within the fastigial CN (Claudi et al., 2021) (Fig. 4.4a). CN neurons did not appear to cluster by connectivity pattern (Fig. 4.4b). However, clustering of CN neurons based on the number of input zones was weakly apparent in our data. To determine whether topographic organization along any axes was observed (Fig. 4.4c, d), we looked at the fit of cells with different numbers of zonal inputs (e.g., 1 zone, 2 zones, 3 zones, 4 zones, Fig. 4.4e). We fit a line for each group of cells receiving input from n zones and determined the R^2 of each. We then performed a bootstrap analysis to randomly sample an equivalent number of cells for each group from our total distribution 50,000 times. To determine how similarly the observed samples' fit matched our data, we assigned significance to any R^2 value from our data that was in the top 5% of distribution of bootstrapped R^2 values (Supplementary Fig. 4.12). A significant R^2 value was only observed once: for 4 zone cells along the rostroventral-caudodorsal axis (Fig. 4.4f), but not along the mediolateral axis (Fig. 4.4g, h). This suggests that 4 zone cells are topographically organized within the fastigial nucleus.

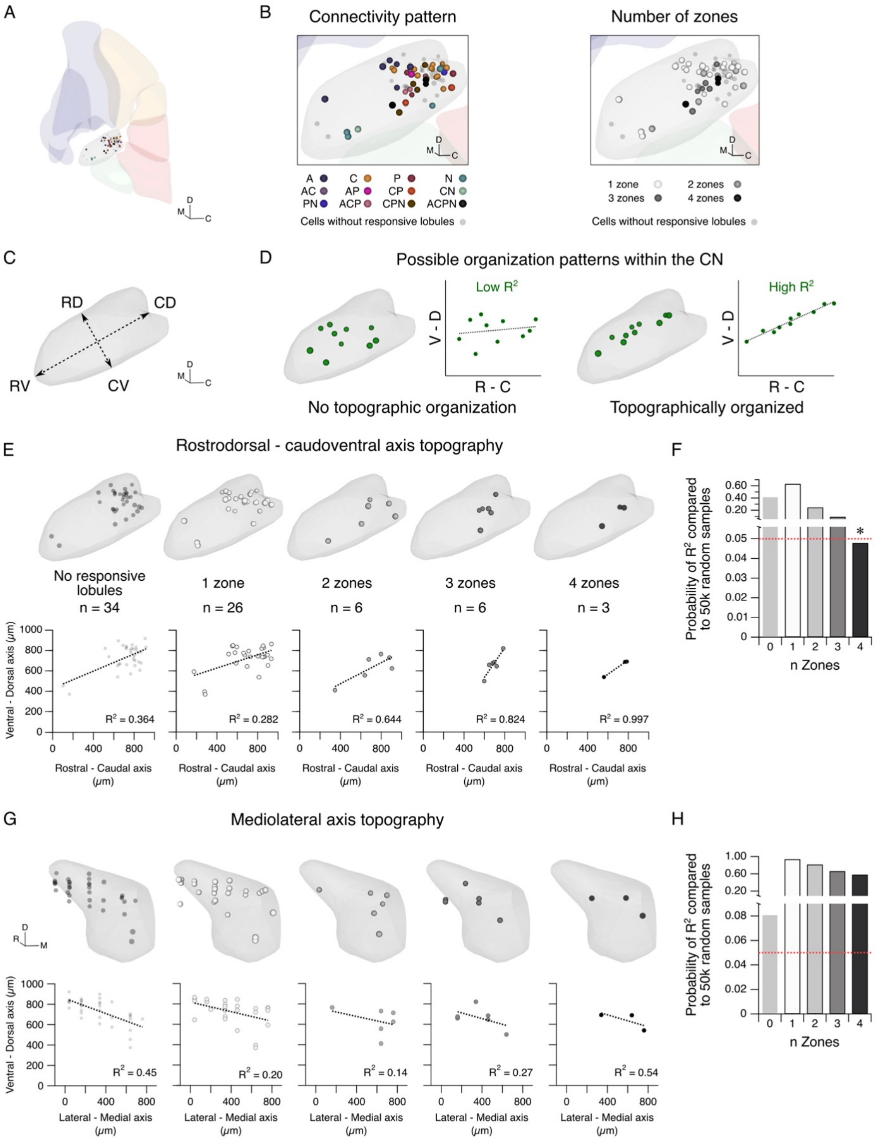

Figure 4.4: **CN neurons receiving input from all four zones are biased along the rostroventral-caudodorsal axis. a**, 3-D representation of recorded cells using coordinates from the Allen Mouse Brain atlas and rendered using Brainrender. (Legend continued on following page.)

Figure 4.4: (Continued) **b**, Left, expansion of inset in a depicting position of cells based on connectivity pattern. Right, cells in **b**, left categorized by number of input zones. **c**, Rendering of fastigial CN depicting the rostroventral-caudodorsal (RV-CD) and rostrodorsal-caudoventral (RD-CV) axes. **d**, Cartoons of two possible organization patterns within the CN, where one type of CN neurons receiving a pattern of Purkinje cell input do not demonstrate topographic organization (left, "Low R^2"), and another where CN neurons receiving a particular pattern of Purkinje cell input exhibit topographic organization biased along a particular axis of the nucleus (right, "High R^2"). **e**, Top (from left to right), rendering of position in the sagittal plane of CN neurons that responded only to axonal terminal stimulation (No responsive lobules), and CN neurons that received input from one zone, two zones, three zones, and four zones. Bottom, same as in **e**, top, but showing the plotted points for each CN neuron in the rostral-caudal and ventral-dorsal axes. Dotted lines and R^2 values in each graph represent the line of best fit for the neurons in each category. **f**, Graph demonstrating the probability that n randomly selected cells from a bootstrapped analysis with 50,000 iterations has an R^2 value $\geq$ the R^2 value from each group of n Zones from **e**, bottom. Only the n Zones = 4 group demonstrates an R^2 value ≥ 0.997 in fewer than 5% of the bootstrapped samples (one-tailed). Red dotted line indicates $P = 0.05$. **g-h**, Same as **e-f** but along the mediolateral and ventral-dorsal axes. $^*P < 0.05$. Unlabeled comparisons = not significantly different. n = 75 cells. C: caudal, D: dorsal, M: medial, R: rostral, V: ventral.

Purkinje cell puncta from different zones terminate onto CN neurons in close proximity

Our finding that CN neurons receive Purkinje cell input from multiple cerebellar zones was surprising, so we next validated our findings using a complementary anatomical approach. We performed stereotactic surgeries to inject up to 3 adeno-associated viruses (AAVs) expressing different fluorophores into different cerebellar zones of adult Pcp2-Cre-negative mice to label Purkinje cells and their axons. This enabled us to visualize the axons and terminals of Purkinje cells from different locations within the CN (Fig. 4.5a). We found that Purkinje cells from different zones (Fig. 4.5b, c) show gross differences in their projection patterns within the nucleus (Fig. 4.5d), as previously reported (Sugihara et al., 2009). However, in many regions of the CN, we observed puncta from different zones in close proximity (Fig. 4.5e). In fact, ∼30% of puncta from Purkinje cells originating in one zone were located within 17.5 μm of puncta from a different zone, which is the average CN soma diameter (Fig. 4.5f, Supplementary Fig. 4.13) and >80% of puncta were located within 97.2 μm, the average length of a CN dendrite (Fig. 4.5f, Supplementary Fig. 4.13). This argues that Purkinje cells from different zones frequently terminate within close enough proximity that they can synapse onto the same CN neuron, supporting our optogenetic and electrophysiological findings of convergence across multiple functional zones (Fig. 4.2).

Simultaneous activation of single-zone Purkinje cell inputs is sufficient to silence CN neurons

It has been suggested that Purkinje cell input synchrony is required to affect the output of CN neurons (Person and Raman, 2012a). Our observation that multiple zones converge on single CN neurons raises the question of whether synchrony is required

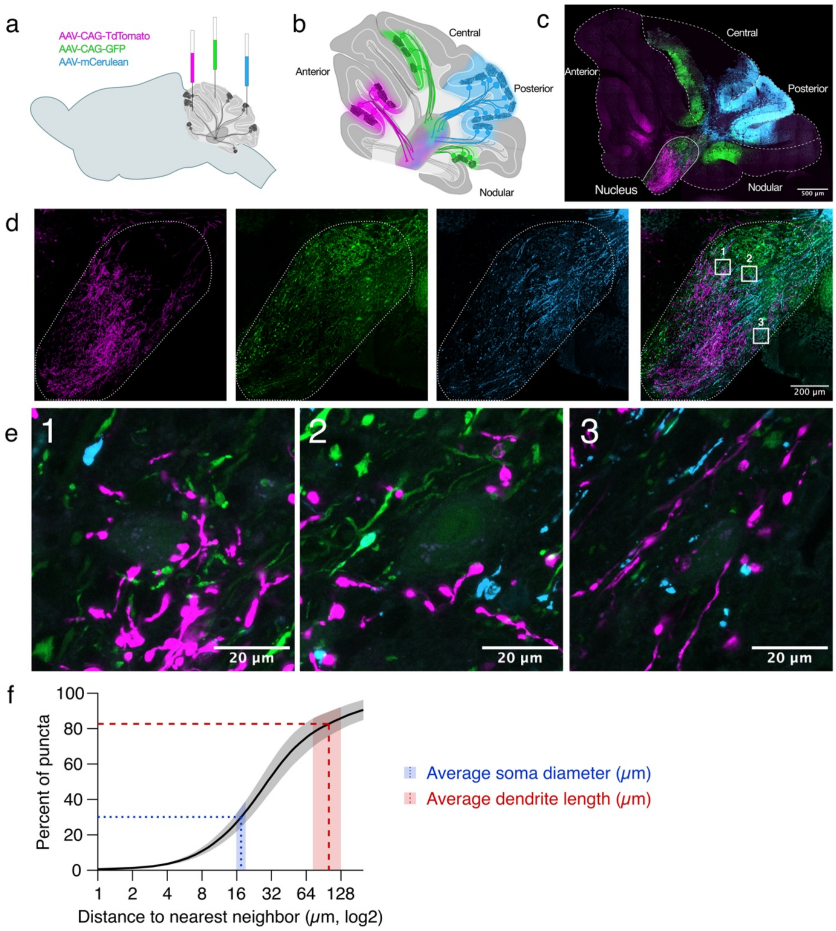

Figure 4.5: **Purkinje cell puncta from different zones terminate in close proximity in the CN. a**, Cartoon representing intracranial injections of fluorescent viruses into different areas of the cerebellar vermis. **b**, Cartoon representing Purkinje cells labeled with virus injections depicted in **c**, representative image of cerebellum with viruses expressed in different zones at 20x magnification. **d**, Expansion of nucleus in c showing that Purkinje cells from different lobules target different areas of the CN, 20x magnification. **e**, Expansion of numbered boxes in **d**, showing Purkinje cell puncta from different zones in apposition to individual CN neurons, 63x magnification. **f**, Cumulative probability of nearest neighbor distance of Purkinje cell puncta from different zones. Dashed lines: average soma diameter (blue, 17.5 microns) and dendrite length (red, 100.5 microns). Shaded rectangles: 95% confidence interval of nearest neighbor puncta across animals. N = 6 animals.

across functional zones to affect cerebellar output. To explore this, we recorded action potentials from CN neurons from a subset of spontaneously active cells (Fig. 4.6a, b) prior to breaking through into voltage-clamp configuration for IPSC recordings (Fig. 4.6c), and stimulated Purkinje cell axons as described above (Fig. 4.1d). This allowed us to measure both the impact of Purkinje cell stimulation on CN firing and the synaptic response within the same CN neuron. We measured the inter-spike intervals (ISI) of CN neurons immediately after the light pulse as a ratio of the average ISI duration per trial for each CN neuron that fired regularly (Fig. 4.6d, "ISI ratio"). We found that the ISI ratio immediately following stimulation of lobules that were connected to the CN neuron was typically higher than that for lobules that were not synaptically connected to the CN neuron, and the ISI ratio for responsive lobules for each cell after

