Motor and non-motor control by neuronal subpopulations within the deep cerebellum

Low, Aloysius Yeow Teck

2018

Low, A. Y. T. (2018). Motor and non-motor control by neuronal subpopulations within the deep cerebellum. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/88172>

<https://doi.org/10.32657/10220/47424>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**MOTOR AND NON-MOTOR CONTROL BY NEURONAL
SUBPOPULATIONS WITHIN THE DEEP CEREBELLUM**

ALOYSIUS Y. T. LOW

**Interdisciplinary Graduate School
NTU Institute for Health Technologies**

2018

MOTOR AND NON-MOTOR CONTROL BY NEURONAL SUBPOPULATIONS WITHIN THE DEEP CEREBELLUM

LOW YEOW TECK, ALOYSIUS

**Interdisciplinary Graduate School
NTU Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of
Doctor of Philosophy

2018

Inspired by Henri Matisse, snow flowers, 1951, cut outs of the white flowers bear great similarity to silhouettes of sagittal cerebellar sections. Boldly blobbed shades of violet within the flower petals represent the deep cerebellar nuclei. Speckles of luminous green akin to snow drops symbolize a specific sub-population of *Ucn3::Cre* recombined neurons described by Low et al.

Artwork by
Aloysius Low

Authorship Attribution Statement

This thesis contains material from a paper published in the following peer-reviewed journal where I was one of the co-first authors who contributed equally.

Chapter 1 of this thesis is published as Low, A.Y.T., Thanawalla, A.R., Yip, A.K.K., Kim, J., Wong, K.L.L., Tantra, M., Augustine, G.J., and Chen, A.I. (2018). Precision of Discrete and Rhythmic Forelimb Movements Requires a Distinct Neuronal Subpopulation in the Interposed Anterior Nucleus. *Cell reports* 22, 2322-2333. DOI: <https://doi.org/10.1016/j.celrep.2018.02.017>

The contributions of the co-authors are as follows:

A.Y.T.L., A.R.T., A.K.K.Y. and A.I.C. designed the studies and prepared the manuscript with comments from all authors. A.Y.T.L., A.R.T. and A.K.K.Y. contributed equally to all the experiments and analysis of the data, and are listed in alphabetical order. K.L.L.W. and M.T. helped with behavioral analysis. J.K. and G.J.A. performed electrophysiological experiments and analyzed the data.

15th August 2018

.....

Date

.....

Aloysius Y.T. Low

ACKNOWLEDGEMENTS

I would like to express my gratitude to Prof. Albert I. Chen for his mentorship throughout my PhD career. His approachability, availability and genuine interest in developing students into professionals with sophisticated set of skills, sharp and independent minds is greatly appreciated.

I am grateful to my co-supervisors, Prof. George J. Augustine and Prof. Annabel S.H. Chen. Their invaluable role in providing critical guidance, advice and support immensely enriched my research training and helped achieved my goals.

I would like to offer my special thanks to Prof. Nicholas J. Betley for hosting me in his laboratory at the University of Pennsylvania during the fall of 2017. His constructive recommendations, enthusiasm and openness to encourage me to explore new research areas broadened my research perspectives and brought about an extremely fruitful externship experience.

I would also like to thank Prof. Ayumu Tashiro and Prof. Ch'ng Toh Hean for their valuable advice and for generously sharing their expertise and resources.

I would like to thank all my collaborators who have been instrumental for the completion of a major part of my thesis. Alaric K.K. Yip, Ayesha R. Thanawalla and Kelly L.L. Wong for the motor behavior experiments. Alaric K.K. Yip and Victoria Turner for the microarray experiment. Dr. Norliyana Zainolabidin and Victoria Turner for the *in situ* hybridization experiment. Dr. Jinsook Kim from Augustine lab for the electrophysiology recording experiment. Dr. Zhenwei Su from the Betley Lab for the fiber photometry recording experiment.

I wish to thank all present and past Chen Lab members: Drs. Helen L. Guo, Martesa Tantra and Sandhya P. Kamath, and every student that have worked with me: Frances X.E. Jin, Helen S.T. Ho, Javier J.H. Tan, Melissa X.Y. Wong, Q.H. Lee, Sabina Y.S. Tan and Sheryl W.N Tay; for being the best colleagues and friends, creating the most conducive environment to talk about and develop scientific ideas whilst having fun. My grateful thanks are also extended to members of the Betley Lab, Dr. Amber Alhadeff, Elen Hernandez, Michelle Klima and Santiago Pulido for being especially helpful and welcoming.

I would like to acknowledge and thank Singapore National Medical Research Council (0075/2014), Singapore Ministry of Education (RG124/15), NTU – Interdisciplinary Graduate School, Institute for Health Technologies, NTU – School of Biological Sciences, University of Warwick (Warwick-NTU Neuroscience Programme), Imperial College-NTU – Lee Kong Chian School of Medicine, NTU – School of Social Sciences College of Humanities, Arts & Social Sciences, and University of Pennsylvania – School of Arts and Sciences, for providing funding and resources for my research.

Finally, and most importantly, I wish to thank my family, Margaret and friends for the love and support given to me during this journey.

PUBLICATION LIST

1. Low, A.Y.T., Thanawalla, A.R., Yip, A.K.K., Kim, J., Wong, K.L.L., Tantra, M., Augustine, G.J., and Chen, A.I. (2018). Precision of Discrete and Rhythmic Forelimb Movements Requires a Distinct Neuronal Subpopulation in the Interposed Anterior Nucleus. *Cell reports* 22, 2322-2333.