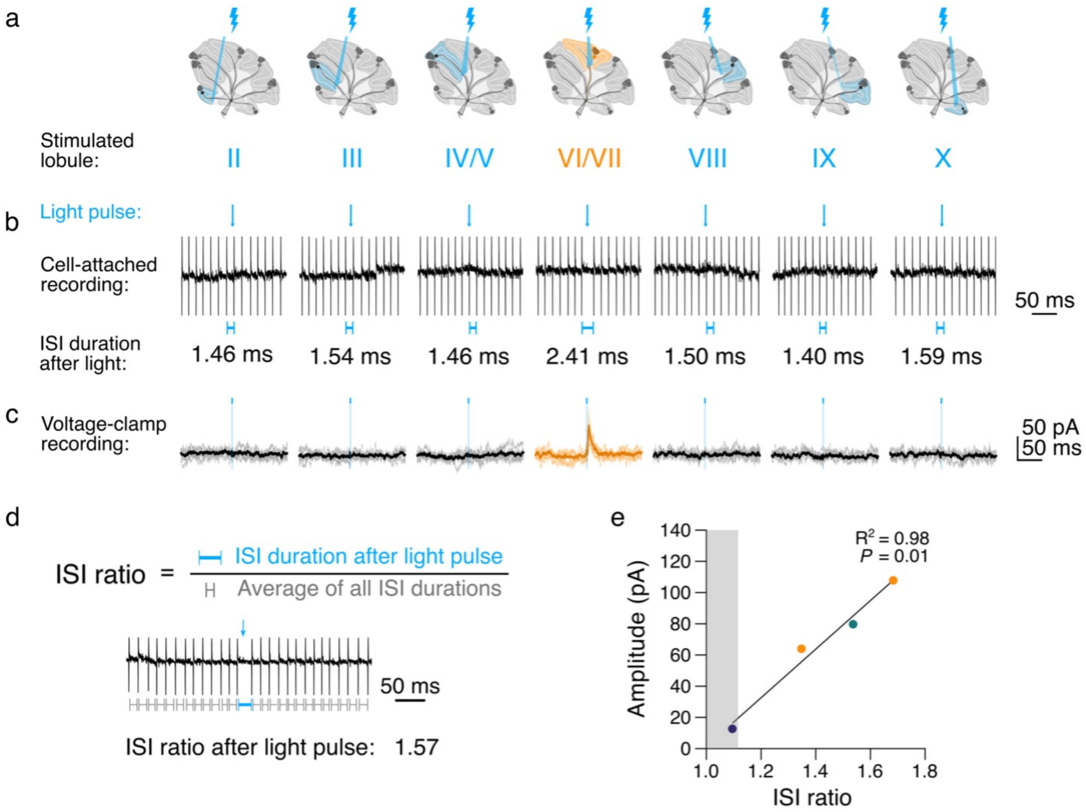

Figure 4.6: **Small inputs from a single lobule are sufficient to alter CN output. a**, Stimulation protocol. **b**, Top, representative traces of spontaneous firing from a CN neuron during lobule stimulation before breaking into whole-cell configuration. Blue line with arrowhead indicates onset and duration of light pulse. Bottom, blue lines indicate duration of inter-spike interval (ISI) immediately following stimulation. **c**, Voltage-clamp traces during stimulation from cell in b after breaking through to whole-cell configuration. **d**, Top, ISI ratio calculated by dividing the ISI duration after stimulation with the average ISI duration per cell per trial. Bottom, example trace from a cell with an ISI ratio of 1.57. **e**, ISI ratio vs. IPSC amplitude per cell, Pearson's R^2 correlation coefficient. Shaded area depicts 95% confidence interval of the ISI ratio of non-responsive lobules (≥ 1). n = 4 cells out of 75 cells.

stimulation was greater than the ISI ratio from randomly sampled spike ISIs from the same trial (Supplementary Fig. 4.14). We found that IPSC amplitude was positively correlated with the ISI ratio (Fig. 4.6e), where small inputs produced small pauses, and larger inputs produced larger pauses. Remarkably, even small inputs ($\sim$60 pA, where $\sim$ 50% of all recorded lobule inputs $\geq$ 60pA) were sufficient to pause CN firing. Thus, although widespread synchrony across Purkinje cell inputs may occur, our findings suggest that is not necessary since even small inputs can alter CN output.

4.5 Discussion

Our results reveal how CN neurons in the fastigial nucleus receive input from Purkinje cells that arise from multiple locations across the cerebellar vermis (Gould, 1979), revealing that CN neurons are an overlooked locus of cerebellar integration. Using electrophysiology combined with focal optogenetic stimulation of Purkinje cell input, we mapped the spatial patterns of inputs onto CN neurons. We found that input from Purkinje cells tended to recapitulate the transverse zones of the cerebellar cortex, with roughly two thirds of CN neurons receiving uni-zonal input. However, some CN neurons received input from multiple zones. In particular, input from all four functional zones was observed more often than predicted by a random model, and these 4-zone cells appeared to be topographically located near the midline of the rostrodorsal - caudoventral axis in the CN. Corroborating these findings, we used stereotactic viral expression of multiple fluorescent proteins to provide morphological evidence that puncta from Purkinje cells in different functional zones are proximal to puncta from other zones within the CN. Lastly, we showed that small synaptic inputs could pause CN neurons, and that the size of the synaptic response was correlated with the length of the pause. These data demonstrate that widespread Purkinje cell synchrony may not be necessary to influence CN neuron output. Taken together, our results reveal that CN neurons are dynamic computational units that contribute more to cerebellar integration than was previously appreciated.

One of the limitations of our study is that connectivity is restricted to axons within the thickness of the slice preparation. While fastigial neurons receive most input from the vermis in a spatially restricted manner (Gould, 1979; Sugihara et al., 2009), this suggests that the connectivity patterns we report correspond to a lower limit, and that some multi-zonal cells may have been misclassified as uni-zonal due to missed input. Notably, the size of the dendritic trees did not differ across cells with different numbers of zonal inputs, suggesting that this was not a systematic artifact of slice preparation. Despite our finding that 4-zone connectivity was over-represented, we may nonetheless underestimate the prevalence of these cells in the CN. Furthermore, it is possible that findings in the fastigial nucleus may not resemble the input connectivity patterns in the interposed and dentate nuclei given the distinct three-dimensional features of each nucleus, as well as their different downstream targets (Fujita, Kodama, and Du Lac, 2020; Sugihara, 2011; Sugihara et al., 2009).

Functional transverse zones have been proposed as an underlying feature of the cerebellar cortex based on cerebellar development, different patterns of gene expression, and Purkinje cell physiology (Apps and Hawkes, 2009; Apps et al., 2018; Ozol et al., 1999; Reeber et al., 2012; Zhou et al., 2015). Our observation that input to CN neurons clusters based on these zones of the cerebellar cortex underscores the importance of zonal organization in the cerebellum. We observed that CN neurons receiving 4-zone inputs from Purkinje cells were observed more than would be expected from a uniformly random model. Multi-zonal convergence onto a CN neuron suggests a previously unknown site for multimodal integration and output. Such multimodal integration has been observed before at the input level of the cerebellum in the granule cells (Ishikawa, Shimuta, and Häusser, 2015), and is also a feature of Purkinje cells, that integrate large numbers of inputs in their extensive dendritic trees (Kitamura and Häusser, 2011; Roome and Kuhn, 2020; Wang, Denk, and Häusser, 2000). However, it has not been clear whether such multimodal integration occurred in the CN. The enrichment of 4-zone input CN neurons in our data suggests that multimodal processing may be a feature of these neurons, and that they function as “integrator neurons” in the cerebellar circuit.

Recently a great deal of work has focused on the different classes of neurons of the CN, based on their projections, molecular expression, and physiological properties (Fujita, Kodama, and Du Lac, 2020; Novello, Bosman, and De Zeeuw, 2022; Uusisaari, Obata, and Knöpfel, 2007). We expected that CN neurons might exhibit distinct features based on their input patterns. Our inability to detect any morphological or physiological distinctions does not mean that they do not exist. While properties such as firing rate have been described to differ for different classes of CN neurons, the distributions of firing rates for each category overlaps, meaning that large numbers are needed to differentiate between these cell types (Uusisaari, Obata, and Knöpfel, 2007). We would be unlikely to discern small differences in overlapping distributions between CN neuron classes because the number of neurons we have characterized in each class is relatively low. However, the topographic enrichment of 4-zone input cells spread along the rostroventral-caudodorsal axis, where 4-zone input cells are tightly clustered in the orthogonal rostrodorsal-caudoventral axis, is likely to have implications for the output circuits of the CN (Fujita, Kodama, and Du Lac, 2020; Sugihara, 2011), although they do not appear to represent any defined output cell population identified to date (e.g. ref. Fujita, Kodama, and Du Lac, 2020).

Several different theories have set out to explain how the cerebellum processes sensorimotor information while producing complex behaviors. In the universal cerebellar transform hypothesis, the cerebellum uses a one-size-fits-all computation to process diverse input and contribute to motor and cognitive functions (Schmahmann, 2000; Schmahmann et al., 2019). Alternatively, the multiple functionality transform hypothesis (Diedrichsen et al., 2019) posits that distinct tasks require distinct algorithms which may be mediated by factors such as Purkinje cell firing rate, molecular profile, or timing rules (Cerminara et al., 2015; Diedrichsen et al., 2019; Suvrathan, Payne, and Raymond, 2016). Since neither theory has incorporated a role for CN neurons in cerebellar processing (Kebschull et al., 2023), our findings suggest that theories of cerebellar function may need to be updated.

Both CN neurons and Purkinje cells fire spontaneous action potentials at high frequencies. If inputs from different Purkinje cells were activated asynchronously, individual inputs may not impact CN output. If, however, Purkinje cell inputs were activated synchronously, the synchronized, stronger inputs may be more able to influence CN output (Person and Raman, 2012a). Interestingly, our data show that even relatively small inputs of $\sim$60 pA can alter CN neuron output, which is smaller than the previously estimated unitary amplitude for Purkinje cell - CN neuron synapses (Person and Raman, 2012a). This suggests that input synchrony from Purkinje cells across different zones may not be strictly necessary, although it does not rule out that it occurs. Purkinje cell synchrony is still possible, since larger amplitude synaptic responses had a stronger impact on CN output. It will be important in the future to determine whether the synchronicity of multi-zonal Purkinje cell input onto individual CN neurons can influence CN neuron output, as this will influence the role of CN information processing in the cerebellum.

4.6 Methods

Animals

We crossed mice hemizygous for Purkinje cell-specific Cre [strain B6.Cg-Tg(Pcp2-cre)3555Jdhu/J; stock number: 010536; PCP2-Cre] with mice with a loxP-flanked stop cassette upstream of Channelrhodopsin-2(ChR2)/H134R fused with enhanced YFP [strain: B6;129S-Gt(ROSA)26So$^{tm32(CAG-COP4*H134R/EYFP)Hze}$/J; stock number 012569; Ai32] to produce hemizygous PCP2-Cre/Ai32 mice (Jackman et al., 2014) that express ChR2 in Purkinje cells, or ChR2(H134R)-EYFP mice. All animal procedures were approved by the McGill Animal Care Committee, in accordance with guidelines established by the Canadian Council on Animal Care.

Acute Slice Preparation

Acute sagittal slices were prepared as previously described (Gruver and Watt, 2019) from 22 female and 25 male mice, aged postnatal day P21 to P32 (Supplementary Fig. 4.15). Mice were deeply anesthetized with isoflurane until unresponsive to a toe pinch and were then rapidly decapitated. Brains were removed and placed in partial sucrose replacement slicing solution (in mM: 50 NaCl, 2.5 KCl, 0.5 CaCl2, 10 MgCl2, 1.25 NaH2PO4, 25 NaHCO3, 25 glucose, and 111 sucrose bubbled with 95% O_2-5% CO_2, to maintain pH at 7.3, osmolality $\sim$320 mOsm) heated to $\sim$37° C (Ankri, Yarom, and Uusisaari, 2014), or in a subset of experiments, in ice-cold slicing solution. We used a Leica VT 1200S vibrating blade microtome (Leica Microsystems, Wetzlar, Germany) to cut parasagittal slices of left cerebellar paravermis at a thickness of 200 μm in a chamber heated to 37° C. Slices were incubated in artificial cerebrospinal fluid (ACSF, in mM: 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 1.25 NaH2PO4, 25 NaHCO3, and 25 glucose, bubbled with carbogen, osmolality ∼320 mOsm) at 37°C for 30-45 min in a darkened slice chamber (Gruver and Watt, 2019), and subsequently moved to room temperature for up to 6 h. Chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) except CaCl2 and MgCl2 from Fisher Scientific (Toronto, ON, Canada).

Electrophysiology

Using acute sagittal slices from mice expressing ChR2 in Purkinje cells, we performed cell-attached and whole-cell patch clamp recordings from CN neurons from the fastigial (medial) cerebellar nucleus. To do this, we pulled borosilicate patch pipettes (2-7 MΩ) using a P-1000 puller (Sutter Instruments, Novato, CA, United States) and filled pipettes with an internal solution containing (in mM): 150 potassium gluconate, 3 KCl, 10 HEPES, 0.5 EGTA, 3 Mg-ATP, 0.5 GTP tris salt, 5 phosphocreatine-(di)tris, with 302 mOsm and pH 7.2 (adjusted with KOH). AlexaFluor 594 (50 mM, ThermoFisher, Burlington, ON, CA) was added to the internal solution to fill patched CN neurons to enable post-recording imaging and morphological reconstruction. Recordings were acquired with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, United States) on a SliceScope Pro 3000 microscope (Scientifica, Uckfield, United Kingdom) from CN neurons in slices maintained at a temperature of 34°C ± 1°C bathed with oxygenated ACSF. For cell-attached recordings, we recorded from spontaneously-firing CN neurons before breaking through into whole-cell patch configuration. For voltage-clamp recordings, CN neurons were clamped at -60 mV and R_{in} and resting membrane potential were monitored, and recordings were excluded if R_{in} fluctuated more than 20%. Data acquisition was performed using custom IGOR Pro acquisition software (Gruver and Watt, 2019) (Wavemetrics, Portland, OR, United States).

Optogenetics and spatial mapping

We used a Polygon400E patterned spatial illuminator with a 470 nm LED light source (Mightex, Toronto, ON, Canada) at an estimated focal plane power density of 100 mW/mm^2 through a 40X water-immersion objective (Olympus LUMPLFLN40XW, Tokyo, Japan) to optically stimulate Purkinje cell axons focally (Gruver and Watt, 2019) using a blue rectangular (85 x 95 μm) light pulse of 3 ms with an inter-trial interval of 10 ms. To assess cell health, we stimulated the patched CN neuron to activate a large proportion of the synaptic inputs onto the cell directly (Purkinje cell "axonal terminals"). We next stimulated the base of each lobule sequentially (e.g. moving from lobule II to III to IV/V, etc. to lobule X, then over the axonal terminals, for ≥ 3 trials per location to identify functionally connected lobules. In a subset of CN neurons, we sampled connectivity by stimulating Purkinje cell axons at additional locations.

To assess the impact of synaptic input on CN neuron spontaneous firing, we performed cell-attached recordings in CN neurons and sequentially stimulated individual lobules as described above before breaking through into voltage-clamp mode. We excluded cells that broke through into whole-cell mode before stimulating each lobule at least once, trials in which spontaneous firing frequency was < 20 Hz, and trials that were marked by bursting rather than tonic firing. After completing the stimulation protocol in cell-attached mode, we broke through and patch-clamped the CN neurons to compare voltage-clamp recorded IPSCs with their corresponding spontaneous firing data.

Analysis of electrophysiological data

All electrophysiological data were analyzed using custom Igor Pro data analysis software (Gruver and Watt, 2019). For IPSCs, the rise time was measured as the time between 20-80% of the peak (standard deviation, SD) (Gruver and Watt, 2019). To assess the decay phases of synaptic currents, we used a double-exponential to fit the decay phases of IPSCs and calculated weighted decay τ time constants by assessing the contribution of each component of the exponential to the peak IPSC amplitude (Vaaga, Brown, and Raman, 2020). Proportion of failures was measured as the number of trials in which a responsive lobule failed to elicit an IPSC while the patch was still successfully in place as determined by the axonal terminal stimulation. For CN neurons that received input from more than one lobule or zone, we compared differences in amplitudes and rise times between individual IPSCs using a disparity index, or a measure of the coefficient of the variation of evoked IPSC amplitudes (Hashimoto and Kano, 2003).

Identifying connectivity motifs

To determine whether structure in connectivity patterns was observed, we performed two analyses. First, we created a binary dataset including each CN neuron's connectivity to lobules III through X, excluding lobule II since several lateral slices did not include lobule II, and excluding neurons from which we did not identify a connected lobule (n = 34 cells). We then performed unsupervised hierarchical clustering of the cells with identified functional connections (n = 41 cells). We used Seaborn to create a clustergram of the binary connections between cells (Fig. 4.2b, rows) and lobules (Fig. 4.2b, columns) based on the Hamming distances between lobules with an unweighted average linkage. Input from lobules II, III, and/or IV/V were categorized as the anterior zone, lobule VI/VII as the central zone, lobule VIII, IX (without lobule X) as the posterior zone, input from lobule IX and X, or only lobule X, as the nodular zone. To identify whether a given pattern of input to a CN neuron constituted a motif, or a statistically overrepresented connectivity pattern in our dataset, we tested the following hypothesis. From adequately sampled CN neurons, we assumed input from each of the four cerebellar zones to occur independently of input from another zone. We calculated the probability of input from each zone within the dataset independent of each other probability. To evaluate multi-zone connectivity patterns, we multiplied the probability of each zone occurring in our dataset to the probability of the other combination(s), multiplied by the probably of non-connection to excluded zones. For example, a Central-Posterior input probability = the probability of input from the central zone (26 cells/75 cells tested) * the probability of input from the posterior zone (16 cells/75 cells tested) * by the probability of not-A anterior zone (1 - 13/75 cells) * the probability of not-N nodular zone (1 - 13/75 cells) = (0.347 * 0.213 * 0.827 * 0.827). We then compared the actual probability of the combinations occurring in our dataset to the predicted using a binomial probability test, where combinations that occurred significantly more or less often than predicted were described as "motifs."

Unless otherwise indicated, analyses performed in Figs. 1-4 and Fig. 4.6 were performed on the same sample of n = 41 cells with lobule connectivity identified, out of 75 CN neurons.

Intracranial viral surgeries

To label Purkinje cell axonal projections to CN neurons, we first injected Pcp2-Cre negative mice (N = 6 female mice, ages 6-14 months) intraperitoneally with a 25% solution of mannitol in PBS (7.5g/Kg, Sigma-Aldrich, Oakville, ON, Canada) in order to enhance virus distribution (Carty et al., 2010; Mastakov et al., 2001). Mice were then anesthetized using isoflurane (2.5% mixture in O_2) before their fur was shaved and they were transferred to a stereotactic frame (Stoelting Co). Carprofen was given subcutaneously, and a lidocaine/bupivacaine mixture was applied locally for analgesia. We performed three injections on each mouse from posterior to anterior. Under stereotactic guidance, a small craniotomy was drilled (< 1 mm) at the first location using a dental drill. A capillary filled with mineral oil (Sigma-Aldrich, Oakville, ON, Canada) attached to a nanoinjector (WPI, Sarasota, Florida, USA) was filled with 150-250 nL of virus, injected into the posterior cerebellar vermis (lobule VIII) and allowed to diffuse for five minutes. Once retracted, the mineral oil-filled capillary was replaced and realigned with Bregma. The second and third injections were performed in the central (lobule VI/VII) and anterior vermis (lobule IV/V) as described. The injection coordinates for surgeries were as follows along the midline (X=0) from Bregma (Y, Z): Posterior vermis: (-7.25, -4.0 or 7.25, -4.25), Central vermis (-6.9, -2.5 or -6.9, -1.8 or -6.65, -1.8), Anterior vermis (-5.65, -3.0 or -5.7, -2.0 or -5.85, -2.5). We used the following viruses to label Purkinje cell axons arising from different cerebellar zones: pENN.AAV.CB7.CI.mCerulean.WPRE.RBG (titer $\geq 5{\times}10^{12}$ vg/mL), pAAV-CAG-tdTomato (codon diversified, titer $\geq 1{\times}10^{13}$ vg/mL) and pAAV-CAG-GFP (titer $\geq 7{\times}10^{12}$ vg/mL) viral preps were purchased from Addgene (Addegene viral prep #: 105557-AAV9, 59462-AAV8 and 37825-AAV8). After surgeries, mice were allowed to recover for 2-3 weeks before being euthanized and perfused as described below.

Immunohistochemistry and viral labeling

We performed immunohistochemistry to demonstrate that CN neurons do not express ChR2 by perfusing mice and labeling for NeuN and anti-green fluorescent protein (GFP). For both immunohistochemistry and viral labeling experiments, we prepared slices from animals by deeply anesthetizing mice with 2,2,2-tribromoethanol (avertin, 0.02 mL/10g) via intraperitoneal injection, followed by intracardiac perfusion (Cook et al., 2022). We performed an initial flush using PBS and 5.6 μg/ml heparin salt, followed by perfusion with 40 ml of 4% PFA in phosphate buffer (PB, pH 7.4). We stored brains in 4% PFA for two days at 4°C on a shaker at 70 RPM, followed by storage in PBS with 0.5% sodium azide prior to slicing. We collected sagittal slices (100 μm thickness) using a Vibratome 3000 sectioning system (Concord, ON, Canada). To label slices with NeuN and anti-GFP, we incubated slices for thirty minutes in blocking solution (1x PBS, 0.1M, pH 7.4, 0.4% Triton X, 5% bovine serum albumin (BSA), and 0.05% sodium azide) which was followed by a 3-day incubation with the primary antibodies in blocking solution. During primary antibody incubation, CN neurons were labeled with guinea pig anti-NeuN primary antibody (1:500, abN90, Millipore, Burlington, MA, United States) and rabbit anti-GFP primary antibody (1:500, ab3080, Millipore, Burlington, MA, United States). Slices were subsequently labeled with DyLight 405 anti-guinea pig secondary antibody (1:500, 106475003, Jackson Immunoresearch) and goat Alexa 594 anti-rabbit secondary antibody (1:1000, A11037, Life Technologies) during a 90-minute incubation in blocking solution. We then rinsed and immediately mounted slices onto glass slides with Prolong Gold Antifade (ThermoFisher Scientific, Waltham, MA, United States) under low light, then stored slides in the dark at 4°C.

Image acquisition and analysis

Immediately after electrophysiology experiments, we performed live two-photon imaging of AlexaFluor 594-filled CN neurons on a custom-built two-photon microscope (Scientifica) imaged with a Ti:Sapphire laser (MaiTai, Spectra Physics, Santa Clara. CA, USA) tuned to 890 nm, as previously described (Lang-Ouellette et al., 2021). Images were acquired at a resolution of 512 by 512 pixels using ScanImage 3.7 running in

MatLab 2011B (Mathworks, Natick, MA, USA). We later re-imaged slices containing filled CN neurons using a LSM800 laser scanning confocal microscope at 10x and 20x using Zeiss Zen software (Zeiss, Oberkochen, Germany) following immunohistochemistry staining and fixation in 4% paraformaldehyde (PFA). Slices labeling Purkinje cell puncta (anti-GFP) onto CN neurons (anti-NeuN) were imaged using the same confocal microscope using a 488 nm and 405 nm diode laser, respectively, to distinguish between the rabbit anti-GFP anti-rabbit Alexa594 labeling (Purkinje cell puncta, pseudocolored in green), and guinea pig anti-NeuN anti-guinea pig DyLite 405 labeling (CN neurons, pseudocolored in blue).