TABLE OF CONTENTS

LIST OF FIGURES	i
LIST OF APPENDIXES	iii
ABBREVIATIONS	iv
ABSTRACT	vi
1. INTRODUCTION	1
1.1 An overview of the organization and function of the cerebellum	1
1.1.1 The local circuitry within the cerebellum	2
1.1.2 Distinctions between different sub-nuclei within the deep cerebellum	4
1.1.3 Major neuronal subtypes within the cerebellar nuclei	6
2. CHAPTER 1	8
2.1 INTRODUCTION	9
2.1.1 Role of the cerebellum in movement and motor control	9
2.1.1.1 The organization and connectivity of the cerebellar nuclei in motor function	9
2.1.1.2 Functional significance of the cerebellar nuclei in movement and motor control	13
2.2 HYPOTHESIS	21
2.3 MATERIALS AND METHODS	23
2.3.1 Animals	23
2.3.2 Microarray	23
2.3.3 Histological procedures	25
2.3.4 Immunohistochemistry	26
2.3.5 <i>In situ</i> hybridization	27
2.3.6 Electrophysiology	29
2.3.7 Tract tracing	30
2.3.8 Diphtheria toxin receptor mediated ablation	32
2.3.9 Fiber implantation	32
2.3.10 Optogenetics	33
2.3.11 Open field	34
2.3.12 Rotarod	34
2.3.13 Freely walking task	34
2.3.14 Skilled reaching task	35
2.3.15 Quantification of the distribution of recombined cell in double transgenic mice	37
2.3.16 Quantification of nuclear protein expression	37
2.3.17 Statistical analysis	38
2.4. RESULTS	40
2.4.1 Role of the cerebellar nuclei role in motor control	40
2.4.1.1 Molecular distinctions between cerebellar nuclei	40

2.4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei	44
2.4.1.3 Recombination pattern of <i>Ucn3::Cre</i> in the developing mouse brain	51
2.4.1.4 Molecular identity of IntA ^{Ucn3} neurons in the cerebellar nuclei	53
2.4.1.5 Electrophysiological properties of <i>Ucn3::Cre</i> neurons in the interposed nucleus	58
2.4.1.6 Intra- and extra-cerebellar projections of <i>Ucn3::Cre</i> neurons in the interposed anterior nucleus....	61
2.4.1.7 Ablation of IntA ^{Ucn3} neurons perturbs the accuracy of skilled forelimb reaching	76
2.4.1.8 Consequences of the ablation of IntA ^{Ucn3} neurons on rhythmic forelimb movement	80
2.5. DISCUSSION.....	88
2.5.1 Accessibility to the cerebellar interposed nucleus with the <i>Ucn3::Cre</i> mouse line	88
2.5.2 Functional significance of the intra-cerebellar connectivity of neurons in the IntA.....	89
2.5.3 Functional significance of the extra-cerebellar connectivity of neurons in the IntA	91
2.5.4 Role of the IntA in coordination of voluntary limb movement	92
2.6. FUTURE DIRECTIONS	96
2.6.1 Anatomical, functional and molecular diversity of neuronal subpopulations in the cerebellar nuclei for motor control.....	96
3. CHAPTER 2.....	98
3.1 INTRODUCTION	99
3.1.1 Role of the cerebellar nuclei in non-motor control.....	99
3.1.1.1 The organization and connectivity of the cerebellar nuclei for control of feeding	102
3.1.1.2 Functional significance of the cerebellar nuclei in food intake	108
3.2 HYPOTHESIS.....	114
3.3 MATERIALS AND METHODS	115
3.3.1 Animals	115
3.3.2 Histological procedures	115
3.3.3 Immunohistochemistry	115
3.3.4 Tract tracing.....	115
3.3.5 Designer Receptors Exclusively Activated by Designer Drugs	115
3.3.6 Fiber photometry.....	116
3.3.7 Freely moving feeding assay	118
3.3.8 Statistical analysis	119
3.4 RESULTS	120
3.4.1 Behavioral impact of manipulating neurons in the interposed nucleus on food intake.....	120
3.4.2 Identification of novel nodes linking the cerebellum and hypothalamus.....	124
3.4.2.1 Identification of direct and indirect targets that link the cerebellar nuclei to known feeding centers: trans-synaptic tracing.....	124
3.4.2.2 Defining the connectivity between the interposed nucleus and the arcuate nucleus of the hypothalamus: antero- and retrograde tracing	128
3.4.3 Cerebellar output neurons are functionally linked to AgRP neurons in the arcuate nucleus.....	132

3.5 DISCUSSION.....	136
3.5.1 Multiple pathways linking the cerebellum to key feeding centers.....	136
3.5.2 Role of the cerebellar interposed nucleus in feeding related behaviors	138
3.6 FUTURE DIRECTIONS	141
3.6.1 Interrogation of the role of the cerebellar nuclei in feeding behavior.....	141
4. REFERENCE	143
APPENDIX	160