After imaging prepared slices, we used Fiji (ImageJ, NIH) (Schindelin et al., 2012) to analyze filled, stained CN neurons from electrophysiology and immunohistochemistry experiments. We confirmed the identity of AlexaFluor 594-filled CN neurons by comparing them with the initial two-photon images acquired following live electrophysiology experiments and identified the dendritic and lobule orientation in slices with filled cells to evaluate the relationship between CN morphology and input patterns. We used Neurolucida software (MBF Biosciences, Williston, VT, USA) to manually perform morphological reconstructions of CN neurons from the two-photon imaging session following patch clamp experiments. We traced cells in 3-D to obtain the cell soma contours, dendrite projections, and axon within the image stack. We imported tracings into Neurolucida Explorer to extract morphological data, including soma area, soma diameter, number, and length of dendrites. Branch index was calculated by dividing the number of dendritic crossings 25 μm from the cell soma by the number of primary dendrites (Uusisaari, Obata, and Knöpfel, 2007). Polar plots were made using IgorPro to evaluate the overlap between dendritic orientation and Purkinje cell input. Dendritic polar plots were normalized to the length of the longest dendrite.

To evaluate the proximity of virally-labeled Purkinje cell puncta originating from different cerebellar zones following intracranial stereotactic surgeries, we imaged mounted 100 μm-thick sections using the LSM-800 confocal microscope at 10x, 20x, and 63x oil immersion and acquired tiled stacks using a 405 nm, 561 nm, and 488 nm laser to distinguish between the mCerulean, TdTomato, and GFP expression. Images were pseudocolored in blue, magenta, and green, respectively, and brightness and contrast values were enhanced for representative images. Raw images were thresholded in ImageJ and used to identify the X and Y coordinates of each puncta in individual channels. Using a custom Python script, we identified the nearest neighbor puncta across different zonal inputs with different imaging channels to identify the Euclidean distance of the nearest puncta across zones. To confirm that there was little to no colocalization of multiple viruses in the same Purkinje cells, we performed thresholding of Purkinje cell puncta for each channel in ImageJ and acquired the positions of puncta in the X-Y plane using ImageJ's plot profile analysis function and used a custom Python script to identify the percentage of colocalized pixels per slice in each stack which determined there was virtually no colocalization between channels (Table 4.1).

Cell topography within fastigial nucleus

The position of each recorded CN neuron (Fig. 4.5) was recorded manually during each experiment and paired with an image of the position of each patch pipette during the recording. We mapped these positions for each slice along the mediolateral axis using the coordinate system from the Allen Mouse Brain Atlas (Wang et al., 2020), and for cells that were successfully filled with Alexa594 during each experiment, we confirmed these positions after confocal imaging of each slice. We then used Brainrender (Claudi et al., 2021) to visualize these cells in 3-D within the nucleus. Next, we represented plotted CN neurons based on their number of input zones (0 to 4 zones) along the Allen Mouse Brain Atlas coordinates and produced graphs along the rostro-caudal and ventral-dorsal axes. We performed a linear regression for each category ($n = 0, 1, 2, 3$, or 4 zones) and identified the R^2 value for each group. To evaluate whether the strength of the best fit line for each category was significant, we performed a bootstrap analysis where we randomly sampled n cells (equivalent to the number of cells in our dataset that received input from n zones) from each category 50,000 times and determined whether the R^2 value from our data occurred within the top 5% of R^2 values from the bootstrapped samples (one-tailed test). We plotted the percentile of the R^2 values from our data compared to the bootstrapped samples as the "probability of R^2 compared to 50,000 random samples" (Fig. 4.4f, g).

Statistics

We used JMP software (SAS, Carey, NC, United States), to perform Student's t-tests on datasets with normal distributions, and Igor Pro software for non-parametric Mann-Whitney U tests for datasets with non-normal distributions, with the level of significance (α) set at $P < 0.05$. Unless otherwise indicated, all statistical tests performed were two-tailed tests. To evaluate connectivity motifs, we used a binomial distribution calculator followed by multiple hypothesis corrections using the Benjamini-Hochberg method. Data are reported as mean ± SEM unless otherwise indicated. For all data, n = number of neurons and N = number of mice.

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Author Contributions

K.M.G. designed and ran experiments for all figures except Fig. 4.5, analyzed data for all figures, and wrote the manuscript., J.W.Y.J. analyzed data for Figs. 3.3-3.4, S4-5, and S7., E.F. designed and ran experiments for Fig. 4.5, S.S. analyzed data for Fig. 4.2, P.J.S. wrote custom Igor software packages for electrophysiology data acquisition and analysis, and analyzed data for Figs. 2 and 4. A.J.W. conceived of the project, designed experiments, analyzed data, supervised the project, and wrote the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Data availability

All relevant data are available from the corresponding author upon reasonable request. The source data for each figure is provided as a Source Data file and is provided with this paper. Datasets analyzed with custom code are also available on Github: (https://github.com/kgruver/StructuredConnectivity).

Code availability

Custom code used to analyze the findings of this study that has not yet been described in published literature is available on GitHub:
(https://github.com/kgruver/StructuredConnectivity).

4.7 Supplementary Material

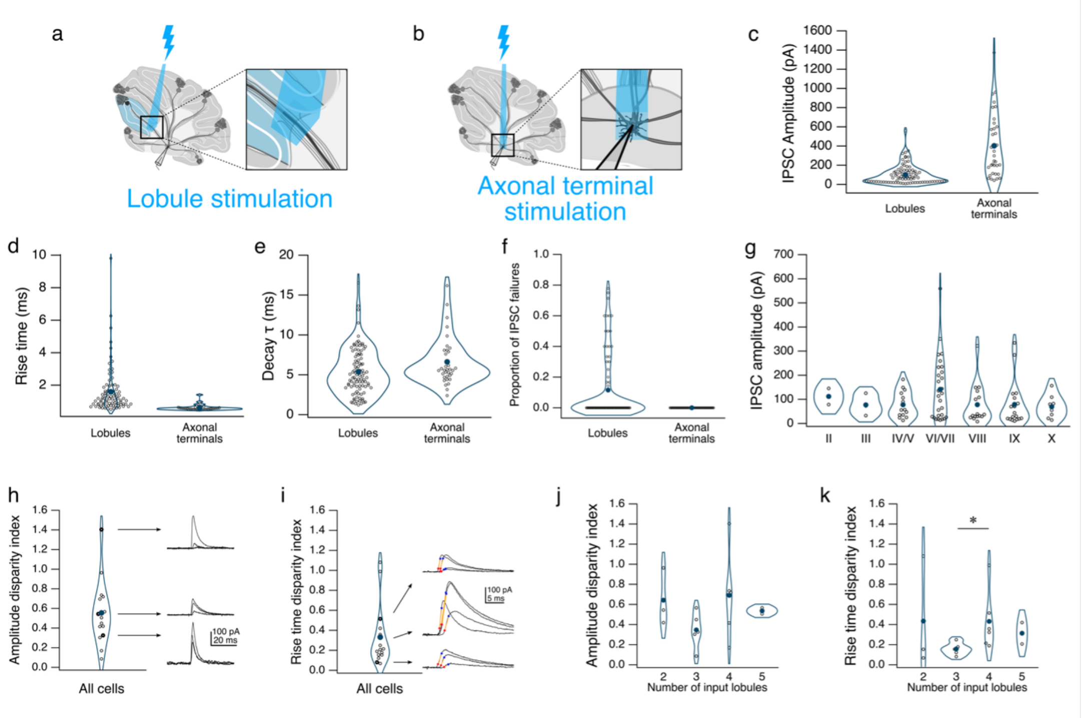

Figure 4.7: **Properties of inhibitory post-synaptic currents (IPSCs) following Purkinje cell stimulation.** **a**, Cartoon showing area of photostimulation for "lobule stimulation," and **b**, "axonal terminal stimulation." **c-f**, IPSC amplitudes, rise times, weighted decay τ time constants, and failures evoked following "lobule" or "axonal terminal" stimulation. **g**, IPSC amplitudes by lobule. **h-i**, Average IPSC amplitude disparity index for all cells with input from > 1 lobule: 0.55 ± 0.08, and average rise time disparity index for all cells with input from > 1 lobule: 0.33 ± 0.08. Traces to the right of each graph displaying examples of IPSCs with different disparity index values. **j-k**, Disparity indices for IPSC amplitudes and rise times by number of lobule inputs. $^*P < 0.05$. Unlabeled comparisons = not significantly different.

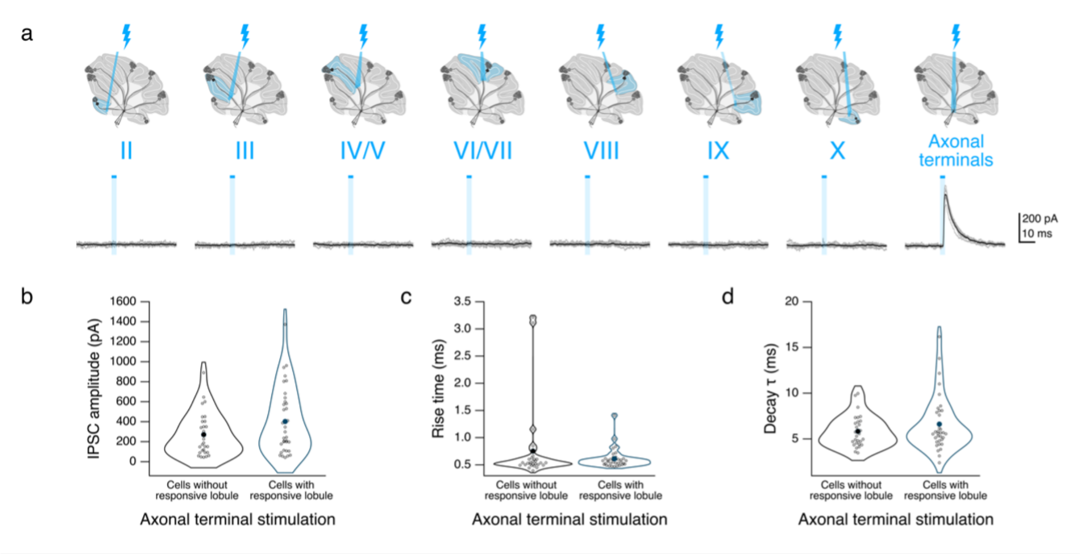

Figure 4.8: **Responses from cells with non-responsive lobules after lobule stimulation.** **a**, Top, stimulation protocol as described in **Fig. 4.1b**. Bottom, example traces following lobule stimulation (left) and axonal terminal stimulation (right). **b**, Average IPSC amplitude for cells without responsive lobules versus cells with responsive lobules (Fig. 4.1) following axonal terminal stimulation. **c**, Rise times for cells without responsive lobules versus cells with responsive lobules following axonal terminal stimulation. **d**, Weighted decay τ time constants of IPSCs from cells without responsive lobules versus cells with responsive lobules. IPSC amplitudes, rise times, and decay τ time constants compared between cells with and without responsive lobules using non-parametric multiple comparison Mann-Whitney U test. Unlabeled comparisons = not significantly different.

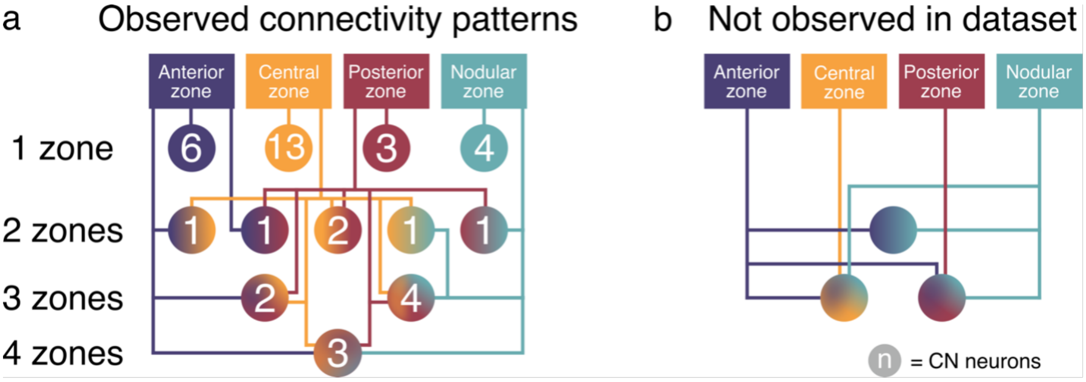

Figure 4.9: **a**, Representation of observed Purkinje cell – CN neuron connectivity patterns based on cerebellar zones (circles). Numbers represent occurrences of each pattern in our dataset. **b**, The following connectivity patterns were not observed in our dataset: "AN," "ACN," and "APN" CN neurons.

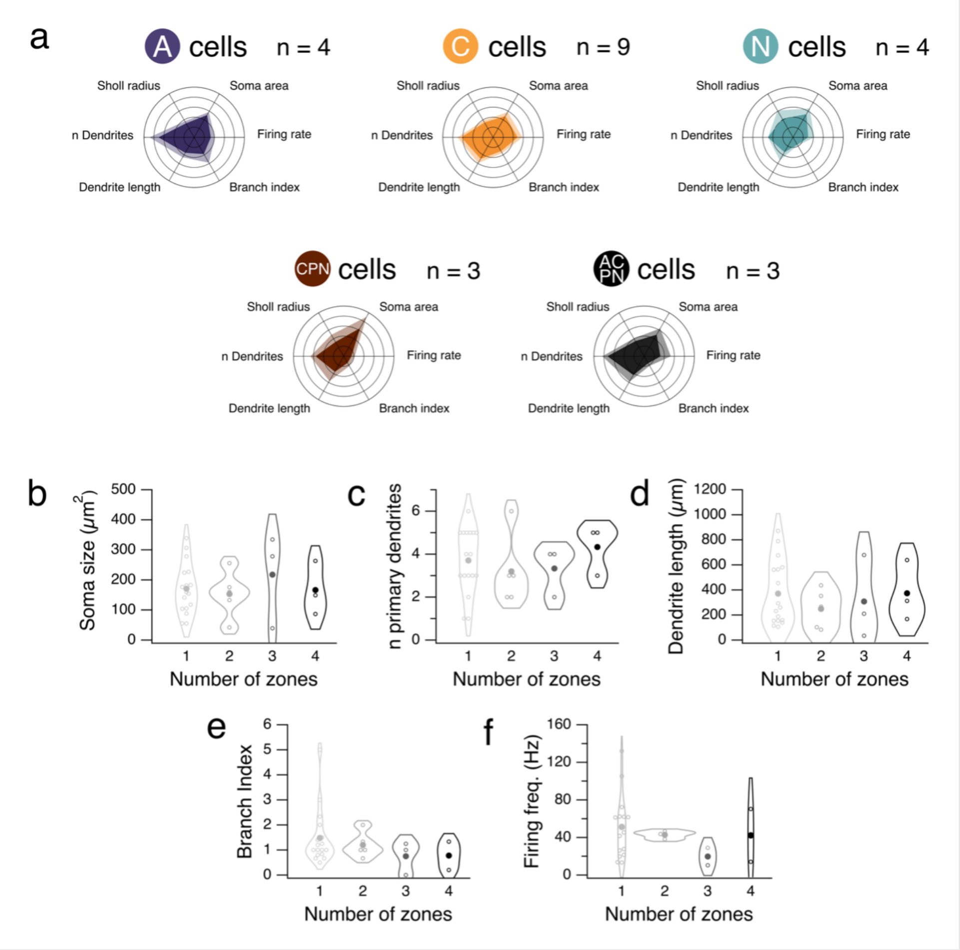

Figure 4.10: **Morphological and physiological properties of CN neurons.** **a**, Polar plots displaying normalized average value of each measure per connectivity pattern for patterns with ≥ 3 cells per group. Shaded region indicates standard error. **b**, Soma size. **c**, Number of primary dendrites. **d**, Total dendrite length. **e**, Branch index at 25 μm. **f**, Firing frequency for cells by number of input zones. Unlabeled comparisons = not significantly different.

Animal	**Number of positive pixels in channel 1**	**Number of positive pixels in channel 2**	**Number of colocalized positive pixels**	**Percent colocalized pixels/total positive pixels**
Animal_ID1	52351	6756	139	0.24
Animal_ID2	75784	10140	88	0.10
Animal_ID3	5758	4844	41	0.39
Animal_ID4	107730	2808	157	0.14
Animal_ID5	35239	12014	140	0.30
Animal_ID6	8181	1392	0	0.0

Table 4.1: **Colocalization of multiple viruses in virally labeled Purkinje cells.** Number of positive pixels across imaging channels for six animals injected with fluorescently labeled viruses. Channels selected for colocalization analysis include the channel with the most viral expression (channel 1) and the channel with the second-most viral expression (channel 2). Numbers of positive pixels per channel represent the total number of positive pixels across >1 slices in imaging stack (2 μm interval).

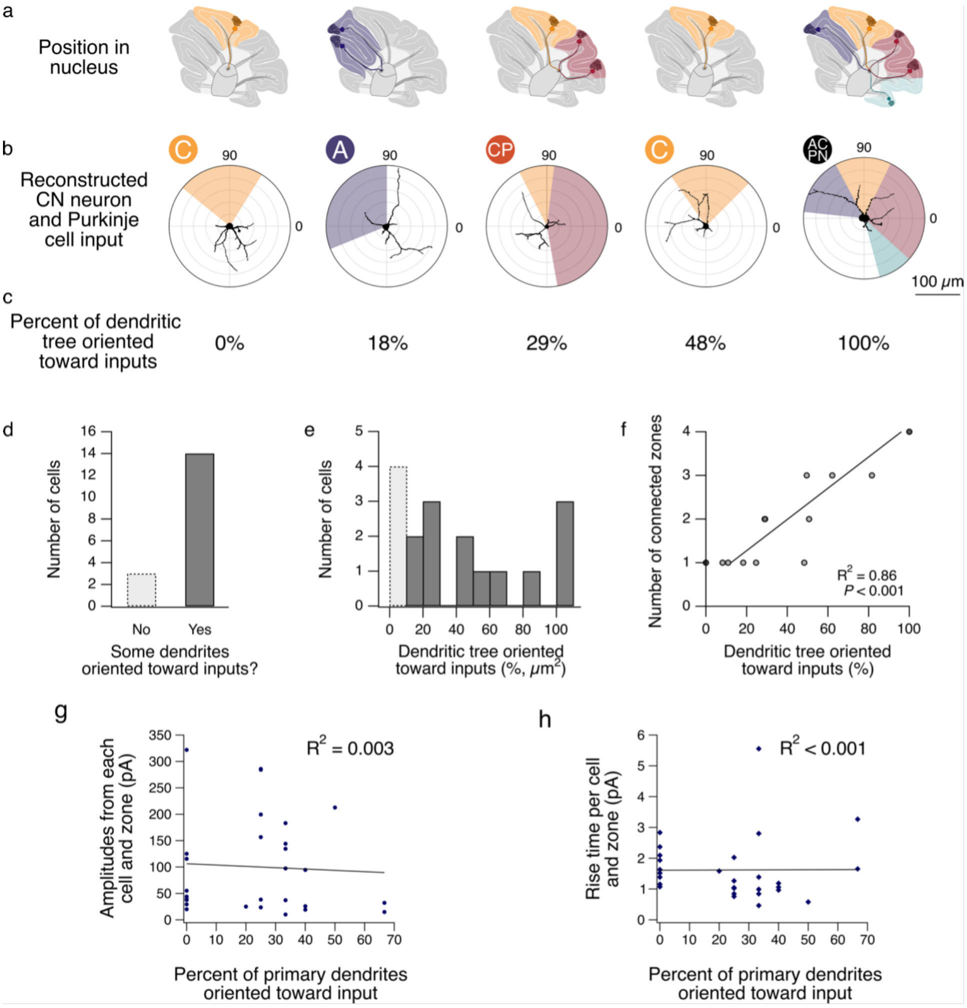

Figure 4.11: **CN dendrites are not preferentially oriented toward their zone inputs.** **a**, Cartoons of cerebellar slices showing the relative position of individual CN neurons with different connectivity patterns. **b**, Polar plots showing the superimposed reconstructions of CN neurons from a with the input directions of the connected zones. **c**, Percent of dendritic tree for cells in b oriented toward their connected zones. **d**, Numbers of cells with and without dendrites oriented toward their inputs. **e**, Percent of CN neuron dendrite surface area oriented toward their connected zones, and **f**, as a function of the number of connected zones. Number of connected zones vs. percent of dendritic tree oriented toward inputs, Pearson's R^2 correlation coefficient. **g**, Amplitudes for each input per cell vs. the number of primary dendrites oriented toward that input zone (e.g. a four-zone cell has four data points). **h**, Same as g, but the rise time for each input.

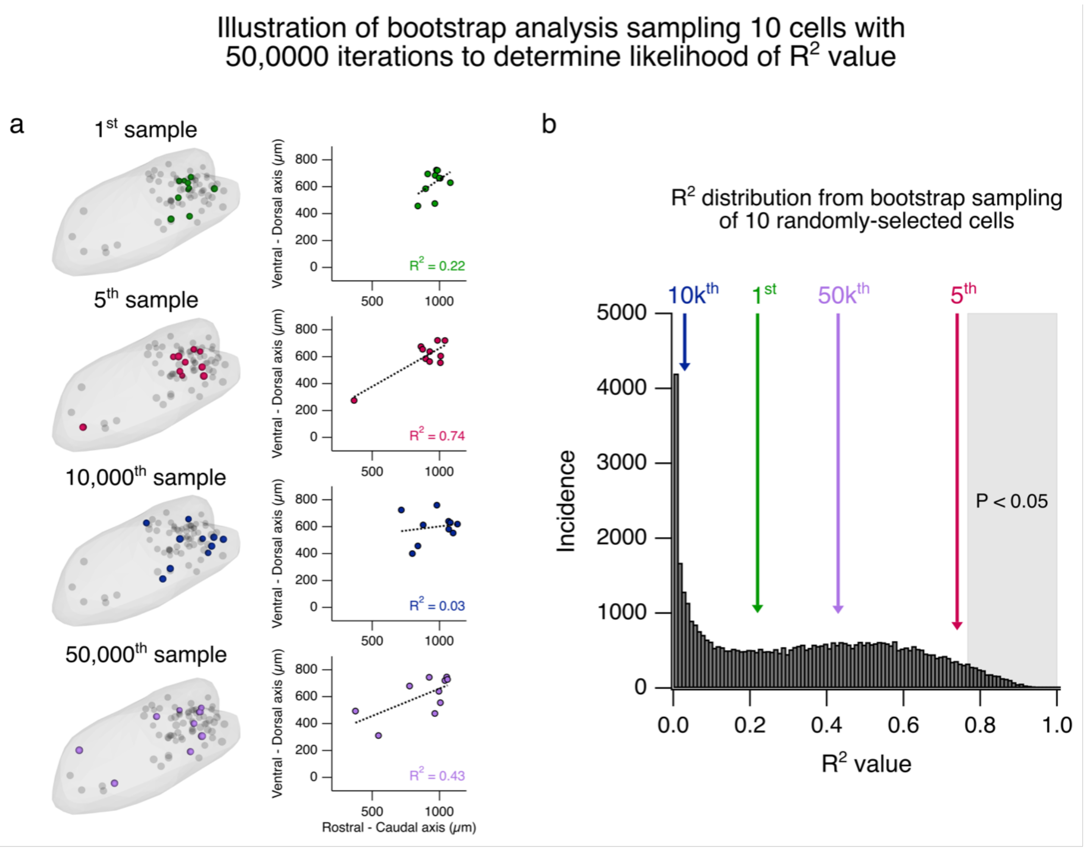

Figure 4.12: **Illustration of bootstrap analysis using 50,000 randomly selected samples of n cells to assess topographic organization within the CN.** **a**, Left, renderings of CN in the sagittal plane depicting 10 randomly selected cells in the 1st, 5th, 10,000th, and 50,000th bootstrapped samples. Right, same as in a, left, but showing the plotted points for each CN neuron in the rostral-caudal and ventral-dorsal axes. Dotted lines and R^2 values in each graph represent the line of best fit for each sample of 10 cells. **b**, Distribution of R^2 values from the 50,000 bootstrapped samples. R^2 values from samples in a are highlighted with arrows. Shaded region depicts R^2 values that occurred in the top 5% of the distribution.