LIST OF FIGURES

Figure 1. Diagram of the cerebellar circuitry	3
Figure 2. A schematic of the cerebellum	5
Figure 3. A schematic of the olivo-cortico-nuclear modules	11
Figure 4. A schematic of the three major cerebellar nuclei nucleofugal efferents	12
Figure 5. Molecular profiling of the individual regions within the cerebellum	41
Figure 6. Recombination patterns and molecular expression of <i>Parvalbumin::Cre; Rosa::lsl.ChR2-eYFP</i>	46
Figure 7. Recombination patterns and molecular expression of <i>GlyT2::Cre; Rosa::lsl.ChR2-eYFP</i>	47
Figure 8. Recombination patterns and molecular expression of <i>Pcp2::Cre; Rosa::lsl.ChR2-eYFP</i>	49
Figure 9. Selective genetic targeting of neurons in the IntA	50
Figure 10. Identification of a distinct sub-population of glutamatergic neurons in the IntA	52
Figure 11. Differential expression levels of TBR1	54
Figure 12. Expression of endogenous <i>Ucn3</i>	55
Figure 13. Localized c-FOS immunoreaction within the nucleus of cells	57
Figure 14. Molecular and electrophysiological properties of IntA ^{Ucn3} neurons	60
Figure 15. Targeting and labeling IntA neurons to distinguish between two potentially distinct sub-populations	62
Figure 16. IntA neurons give rise to NC-MFs	64
Figure 17. IntA ^{Ucn3} neurons give rise to nucleocortical mossy fibers restricted to the anterior forelimb associated region	66
Figure 18. Genetic and viral tracing reveal extracerebellar regions targeted by IntA ^{Ucn3} neurons	68
Figure 19. Genetic and viral tracing reveal extracerebellar regions targeted by IntA ^{Ucn3} neurons	70
Figure 20. Cre expressing puncta within the VA-VL is distinctively derived from IntA	72
Figure 21. IntA ^{Ucn3} neurons constitute a specific element of the cerebello-thalamo-cortical network	74
Figure 22. Vestibular neurons give rise to vestibulospinal projection and vestibulocerebellum mossy fiber in the cerebellar vermis	75
Figure 23. Ablation of IntA ^{Ucn3} neurons disrupts the accuracy of forelimb-skilled reaching	77
Figure 24. Ablation of IntA ^{Ucn3} does not perturb the x-axis of strides in freely walking mice	81
Figure 25. Manipulation of IntA ^{Ucn3} perturbs specific phases of forelimb trajectory in freely walking mice	85

Figure 26. A schematic of the hypothalamo-cerebellar and cerebello-hypothalamic pathways	104
Figure 27. Schematic of hypothetical indirect connections between the hypothalamus and the cerebellum	106
Figure 28. Subpopulations of neurons in the cerebellar interposed nucleus neurons regulate food intake	121
Figure 29. Transsynaptic tracing reveals efficient uptake of WGA-Cre at the ZIV and InG	125
Figure 30. Transsynaptic tracing reveals connectivity between the cerebellar interposed nucleus and the hypothalamus	127
Figure 31. Cerebellar interposed anterior nucleus does not contribute to direct cerebello-hypothalamic pathway	130
Figure 32. Cerebellar interposed nucleus contributes to the cerebello-zona incerta-hypothalamic pathway	132
Figure 33. Activation of the cerebellar nuclei via hM3D decreases calcium activity of AgRP neurons in fasted state	134

LIST OF APPENDIXES

Appendix A. Cre and reporter mouse lines	160
Appendix B. List of genes that are distinctively expressed in the cerebellar nuclei within the cerebellum	161
Appendix C. Expression patterns of recombined cells in six individual double-transgenic mouse lines and a reporter mouse	163
Appendix D. Expression patterns of recombined cells at three different postnatal ages	164
Appendix E. Analysis of the expression of glutamatergic molecular markers in YFP ⁺ neurons in the cerebellar nuclei	165
Appendix F. Electrophysiological and morphological properties of neurons in the IntA of <i>Ucn3::Cre; Rosa::ChR2-eYFP</i> mice	166
Appendix G. Analysis of the transduction efficiency of AAV2- <i>hSyn::mCherry</i> in <i>Ucn3::Cre; Rosa::ChR2</i> mice	167
Appendix H. Analysis of the percentage distribution of nucleocortical mossy fibers in each cerebellar associated limb region	168
Appendix I. Analysis of the distribution of labeled puncta across all cerebellar-cerebrum traced regions in <i>Ucn3::Cre; Rosa::ChR2</i> mice injected with AAV2- <i>hSyn::mCherry</i> at the IntA	169
Appendix J. Locomotion parameters of the ipsilateral forelimb in freely moving mice	170
Appendix K. Behavioral consequences of manipulating the cerebellar interposed nucleus using DREADD	171

ABBREVIATIONS

AgRP	Agouti-related peptide
Arc	Arcuate nucleus
BAC	Bacteria artificial chromosome
C1QL3	Complement component 1, q subcomponent-like
CALB1	Calbindin
CFA	Caudal forelimb area
ChR2	Channelrhodopsin2
CNO	Clozapine-N-oxide
CRF-R2	Corticotropin-releasing factor receptor type-2
CRH	Corticotropin-releasing hormone
CTB	Cholera toxin subunit B
DA	Dorsal hypothalamic area
DM	Dorsomedial hypothalamic nucleus
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
DTR	Diphtheria toxin receptor
EF1a	Eukaryotic translation elongation factor 1 alpha
FG	Fluoro-Gold
GABA	Gamma aminobutyric acid
GAD+	Mixed GABAergic/glycinergic interneuron
GAD67	Glutamic acid decarboxylase 67
GADnL	Putatively glutamatergic projection neuron
GADnS	Putatively glutamatergic interneuron
Gly-F	Glycinergic projection neuron
GlyT2	Glycine transporter 2
hM3D	human M3 muscarinic receptor
hM4D	human M4 muscarinic receptor
IGL	Internal granular layer
iNC	Inhibitory nucleo-cortical GABAergic/glycinergic projection neuron
InG	Intermediate gray region of the superior colliculus
IntA	Interposed anterior nucleus of the cerebellum
IntA ^{Ucn3}	Interposed anterior neurons targeted by <i>Ucn3::Cre</i> mouse
IntDL	Interposed dorsolateral
IntP	Interposed posterior
IntPPC	Interposed posterior parvicellular
IOD	Dorsal nucleus of the inferior olivary complex
Lat	Lateral
LatPC	Lateral parvicellular
LH	Lateral hypothalamus
LRt	lateral reticular nucleus
M1	Primary motor cortex
Med	Medial
MedDL	Medial dorsolateral
MedL	Medial lateral
ML	Molecular layer
MLI	Molecular layer interneuron
NO	Nucleo-olivary GABAergic projection neuron
P	Postnatal days