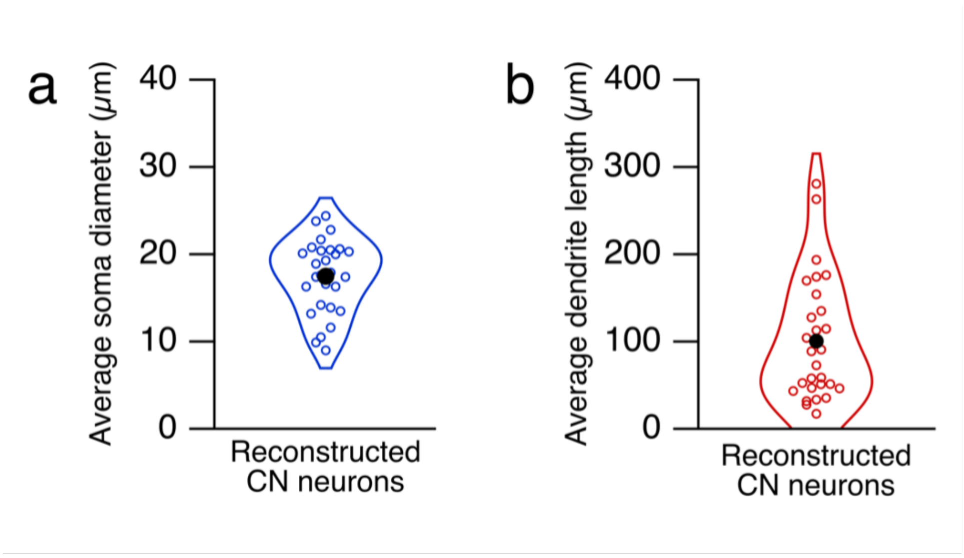

Figure 4.13: **Morphological properties from filled, reconstructed CN neurons.** **a**, Average feret maximum soma diameter from Alexa-594-filled CN neurons. **b**, Average dendrite length from Alexa-594-filled CN neurons. n = 18 cells.

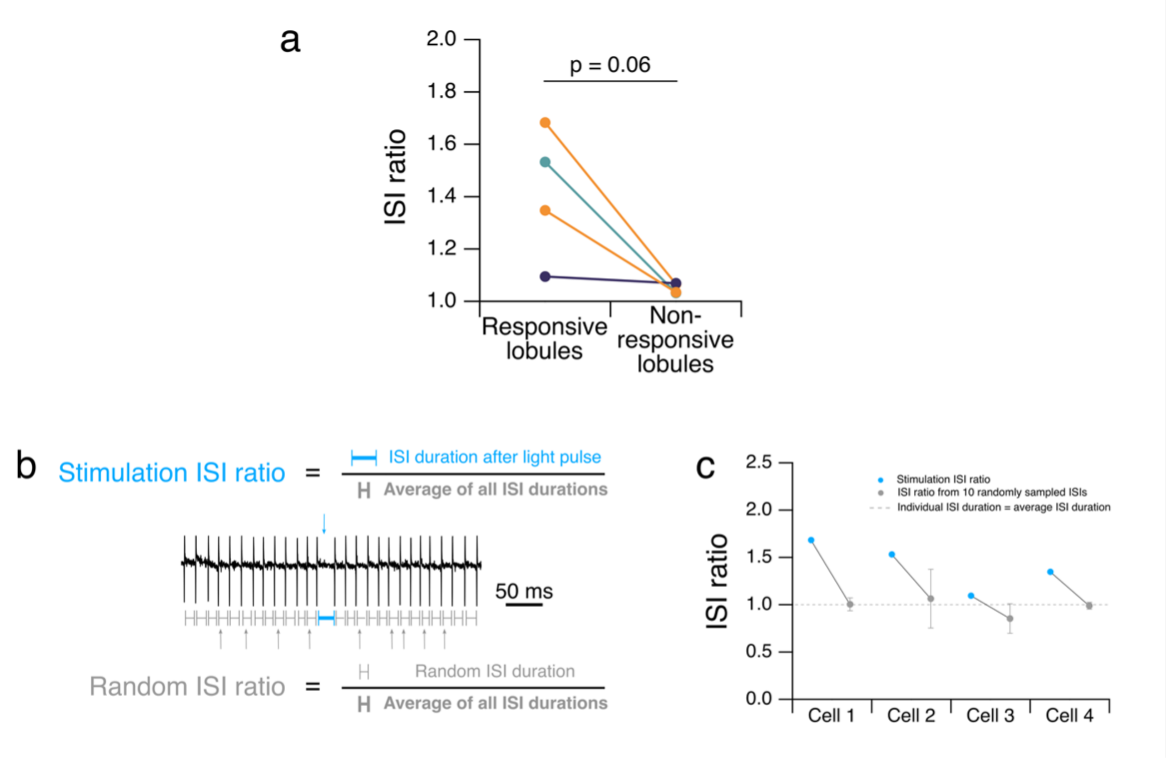

Figure 4.14: **The ISI ratio following stimulation of responsive lobules is higher in all tested cells compared to randomly sampled spike ISIs from the same trial.** **a**, ISI ratios per cell for responsive lobules: 1.42 ± 0.25, non-responsive lobules: 1.05 ± 0.02. **b**, Top: ISI ratio calculated by dividing the ISI duration after stimulation with the average ISI duration per cell per trial. Middle: Example trace with blue and grey arrows highlighting the immediate post-stimulation ISI and other random ISIs, respectively. Bottom: Random ISI ratio calculated by dividing the ISI duration from a randomly sampled spike pause in the same trial as the stimulation, by the average ISI duration across the entire trial. **c**, Comparison of stimulation ISI ratios (blue) and 10 randomly selected ISI ratios (grey) for each cell. Error bars show the 95% confidence interval for ISI ratio acquired from the 10 randomly selected ISIs for each cell. Dotted line indicates ISI ratio of 1, where the selected ISI duration is equivalent to the average ISI duration for the entire trial. Unlabeled comparisons = not significantly different.

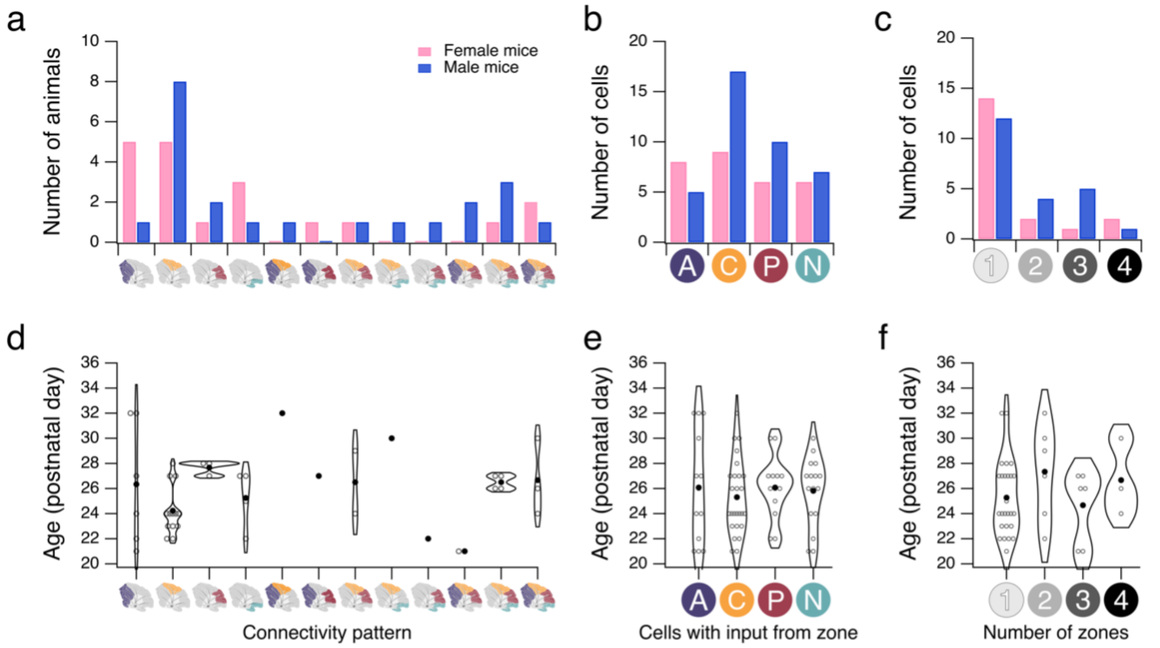

Figure 4.15: **No sex or age differences observed across different connectivity patterns.** **a**, Distribution of observed connectivity patterns for female (pink) and male (blue) mice. **b**, Number of cells from female and male animals receiving input from each zone. **c**, Number of cells from female and male animals receiving input from n number of zones. **d**, Distribution of observed connectivity patterns across different ages. **e**, Distribution of different ages receiving input from each zone. **f**, Distribution of different ages receiving input from n number of zones. N = 22 female mice, 25 male mice. Unlabeled comparisons = not significantly different.

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Chapter 5

Discussion

Mapping out connections between neurons has been a major focus in neuroscience research. Large-scale initiatives have been undertaken to produce comprehensive maps of connectivity (or "connectomes") within the brains of species like *Drosophila* (Scheffer et al., 2020), mice (Oh et al., 2014), and humans (Elam et al., 2021). Other projects like the Blue Brain Project (Markram, 2006) and Human Brain Project (Amunts et al., 2019) set out to build biologically-accurate simulations of the brains of mice and humans, respectively. By 2011, $40 million of funding from the National Institutes of Health had already been allocated to the Human Connectome Project (Vogelstein, 2011). The Blue Brain Project is slated to receive nearly a quarter of a billion dollars between 2013 and 2024 from the Swiss government (*EPFL Blue Brain FAQ* n.d.). At present, only the *C. elegans* nervous system has thus far been completely mapped (White et al., 1997), likely due to its modest circuitry featuring just 302 neurons.

Small-scale research that identifies properties of neural circuits also contributes, however modestly, to the herculean effort of understanding how the brain works by uncovering its wiring. The work presented in this thesis contributes to our understanding of a critical component of the cerebellar circuit: the synapse between Purkinje cells and cerebellar nuclear neurons.

5.1 Summary

In the preceding chapters I described our exploration of the spatial connectivity patterns that bridge cerebellar input and output at the Purkinje cell – cerebellar nuclear neuron synapse. During my PhD, we developed an optogenetic stimulation paradigm that allowed for robust and reliable identification of functional connections between Purkinje cells and cerebellar nuclear neurons, described in Chapter 3. We optimized the photostimulation parameters required to reliably evoke single action potentials by stimulating Purkinje cell axons with light (Fig. 3.2). We found photostimulation was highly spatially restricted, where shifting the stimulated region by $\sim 35\ \mu$m could result in a failure to induce an action potential from Purkinje cell axons, suggesting that there is little unintended activation outside of the designated stimulation area (Fig. 3.1B). Further, axonal photostimulation consistently elicited IPSCs from cerebellar nuclear neurons (Fig. 3.4), demonstrating that our optogenetic stimulation paradigm is a useful tool for mapping the connections between Purkinje cells and cerebellar nuclear neurons.

We next used this approach to identify the patterns that underlie Purkinje cell – cerebellar nuclear neuron functional connections (Chapter 4), which are essential for cerebellar information transfer and subsequent behavior. Overall, we found non-random connectivity patterns between Purkinje cells and cerebellar nuclear neurons along the rostrocaudal plane, orthogonal to the mediolateral gradient of corticonuclear connectivity that has been previously described in the field (Voogd and Ruigrok, 2004). Cerebellar nuclear neurons that receive input from all four zones exhibit a topographic organization within the fastigial nucleus, supporting the notion that a functional topography governs general cerebellar processing in both the cerebellar cortex (Sugihara and Shinoda, 2004) and the nuclei (Fujita, Kodama, and Du Lac, 2020).