PCL	Purkinje cell layer
PCP2	Purkinje cell protein 2
PH	Posterior hypothalamic area
POMC	Opiomelanocortin
Pv	Parvalbumin
PV	Paraventricular nucleus
RMC	Magnocellular part of the red nucleus
RT-PCR	Real time-polymerase chain reaction
SMI32	Anti-neurofilament H non-phosphorylated
TBR1	T-box brain 1
Tfap2	Activating enhancer binding protein 2 alpha
TM	Tuberomammillary nucleus
Ucn	Urocortin
Ucn3	Urocortin 3
vGluT2	Vesicular glutamate transporter 2
VM	Ventromedial thalamic nucleus
YFP ^{OFF}	Non-IntA ^{Ucn3} neurons
YFP ^{ON}	YFP+ IntA ^{Ucn3} neurons
ZI	Zona incerta
ZIV	Zona incerta ventral

ABSTRACT

Motor and non-motor control by neuronal subpopulations within the deep cerebellum

The cerebellum is linked to a multitude of regions in the brain and spinal cord, projecting to at least twenty-seven brain regions, to control a diverse array of motor and non-motor processes. Embedded within lies the cerebellar nuclei which receive, integrate and transmit diverse sensorimotor information to relevant brain regions. However, the functional relevance of identifiable neuronal subpopulations within the cerebellar nuclei remains unclear. To explore the mechanisms underlying the function of the cerebellar nuclei, we examined a subset of genetically tractable neurons in the mouse cerebellar interposed nucleus, and studied the involvement of these neurons in discrete and rhythmic limb movements, and in feeding-related tasks.

Analysis of the connectivity and function of these neurons through tract tracing and optogenetic experiments revealed that a subset of glutamatergic neurons in the anterior cerebellar interposed nucleus can be labeled with the *Urocortin 3::Cre* mouse line, and that these neurons constitute a specific element of an internal feedback circuit within the cerebellum and cerebello-thalamo-cortical pathway associated with limb control. Additionally, through trans-synaptic and anterograde/retrograde tracing analysis, we found that neurons in the interposed nucleus also belong to the cerebello-subthalamic-hypothalamic pathway which may be involved in non-motor processes. Chemogenetic manipulation of neurons in the interposed nucleus revealed that the activity of neurons in the arcuate nucleus and feeding behavior also depend on neurons in the interposed nucleus.

In summary, we show that target selectivity may be a key distinguishing property among neuronal subpopulations in the cerebellar nuclei, and illustrate the diversity and

complexity of intra- and extra-cerebellar connections made by neurons in the cerebellar nuclei. In agreement with the notion that the cerebellum modulates both motor and non-motor functions, our study links different subpopulations of glutamatergic neurons in the interposed nucleus and their unique circuitry with coordination of precise and effective movements, as well as mediation of feeding-related response.

1. INTRODUCTION

1.1 An overview of the organization and function of the cerebellum

The focus of this thesis is to define the function of the deep cerebellar nuclei in controlling motor and non-motor behaviors. Before describing details about the organization and function of the deep cerebellum, I will first introduce our current understanding of the distinctions and local circuitry of different subtypes of neurons in the cerebellum, and how the cerebellum is linked to extra-cerebellar brain regions through output neurons in the cerebellar nuclei. Although the general intra- and extra-cerebellar connectivity within the cerebellum and the function of the cerebellum are emerging, the lack of identifiable and accessible cerebellar neuronal subtypes limits our ability to assess how specific cerebellar-mediated pathways control movement and innate activities. Therefore, this thesis aims to investigate the role of output neurons in the deep cerebellar nuclei and associated circuit elements in order to better understand how the cerebellum controls discrete and rhythmic movements and feeding related behaviors.

The cerebellar cortex consists of a laminated geometric structure with multiple wave-like folds from the anterior to the posterior producing cerebellar lobules (Altman and Bayer, 1997). Broadly, the cerebellum can be modularly divided into the vermal, paravermal and hemispheric regions (Stoodley and Schmahmann, 2018). Respectively, these regions are located closer to the midline and farther towards the lateral regions, with a functional topography of limbic, sensorimotor and cognitive regions (Stoodley and Schmahmann, 2018). The cerebellum connects to extra-cerebellar regions via three types of cerebellar peduncles (Stoodley and Schmahmann, 2018). The superior cerebellar peduncle is made up of efferent projections from the cerebellum to the cerebral cortex and in turn, afferent

projections from the cerebral cortex are relayed through the pontine nuclei and the middle cerebellar peduncle to the cerebellum (Stoodley and Schmahmann, 2018). The inferior cerebellar peduncle carries afferents and efferents of the cerebellum to the spinal cord, as well as cerebellar afferents from the inferior olive and vestibular system (Stoodley and Schmahmann, 2018). Despite detailed studies of the anatomical and topographical organization within the cerebellum (Apps and Hawkes, 2009; Uusisaari and De Schutter, 2011) (Figure 1), how the integrated information in the cerebellum is transmitted to the other brain regions as well as the spinal cord remains unclear. Moreover, whether distinct neuronal subpopulations project to different extra-cerebellar targets is not known.

1.1.1 The local circuitry within the cerebellum

The cerebellum consists of two major subdivisions – 1) the cerebellar cortex, an organized layered structure which sandwiches a sheet of Purkinje cells between the molecular layer (ML) and internal granular layer (IGL), and 2) the cerebellar nuclei (Voogd and Glickstein, 1998) (Figure 1). Within the IGL, excitatory mossy fibers arising from peripheral nerves, spinal cord, and brainstem terminate onto dendrites of granule cells and Golgi cells (Chadderton et al., 2004; Eccles et al., 1967). In addition to mossy fiber input, granule cells receive inhibitory input from Golgi cell axon terminals (Ito, 2006). Granule cells in turn extend axons into the ML and bifurcate into parallel fibers (Pichitpornchai et al., 1994). In rodents, parallel fibers form excitatory synaptic contacts with more than half of the Purkinje cells they pass through (Barbour, 1993). Besides parallel fiber inputs, Purkinje cells receive excitatory climbing fiber impulses arising from the inferior olive (Nieto-bona et al., 1997), and inhibitory basket cells and stellate cells inputs (Ito, 2006). Together, these studies have shown that a great deal of signal processing takes place within the cerebellar cortex

before ultimately converging onto the sole output neuronal subtype in the cerebellar cortex, the Purkinje cells (Heiney et al., 2014; Voogd and Glickstein, 1998). The majority of the axons of Purkinje cells terminate within the cerebellar nuclei, except for a small subset of Purkinje cells residing in the cerebellar flocculus which terminates in the vestibular nuclei (De Zeeuw and Berrebi, 1995; Dezeuw et al., 1994). As such, there is an additional convergence of cerebellar efferents onto the cerebellar nuclei, supporting the notion that the cerebellar nuclei are the ultimate output structures of the cerebellum (Ito, 1984).

Figure 1. Diagram of the cerebellar circuitry. The cerebellar cortex consists of three layers: the molecular layer, Purkinje cell layer and internal granule layer. Both the cerebellar cortex and the cerebellar nuclei receives input from two excitatory pathways, first are the mossy fibers projections and the climbing fibers that originated

from the inferior olivary nucleus. Most synapses formed in the cerebellar nuclei are by Purkinje cell axon terminals. The cerebellar outputs roughly consist of the inhibitory nucleo-olivary projection, and the excitatory efferents projecting to the thalamus and brainstem. The cerebellar nuclei in this schematic is drawn without differentiating the three sub nuclei namely, lateral, interposed and medial nuclei. There are six currently known distinct neurons within the cerebellar nuclei: large glutamatergic projection neurons (GADnL projection neuron), small gamma aminobutyric acid (GABA) -ergic projection neurons connected to the IO (NO projection neuron), medium-sized GABAergic glycinergic projection neurons projecting into the cerebellar cortex (iNC projection neuron), glycinergic neuron found only in the medial nucleus projecting into the vestibular nuclei (VN) (Gly-F projection neuron) and two interneuronal subtypes which are GABAergic (GAD+ interneuron) and non-GABAergic, putatively glutamatergic (GADnS interneuron) (Uusisaari and De Schutter, 2011).

Positioned between the cerebellar cortex and the rest of the brain and spinal cord, the cerebellar nuclei represent crucial nodes for mediating cerebellar signal transmissions. In order to begin to dissect the functional relevance of cerebellar nuclear neurons in motor and non-motor processes, it is crucial to first define the molecular, anatomical and functional distinctions between neuronal subpopulations in the cerebellar nuclei.

1.1.2 Distinctions between different sub-nuclei within the deep cerebellum

The cerebellar nuclei in the mouse are conventionally divided into three nuclei – the lateral, interposed and medial, each of the nucleus is geographically situated at the hemisphere, paravermis and vermis, respectively (Apps and Garwicz, 2005; Voogd et al., 1981) (Figure 2). Whereas, in humans, the cerebellar nuclei consist of four nuclei: the dentate, emboliform and globose, and fastigial, with the interposed-equivalent nucleus subdivided into emboliform and globose (Elsen et al., 2013). The three major cerebellar nuclei in the mouse are sub-divided into nine intranuclear subdivisions based on histology,

and include the Lat-lateral, LatPC-lateral parvicellular, IntDL-interposed dorsolateral, IntA-interposed anterior, IntP-interposed posterior, IntPPC-interposed posterior parvicellular, MedDL-medial dorsolateral, Med-medial, and MedL-medial lateral (Paxinos and Franklin, 2007) (Figure 2). Due to a lack of laminar organization, the cellular topography within the cerebellar nuclei is seemingly disorganized.

Figure 2. A schematic of the cerebellum. The cerebellum consists of the three major nuclei and nine subnuclei in the cerebellar nuclei, based on anatomical classification (Low et al., 2018; Paxinos and Franklin, 2007).

To date, two important studies have provided non-histological strategies to delineate the boundaries of the distinct sub-nuclei within the cerebellar nuclei. First, a study using tract tracing and immunohistochemistry in rats proposed that the cerebellar nuclei can be topographically divided into five functional groups based on aldolase C expression in Purkinje cell terminals which are closely correlated to the patterns of olivocerebellar climbing fiber projections (Sugihara and Shinoda, 2007). Second, a study using antibodies T-

box brain-1 (TBR1), human natural killer cell antigen 1, and phospholipase C β 4 antigens on mouse brain slices of three genetically labeled mouse lines expressing reporter markers under the glycine transporter 2 (GlyT2)-, Thy-1 Cell Surface Antigen- and early B-cell factor – promoter proposed that the cerebellar nuclei can be topographically divided into 12 expression domains within the cerebellar nuclei (Chung et al., 2009). However, the identification of these subnuclear regions requires the use of a combination of many antibodies, most of which are not commercially available, and multiple transgenic mouse lines as well as anterograde anatomical tract tracing. Thus, there is a need to develop a simpler and more effective characterization scheme for a systematic delineation of the different subdivision of the cerebellar nuclei using tools that are more accessible and reproducible.