Our strategy of performing whole-cell patch clamp recordings from cerebellar nuclear neurons also allowed us to identify micro-scale features of the Purkinje cell - cerebellar nuclear neuron synapse. We described fast kinetic properties of this synapse (Supplementary Fig. 4.7), as well as evaluated the synaptic strengths of localized Purkinje cell convergence on individual nuclear neurons at the level of cerebellar lobules (Supplementary Fig. 4.7) and zones (Fig. 4.1). Purkinje cells from across zones converge on individual cerebellar nuclear neurons with combined IPSC amplitudes in the range of several hundred picoamps (Fig. 4.1). Considering this convergence, we surprisingly observed that small synaptic inputs from single lobules were sufficient to pause cerebellar nuclear neuron firing, and that this pause was strongly correlated with IPSC amplitude (Fig. 4.6). This suggests that coincidental activation or synchronous activity across convergent Purkinje cells may have a graded effect on cerebellar output.

5.2 Interpretation

Circuit mapping of functional synapses in the cerebellum

Our circuit mapping strategy focused primarily on identifying functional connections between Purkinje cells and cerebellar nuclear neurons through paired optogenetic and electrophysiology techniques, with support from AAV-mediated anatomical circuit mapping (Fig. 4.5). Large-scale circuit mapping studies commonly use viral tools such as AAV-mediated labeling following localized injections (Oh et al., 2014) or trans-synaptic labeling with rabies virus (Yao et al., 2023) to identify connections between cells. Indeed, these viral labeling strategies can be combined with genetic tools to induce labeling in specific cell types to produce large high-resolution datasets of mono- or polysynaptic connections (Yao et al., 2023). However, some critical properties of neural circuits cannot be assessed using tracing strategies alone. Among these properties are the characteristics of individual synapses, which are the essence of connections between neurons (Campagnola et al., 2022). Indeed, much of the complexity in functioning circuits can be attributed to the dynamic features of the synapses that connect neurons, since these synaptic features dictate how a circuit performs in a living organism. Models and simulations of the brain, such as the Blue Brain Project, must incorporate accurate characterizations of the synaptic properties that underlie circuits to be truly biologically accurate (Campagnola et al., 2022).

By using information-rich strategies like whole-cell patch clamp recordings (Gouwens et al., 2019) to assess Purkinje cell - cerebellar nuclear neuron connectivity, we not only identified connections between Purkinje cells and cerebellar nuclear neurons (Fig. 4.2), but also characterized the synaptic (Supplementary Fig. 4.7), morphological (Fig. 4.3, Supplementary Fig. 4.10), and topographic (Fig. 4.4, Supplementary Fig. 4.11) features of postsynaptic cerebellar nuclear neurons in the fastigial nucleus (Chapter 4). Therefore, our work contributes to the overarching goal in the field of neuroscience to map the brain while also providing insight into the micro-scale features of synaptic connectivity necessary to create biologically realistic models of the cerebellum.

Compartmentalization of the cerebellum

The cerebellum has long been compartmentalized into a series of longitudinal zones or "modules" (see Chapter 2 section 2.4.3.1), where parasagittally-oriented stripes of Purkinje cells converge on a particular region in the cerebellar nuclei and receive input from a particular region in the inferior olive (Voogd and Ruigrok, 2004). These modules, running parallel along the mediolateral axis, are thought to serve as the basic computational units of the cerebellum (Apps et al., 2018). Our study in Chapter 4 focused on the connections between Purkinje cells in the vermis and cerebellar nuclear neurons in the fastigial nucleus, the most medial of the cerebellar nuclei. These connections likely correspond to the "A" module of the longitudinal zone compartmentalization schema (Apps et al., 2018). Rather than contradicting the mediolateral compartmentalization of the cerebellum, our work complements this organization by exploring the nuance of connectivity within an individual cerebellar module. We demonstrate that a diversity of Purkinje cell convergence patterns exists within the cerebellar vermis. This diversity may endow the cerebellum with greater computational flexibility than if Purkinje cell convergence on cerebellar nuclear neurons were uniform within a cerebellar module.

Different regions of the cerebellum have been associated with different behavioral functions (see Chapter 2 section 2.2.4). Data from neuroimaging studies highlight two localized motor representations in the cerebellum: in lobules I-VI (primarily the anterior zone) and in lobule VIII (posterior zone) (Guell et al., 2018). While we did observe connectivity between lobules in the anterior and posterior zones in individual cerebellar nuclear neurons, we never observed convergence between lobules III (anterior zone) and VIII (posterior zone) in our study, though we did observe convergence between lobules IV/V and VIII (Fig. 4.2). This suggests an additional level of complexity in Purkinje cell convergence, where there is also variability in the lobules that are functionally connected to a cerebellar nuclear neuron. These variations in spatial connectivity maps may contribute to the complexity of information transfer in the cerebellum.

Non-random connectivity in the cerebellar circuit

Connectivity within a circuit can range from highly probabilistic to highly regular (Watts and Strogatz, 1998). Although probabilistic, or “random,” connectivity between neurons in a circuit has been observed in some biological circuits (Miao et al., 2016; Avermann et al., 2012) and is often used to model neural networks (Senk et al., 2022; Maoz et al., 2020), non-random connectivity features more prominently in many circuits of the brain than purely random connectivity (Song et al., 2005; Morgan et al., 2011; Rieubland, Roth, and Häusser, 2014). At the Purkinje cell - cerebellar nuclear neuron synapse, we observed a regular, or "non-random," connectivity motif: four-zone convergence appeared significantly more often than if Purkinje cell convergence occurred probablistically. Our findings show that non-random connectivity underlies Purkinje cell - cerebellar nuclear neuron connectivity (Fig. 4.2e) where Purkinje cell convergence is enriched at the level of transverse zones (Fig. 4.2b).

Non-random connectivity motifs have also been observed in other cell populations in the cerebellum. Connectivity between molecular-layer interneurons is highly influenced by their proximity to one another, where neighbors form clusters of reciprocally connected cells (Rieubland, Roth, and Häusser, 2014). Molecular-layer interneurons provide strong inhibition to Purkinje cells thereby modulating the output of the cerebellar cortex. Cerebellar granule cells, which provide strong excitatory input to Purkinje cells, also demonstrate a topography in their projection patterns (Spaeth et al., 2022). Granule cell – Purkinje cell connectivity exhibits a stereotyped yet plastic organization that is consistent across animals and can change with behavior (Spaeth et al., 2022). Thus, non-random connectivity patterns have been shown to coordinate information transfer at multiple levels of the cerebellar circuit. It would be interesting to explore whether the Purkinje cell – cerebellar nuclear neuron connectivity patterns we described in Chapter 4 are also behavior- or experience-dependent.

Potential roles for different types of fastigial nuclear neurons

We show that cerebellar nuclear neurons represent a diverse group of cells which likely enriches the computational capabilities of the cerebellum. We wonder how different Purkinje cell convergence patterns on individual nuclear neurons might impact their role in brain function. Cerebellar nuclear neurons that receive input from a single transverse zone may act as "specialist" cells that perform linear encoding of Purkinje cell signaling related to a "unimodal" function or behavior, such as gait or postural control. Multi-zonal cerebellar nuclear neurons on the other hand may perform more complex, non-linear encoding of Purkinje cell input. In the granule cell layer, multimodal granule cells that receive, for example, both somatosensory and auditory information, have been hypothesized to "recode" input signals, thereby expanding the representational possibilities for cerebellar information processing (Ishikawa, Shimuta, and Häusser, 2015). Multi-zonal cerebellar nuclear neurons that receive input from Purkinje cells in functionally different (e.g., motor, ocular, and cognitive) regions of the cerebellum may offer a streamlined system to encode complex information as "integrators" without requiring simultaneous activation of multiple uni-zonal specialist cells. Alternatively, multi-zonal cerebellar nuclear neurons may be "generalist" cells that are suited to respond in a variety of contexts, possibly maintaining normal levels of cerebellar output if "specialist" uni-zonal cells are only recruited during specific behaviors.

5.3 Limitations

Acute slice preparation

An important limitation of the work presented in this thesis is that our experiments were performed in 200 μm-thick acute cerebellar slices. This thickness allowed us to more easily visualize and access cerebellar nuclear neurons in adult mice than is possible in thicker slices, since cerebellar nuclear neurons are encircled by dense webs of myelinated axons, as well as by robust perineuronal nets at the ages when we perform recordings (see Chapter 2 section 2.4.2). While many Purkinje cell axons are cut during slicing, recording from the fastigial nucleus granted us an advantage in that most Purkinje cell input to this nucleus is thought to arise from roughly the same or neighboring parasagittal plane. We also stimulated Purkinje cell axons at the base of each lobule, increasing the likelihood that the Purkinje cell axons from a given lobule would be intact in the tissue and available for photostimulation. However, some fastigial nucleus neurons have been shown to also receive input from the cerebellar hemispheres (Armstrong and Schild, 1978b), and these inputs would very likely be severed in our slices. Indeed, nearly half of the cerebellar nuclear neurons in our study did not respond to lobule stimulation (34/75 cells, Supplementary Fig. 4.8).

Additionally, individual longitudinal zones are thought to span several hundreds of microns, with individual zebrin II stripe widths ranging from 100 to 200 μm wide. Since our slices are 200 μm thick, an entire longitudinal zone, which is made up of multiple stripes, is not likely to be preserved within our individual slices. We recorded from three slices of vermis and paravermis containing the fastigial nucleus for a total width of 600 μm, and the individual connectivity patterns we observed were not correlated with the mediolateral position of the slice (Fig. 4.4g shows mediolateral position of cells based on number of connected zones; data not shown for each connectivity pattern). Overall, though our findings represent a lower bound of possible convergence between Purkinje cells and cerebellar nuclear neurons that can be assessed within an acute slice, we nonetheless observed robust connectivity between Purkinje cells and cerebellar nuclear neurons in a variety of combinations. This suggests that slice electrophysiology is a useful tool for evaluating cerebellar connectivity at this synapse.

Sampling bias of cerebellar nuclear neurons

Most of the cerebellar nuclear neurons we recorded from were located in the caudodorsal region of the cerebellum (Fig. 4.4). While we performed the same line-by-line scanning process to visually identify cerebellar nuclear neurons available for patching, moving from ventral to dorsal, we were less successful at recording from neurons located in the rostroventral area of the fastigial nucleus. Cerebellar nuclear neurons in this area likely belong to Group III as described by Sugihara and Shinoda, 2004, and the $F1_R$ region recently described by Fujita, Kodama, and Du Lac, 2020. Neurons in this area include the largest glutamatergic projection neurons (Fujita, Kodama, and Du Lac, 2020) as well as large glycinergic projection neurons (Bagnall et al., 2009), and are thought to be involved in posturomotor control.

We hypothesize two potential reasons for this difficulty in sampling from rostroventrally-located neurons. First, the large putative-glutamatergic projection neurons in the cerebellar nuclei are preferentially enwrapped by thick perineuronal nets (Carulli et al., 2006), though this population may also include the large glycinergic projection neurons located in this region. Secondly, due to their ventral position within the nucleus, these cells may be surrounded by a higher density of axons entering and leaving the cerebellum, impeding our ability to successfully visualize and target these cells for recording. In the future, it may be useful to transgenically label cerebellar nuclear neurons with a fluorescent tag to more effectively visualize and record from these cells to avoid further sampling bias.