1.1.3 Major neuronal subtypes within the cerebellar nuclei

Although the organization and connectivity of neuronal subpopulations within the cerebellar cortex are well-defined (Lainé and Axelrad, 1994; Mugnaini et al., 1997; Sillitoe and Joyner, 2007; Voogd and Glickstein, 1998), the organization and connectivity of neuronal subpopulations in the cerebellar nuclei remain limited, for the most part, to the excitatory projection neurons and inhibitory interneurons (Chen and Hillman, 1993; De Zeeuw and Berrebi, 1995; Teune et al., 1998). In order to bridge this gap, different tools and strategies have been employed in several recent studies. A series of elegant studies took advantage of mice with genetically targeted fluorescent proteins and electrophysiology (Lainé and Axelrad, 1994; Mugnaini et al., 1997; Sillitoe and Joyner, 2007; Voogd and Glickstein, 1998). Together, results from these studies have identified up to seven distinct neuronal subpopulations; five in the lateral nucleus, one in the interposed nucleus, and one in

the medial nucleus (Ankri et al., 2015; Bagnall et al., 2009; Gao et al., 2016; Uusisaari and Knopfel, 2012).

To identify distinct populations of neurons in the cerebellar nuclei, these groups employed electrophysiological, morphological, as well as molecular strategies (Ankri et al., 2015; Bagnall et al., 2009; Gao et al., 2016; Uusisaari and Knopfel, 2012). The seven identified neuronal subtypes include – GAD+, mixed gamma aminobutyric acid (GABA) - ergic/glycinergic interneuron; GADnS, putatively glutamatergic interneuron; NO, nucleoolivary GABAergic projection neuron; iNC, inhibitory nucleo-cortical GABAergic/glycinergic projection neuron which projects to Golgi cells (GC) expressing high GABA but low glycine in the IGL; Gly-F, glycinergic projection neuron found exclusively in the medial nucleus projecting into the vestibular nuclei; GADnL, putatively glutamatergic projection neuron with nucleocortical projecting subtypes and non-nucleocortical projecting subtypes (Ankri et al., 2015; Bagnall et al., 2009; Gao et al., 2016; Uusisaari and Knopfel, 2012) (Figure 1). The characterization of these seven neuronal subtypes relied on mainly two methods. First, neurons were divided into two large populations according to molecular expressions via transgenic fluorescent labeling. Second, neuronal electrophysiological properties such as spontaneous firing frequency and action potential shape were recorded for further characterization. Taken together, results from these two methods revealed morphological, electrophysiological and molecular properties of cerebellar nuclear neurons and provided important groundwork for future analysis of neurons in the cerebellar nuclei. Despite advances in our understanding of the distinctions within the cerebellar nuclei, the lack of genetic strategies to specifically monitor and manipulate a specific neuronal subpopulation within a heterogeneous group of cerebellar nuclear neurons.

CHAPTER 1:

MOTOR CONTROL BY NEURONAL SUBPOPULATIONS WITHIN THE DEEP CEREBELLUM

Reprinted (and adapted) with permission from

Low, A.Y.T., Thanawalla, A.R., Yip, A.K.K., Kim, J., Wong, K.L.L., Tantra, M., Augustine, G.J., and Chen, A.I. (2018). “Precision of Discrete and Rhythmic Forelimb Movements Requires a Distinct Neuronal Subpopulation in the Interposed Anterior Nucleus.” Cell reports Volume 22, Issue 9, ISSN: 2322-2333. Copyright Elsevier Limited (2018).

2. CHAPTER 1

2.1 INTRODUCTION

2.1.1 Role of the cerebellum in movement and motor control

There is a substantial amount of clinical and experimental evidence that have revealed the significance and functional relationship between the cerebellum and cerebral cortex in the influence of motor function (Eccles et al., 1967; Ito, 2006). To execute a movement, the cerebellum receives sensorimotor inputs from peripheral and internal sources via mossy and climbing fiber input, and integrates this information to provide relevant efferent output via the cerebellar nuclei to extra-cerebellar brain regions (Eccles et al., 1967; Ito, 2006). While the cerebral cortex is the primary executioner of voluntary movements, the main outflow of the cerebral cortex contains only a small percentage of corticospinal neurons. Approximately, 97% of all cerebral cortical neurons do not project to the spinal cord, and instead they collateralize within the brainstem nuclei (Allen and Tsukahara, 1974). Notably, corticobulbar fibers innervate several major regions in the brainstem, including the pontine nuclei, inferior olive and lateral reticular nucleus (LRt), that in turn provide input signals to the cerebellum (Leergaard and Bjaalie, 2007; Valle et al., 2001). Thus, this strong cerebrocerebellar feedback loop exemplifies the importance of understanding how the cerebellum processes a range of motor and sensory information from internal and external sources and conveying the integrated information to relevant brain regions.

2.1.1.1 The organization and connectivity of the cerebellar nuclei in motor function

The afferent and efferent connectivity of the cerebellar nuclei place them in a key position to decipher integrated sensory signals from the cerebellar cortex and relay this information to relevant brain regions. In view of the olivo-cortico-nuclear connections, the

cerebellar cortex is topographically organized into longitudinal zones of distinct Purkinje cells (Apps and Garwicz, 2005; Voogd, 2004). Correspondingly, the cerebellar nuclei receive axonal input according to the longitudinal zones of Purkinje cells (Voogd and Glickstein, 1998). The cerebellar lateral nucleus, interposed nucleus and medial nucleus receive projections from the olivo-cortico modules of principal olive to cortical D-zone of the cerebellar cortex, rostral parts of dorsal and medial accessory olive to the C- and Y- zones of the cerebellar cortex, and caudal part of the medial accessory olive to A zone of the cerebellar cortex (Voogd and Glickstein, 1998) (Figure 3). Together, the olivo-cortico-nuclear modules have been proposed to determine putative functional relevance of the cerebellar sub-nuclei based on specific input-output relationship. Despite these advances, in order to better understand the organization and function of the cerebellar nuclei, a systematic evaluation and delineation of the functional significance of these broadly organized modules need to be carried out.