Departures from previously reported cerebellar nuclear neuron properties

Differences between cerebellar nuclei

It is important to note that several of our findings differ from those reported elsewhere in the literature. For example, the decay τ time constants we report ($\sim$ 5 ms: Supplementary Fig. 4.7) are longer than what has been reported previously (2.5 ms: Person and Raman, 2012a). The recordings in the Person and Raman study were performed in the interposed nuclei, and may represent a different population of cerebellar nuclear neurons than those found in the fastigial nucleus (see Chapter 2 section 2.3.3). The synaptic decay kinetics we reported in Chapter 4 are similar to those reported from a different study recording from neurons in the fastigial nucleus (6.7 ms: Vaaga, Brown, and Raman, 2020). Other studies specifically focused on nuclei other than the fastigial nucleus (Uusisaari and Knöpfel, 2010; Najac and Raman, 2015), or recorded from cerebellar nuclear neurons without distinguishing between nuclei (Uusisaari, Obata, and Knöpfel, 2007; Turecek, Jackman, and Regehr, 2016). Thus, results may differ across studies depending on the cerebellar nucleus that is sampled.

Differences between developing and adult mice

Further complicating comparison, many of the early studies characterizing cerebellar nuclear neuron physiology were performed in young rodents (< P21) (Uusisaari, Obata, and Knöpfel, 2007; Pedroarena and Schwarz, 2003; Aizenman and Linden, 1999). While recording from cerebellar nuclear neurons at these young ages facilitates recording likely due to minimal perineuronal net formation and myelination, the properties of this synapse in development are not fully representative of the synaptic properties in adults (Turecek, Jackman, and Regehr, 2016). Our recordings were performed in young adult animals between P21-32, an age range where Purkinje cell - cerebellar nuclear neuron synapses show less depression than those at younger ages (Turecek, Jackman, and Regehr, 2016), and may therefore exhibit other differences in synaptic properties, such as synaptic strength and kinetics.

5.4 Future directions

In vitro studies

The work presented in this thesis leads to many exciting questions and potential avenues for future research. We identified how Purkinje cells in the vermis and paravermis converge on cerebellar nuclear neurons in the fastigial nucleus. Identifying how Purkinje cells diverge from the same lobule or zone on cerebellar nuclear neurons is also important to understand information transfer within the cerebellum. In a study that performed reconstruction of individual Purkinje cell axons, the authors were unable to assess Purkinje cell divergence, i.e., the number of cerebellar nuclear neurons directly contacted by a single Purkinje cell (Sugihara et al., 2009). Therefore, different methods may be needed to address this question.

Performing dual whole-cell patch clamp recordings of cerebellar nuclear neurons is one potential strategy to evaluate Purkinje cell divergence. Based on a previous topographic analysis of the fastigial nucleus (Fujita, Kodama, and Du Lac, 2020), neighboring cerebellar nuclear neurons are likely to receive similar input while cerebellar nuclear neurons in different regions of the nucleus may not. Our observations are largely in agreement with this hypothesis, though we did observe some instances of neighboring uni-zonal nuclear neurons that did not receive Purkinje cell input from the same zone. It is also important to note that, while immensely informative, these experiments may be prohibitively challenging, as recording from single adult cerebellar nuclear neurons alone is a fairly low-yield endeavor.

It would also be informative to compare Purkinje cell - cerebellar nuclear neuron connectivity between the fastigial nucleus and the interposed and dentate nuclei. Since neurons in these nuclei are both transcriptionally (Kebschull et al., 2020) and physiologically (Person and Raman, 2012a) different from those in the fastigial nucleus, I hypothesize that neurons from these cerebellar nuclei likely receive different patterns of convergent Purkinje cell input than those in the fastigial nucleus. However, due to the greater lateral convergence of Purkinje cells to neurons in the interposed and dentate nuclei, acute sagittal slices may not be conducive to identifying connectivity patterns to those nuclei.

How and when do Purkinje cell - cerebellar nuclear neuron convergence patterns crystallize in development? At present, this process is poorly understood (Kebschull et al., 2023). These synapses become functional in the first week of postnatal life in rodents followed by perineuronal net formation in the following weeks (see Chapter 2 section 2.4.2), but whether multi-zonal connectivity patterns are abundant in early life and later pruned back is unclear. Performing these experiments in juvenile mice would likely be simpler than in adults due to the immature myelination of this region and its lack of perineuronal nets. Granule cell - Purkinje cell connectivity maps have recently been shown to undergo critical period rearrangement in the first few weeks of life that stabilizes around P30 (Spaeth et al., 2022). Perhaps connectivity map reorganization is a common feature of the cerebellar circuit.

Lastly, localized Purkinje cell dysfunction and degeneration in disease (see Chapter 2 section 2.2.3.3) is likely to impact cerebellar nuclear neuron integration of Purkinje cell convergence, and could potentially contribute to disease pathology. Purkinje cell death is almost certain to result in imbalanced connectivity patterns compared to healthy animals. However, Purkinje cell dysfunction often precedes cell death (Márquez et al., 2023). Whether the range of Purkinje cell convergence patterns observed in healthy animals is reduced in disease models before Purkinje cell death occurs is not known. Characterizing how a potentially limited range of convergence patterns at the Purkinje cell - cerebellar nuclear neuron affects behavior would be highly informative.

In vivo studies

If uni-zonal cells act as "specialists," and multi-zonal cells as either "integrators" or "generalists" (see Section 5.2, "Potential roles for different types of fastigial nuclear neurons"), could we expect them to exhibit different physiological properties *in vivo*? For example, perhaps uni-zonal cells exhibit higher firing rates *in vivo* if they potentially receive less convergent inhibitory Purkinje cell input, while multi-zonal cells may tend to have lower firing rates if they typically are subject to more Purkinje cell inhibition. We did not observe differences in spontaneous firing rates between these cells in our *in vitro* preparation (Appendix Fig. A.1), but it would be interesting to see if these cells exhibit different properties in awake, behaving animals.

Although we demonstrated that cerebellar nuclear neurons can receive Purkinje cell input from multiple zones, whether multi-zonal cerebellar nuclear neurons receive input from each connected zone simultaneously *in vivo* is not known. Might different combinations of synchronous convergent Purkinje cell input differentially impact cerebellar nuclear neuron activity and resulting behavior? These are open questions that would be useful to explore to better understand the contribution of cerebellar information transfer in behavior.

Finally, exploring the relationship between Purkinje cell - cerebellar nuclear neuron connectivity and ongoing behavior would be an exciting direction of future research. I hypothesize that uni-zonal cerebellar nuclear neurons may be more active during simple, "unimodal" tasks like the vestibulo-ocular reflex (Suvrathan, Payne, and Raymond, 2016), while multi-zonal neurons may be recruited during more complex tasks that require integration of information from across modalities, such as performing a locomotor task in response to a visual or auditory cue. These experiments could be performed using *in vivo* tools such as Neuropixel probes where, depending on the probe placement, activity from cerebellar nuclear neurons located in different areas of the fastigial nucleus could be recorded. Importantly, identification of the Purkinje cell convergence patterns to these individual cerebellar nuclear neurons may first need to be assessed experimentally. However, since cerebellar nuclear neurons with increasing numbers of connected zones appear to cluster near the midline of the nucleus, positional information of the implanted probe may be a starting point for identifying the cerebellar nuclear neuron contribution to complex, ongoing behavior.

Chapter 6

Conclusion

6.1 Objectives of the research

The main objective of my doctoral thesis work has been to explore how Purkinje cells functionally converge on cerebellar nuclear neurons. In Chapter 3, I demonstrated that our optogenetic stimulation paradigm is a suitable and reliable tool for performing circuit mapping of Purkinje cell - cerebellar nuclear neuron connections. Using this technique, I was able to elicit single action potentials from Purkinje cell axonal stimulation and consistently evoke postsynaptic responses from their cerebellar nuclear neuron targets. In Chapter 4, I used this optogenetic stimulation paradigm to identify functional convergence patterns between Purkinje cells and cerebellar nuclear neurons in the fastigial nucleus. I showed that the Purkinje cell - cerebellar nuclear neuron synapse is an important site of integration in the cerebellum: cerebellar nuclear neurons exhibit a wide variety of input patterns from Purkinje cells, with both "specialist" and "integrator" cells. Thus, cerebellar nuclear neurons are not simply relay stations, but are instead capable of contributing richly to overall cerebellar information processing based on the different kinds of information they integrate and transmit to downstream targets.

6.2 Implications of our findings

The findings described in this thesis have several implications. My PhD work expands our understanding of information transfer in the cerebellum: previously, Purkinje cell convergence on cerebellar nuclear neurons was predominantly explored in the mediolateral axis of the cerebellum using structural labeling techniques. In our study, we described a diversity of convergence patterns in the rostrocaudal axis, orthogonal to the mediolateral axis, which likely contribute to the cerebellum's capacity to participate in a wide range of functions and behaviors. Our results provide the neuroscience field with greater insight into cerebellar computation.

Further, our work characterizes Purkinje cell - cerebellar nuclear neuron connectivity in young, healthy mice. Identifying typical connectivity patterns in healthy brains is imperative for understanding what goes wrong in disease. Our findings highlight the Purkinje cell - cerebellar nuclear neuron synapse as an important locus for cerebellar processing. As such, this locus may be implicated in cerebellar dysfunction associated with disease. Overall, the findings of my doctoral work expand our understanding of cerebellar information processing.

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Appendix A

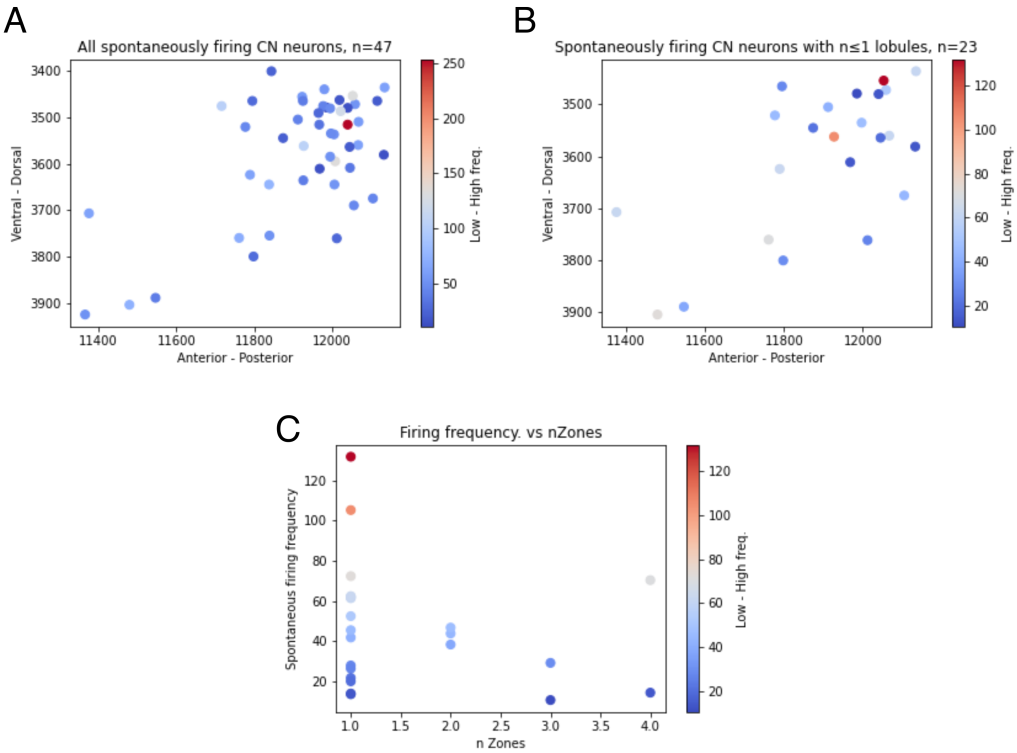

Figure A.1: **Firing rates of spontaneously active cerebellar nuclear neurons.** **A**, Association between cerebellar nuclear neuron location in the sagittal plane and spontaneous firing rate for all recorded spontaneously active cerebellar nuclear neurons. **B**, Association between cerebellar nuclear neuron location in the sagittal plane and spontaneous firing rate for cerebellar nuclear neurons with one or more lobule connections identified. **C**, Association between spontaneous firing rate and number of connected zones for cerebellar nuclear neurons with one or more zone connections identified. Color maps showing range of spontaneous firing frequencies in Hz.

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