A combination of molecular markers in the cerebellar cortex and the presumptive olivo-cortico-nuclear modules have provided additional insight into cerebellar nuclei organization. For instance, the selective and distinct expression pattern of Zebrin, an enzyme expressed by subpopulations of Purkinje cells, has been shown to correlate with the topographical modules defined by olivo-cortico-nuclear connections (Hawkes and Leclerc, 1987). Based on Zebrin staining, individual modules can be further compartmentalized into alternating Zebrin-positive and Zebrin-negative bands (Hawkes and Leclerc, 1987). In general, zebrin-negative zones have been shown to connect with brain regions associated with motor control whereas zebrin-positive zones connect to various cerebral cortical areas thought to be non-motor associated (Stoodley and Schmahmann, 2009; Voogd, 2014). Thus,

the anatomical, molecular and functional organization of the cerebellar cortical Purkinje cell zones and Zebrin expression bands provides an opportunity for additional analysis of the organization and function of distinct regions of the cerebellar nuclei.

Figure 3. A schematic of the olivo-cortico-nuclear modules. The three major topographical organization of the olivo-cortico-nuclear pathways labeled green, red and blue (Paxinos and Franklin, 2007; Voogd, 2014).

Nucleofugal efferents of the cerebellar nuclei consist of GABAergic inhibitory neurons and the glutamatergic excitatory projection neurons (Chan-Palay, 1977; Tolbert et al., 1978). Inhibitory projection neurons, namely the nucleo-olivary GABAergic projection neurons, exclusively target the inferior olive (Bengtsson and Hesslow, 2006; Tolbert et al., 1976). In turn, inferior olive excitatory neurons project to the Purkinje cells to modulate their activity (Ito, 2001), or relay motor associated signals to control motor performance (Welsh et al., 1995). In contrast, glutamatergic projection neurons have been shown to innervate a wide

array of extra-cerebellar regions (Schwarz and Schmitz, 1997) (Figure 4). By no means an exhaustive list, glutamatergic projection neurons target the red nucleus, vestibular nuclei, superior colliculus, reticular formation and the ventral lateral thalamic nuclei (Teune et al., 2000) (Figure 4). Despite the identification of key brain regions linked to cerebellar nuclear output neurons, the lack of genetic tools has precluded mapping of the precise connectivity pattern of specific subsets of cerebellar nuclear neurons.

Figure 4. A schematic of the three major cerebellar nuclei nucleofugal efferents (Teune et al., 2000).

2.1.1.2 Functional significance of the cerebellar nuclei in movement and motor control

The role of the cerebellum in the context of motor control is well-established given that cerebellar lesions result in movement deficits (Subramony, 1994; Thach et al., 1992). Moreover, the increase in activity of cerebellar neurons during motor tasks such as voluntary forelimb and hindlimb movement, and motor learning-tasks has been demonstrated by electrophysiological recordings (Keifer and Houk, 1994; Ojakangas and Ebner, 1992, 1994) and by functional imaging in humans (Ellerman et al., 1994). The extensive involvement of the cerebellum in coordinating movement has raised numerous hypotheses regarding how cerebellar neurons are organized to engage specific functional context such as cerebellar motor learning (Gilbert and Thach, 1977; Ito, 2002; Marr, 1969), error detection and correction (Ito, 2013), movement timing (Keele and Ivry, 1990; O'Reilly et al., 2008).

A number of electrophysiology recording combined with tract tracing studies have demonstrated that the cerebellar nuclei receive direct afferent inputs from extracerebellar regions (Beitzel et al., 2017; Luo and Sugihara, 2014; Shinoda et al., 1997; Wu et al., 1999), and is a source of nucleocortical mossy fiber to the cerebellar cortex (Ankri et al., 2015; Gao et al., 2016; Houck and Person, 2015). The sources of direct axonal terminations within the cerebellar nuclei have been shown to arise from regions receiving motor cortical innervations such as the magnocellular part of the red nucleus (RMC) (Beitzel et al., 2017), pontine nucleus (Shinoda et al., 1997), LRt (Wu et al., 1999), and the caudal raphe interpositus area of the median reticular formation (Luo and Sugihara, 2014). In addition to receiving afferent inputs, in vitro electrophysiological studies have demonstrated that both glutamatergic (Gao et al., 2016) and GABA/glycinergic (Ankri et al., 2015) cerebellar nuclear neurons send nucleocortical mossy fiber projections onto cerebellar cortical neurons. Furthermore, a

subpopulation of cerebellar nucleocortical neurons collateralize with the ventrolateral thalamus and the red nucleus (Houck and Person, 2015). Taken together, these studies indicate that the cerebellar nuclei represent a key component of multiple close-loop circuits, which have been thought to be critical for finalizing accurate output patterns (Ahissar and Kleinfeld, 2003; Kelly and Strick, 2003; McCormick et al., 2015; Shepherd, 2013; Strick et al., 2009); and is potentially an information processor independent of the cerebellar cortex.

Lateral nucleus

The involvement of the cerebellar lateral nucleus in motor functions has been extensively studied and well-recognized (Asanuma et al., 1983; Hoover and Strick, 1999). Inactivation of the cerebellar lateral nucleus leads to ataxia, a phenotype characterized by disrupted fine motor coordination (Vilis and Hore, 1977). The cerebellar lateral nucleus influences control of movement through the primary motor cortex (M1) (Hoover and Strick, 1999). This connection between the cerebellum and the motor cortex is linked by the thalamus, which projects to specific cerebral cortical areas (Asanuma et al., 1983; Hoover and Strick, 1999). Distinct regions of the M1 cortex are targeted by neurons from specific regions of the cerebellar lateral nucleus (Dum and Strick, 2003). These pathways were identified by injecting HSV1, Herpes Simplex Virus 1, into different regions of the M1 cortex corresponding to arm, face and leg representations (Dum and Strick, 2003). Because HSV1 is capable of retrograde and trans-synaptic labeling (Clower et al., 2001), injection of HSV1 in the M1 resulted in retrogradely transduced neurons in the mid-rostrocaudal, caudal and dorsal portions of the cerebellar lateral nucleus (Dum and Strick, 2003). Together, these results revealed the existence of several spatially segregated neuronal populations organized

in a rostral-to-caudal manner in the cerebellar lateral nucleus that may influencing specific body movement.

Interposed nucleus

The role of the intermediate cerebellum in control of motor functions has been well described (Ekerot et al., 1997). With respect to the olivo-cortico-nuclear organization, the cerebellar interposed nucleus receives converging projections from motor function associated zebrin-negative C1, C3 and Y zones of the cerebellar cortex (Apps and Garwicz, 2000). Integrated signals in the cerebellar interposed nucleus is transmitted primarily to the RMC (Beitzel et al., 2017), and the anterior-lateral thalamic nuclear complex (Aumann et al., 1994; Kuramoto et al., 2011). Through the thalamocortical pathways, anterior-lateral thalamic neurons provide abundant afferents to several target fields in the cerebral cortical areas, including primary motor cortex, secondary motor cortex, forelimb area, hindlimb area and primary somatosensory area (Jörntell and Ekerot, 1999; Kievit and Kuypers, 1977; Kuramoto et al., 2009). In turn, corticospinal neurons either project directly onto motor neurons or interneurons within the spinal cord to mediate movement (Bortoff and Strick, 1993), whereas a population of cerebral cortical neurons collateralizes with magnocellular nucleus via pyramidal tract (Akintunde and Buxton, 1992). In primates, anterograde tract tracing studies have shown that both corticospinal and rubrospinal neurons terminate within cervical eight to thoracic one segments of the spinal cord, suggesting that they play a role in controlling forelimb muscles (McCurdy et al., 1987). While these anatomical results revealed the neural substrates that might permit the cerebellar nuclei to control motor movement, it remains unknown through which exact pathway the cerebellar nuclei employ to control specific parameters of movement.

The role of the cerebellar interposed nucleus in movement and motor learning has been assessed primarily using eyeblink classical conditioning, an experimental paradigm that examines the mechanism of associative memory (Aksenov et al., 2004). Unconditioned and conditioned stimuli consisting of auditory, visual and somatosensory signals are transmitted to the cerebellum via the mossy fibers of the pontine nuclei and climbing fibers derived from the dorsal accessory inferior olive (Mihailoff, 1993). Unconditioned and conditioned responses elicited by these stimuli are measurable by eyeblink movement (Disterhoft et al., 1985). When an unconditioned stimulus is introduced, the pontine mossy fibers send excitatory inputs to the cerebellar interposed nucleus, in turn, innervates the red nucleus to cause an eyeblink (Disterhoft et al., 1985). While conditioned stimulus does not excite the cerebellar interposed nucleus, an association between conditioned and unconditioned stimuli decreases climbing fiber excitation on Purkinje cells (Perciavalle et al., 2013; Sánchez-Campusano et al., 2011; Sánchez-Campusano et al., 2012). Thus, a learned conditioned response is derived from the disinhibition of cerebellar interposed neurons. This well-defined functional circuit is indicative of the role cerebellar interposed neurons could play in mediating learned movements. Moreover, whether and how these pathways mediate skilled and locomotor movements have not been examined in detail.

Recent studies utilizing optogenetics in mice have provided evidence that the cerebellar interposed nucleus is involved in eyelid (Gao et al., 2016; Heiney et al., 2014) and limb (Lee et al., 2015; Witter et al., 2013) movements. The effects of indirect disinhibition of cerebellar interposed nucleus was studied by inhibiting Purkinje neurons through optogenetic stimulation of molecular interneurons at paravermal, simplex and Crus1 lobules of the cerebellar cortex (Heiney et al., 2014). Graded disinhibition of cerebellar interposed nucleus

neurons was observed to be linked to the size, speed and acceleration of eyelid movement (Heiney et al., 2014). Additionally, *in vivo* photostimulation of nucleocortical mossy fibers arising from glutamatergic neurons in the cerebellar interposed nucleus improved the learning rate of conditioned eyeblink responses (Gao et al., 2016). These studies suggest that the cerebellar interposed nucleus is capable of fine tuning motor responses, and is involved in motor learning.

The cerebellar interposed nucleus is implicated in reaching and grasping in humans and non-human primates (Kuper et al., 2011; Monzee et al., 2004). The association of prehensile abilities to lesioned brain regions was revealed in patients with surgical cerebellar lesions at the intermediate cerebellum including the cerebellar interposed nucleus using MRI imaging analysis (Kuper et al., 2011). These results support that the cerebellar interposed nucleus lesion is linked to dexterous exertion of grip forces during prehensile movements (Kuper et al., 2011). In non-human primates, muscimol inactivation of the cerebellar anterior interposed nucleus results in dysmetria and dynamic tremors during voluntary reaching and grasping movements (Monzee et al., 2004). Together, these studies indicate that the cerebellar interposed nucleus plays a major role in skilled and smooth prehensile activities.

Similar to the functional role of primate's cerebellar interposed nucleus in limb movement, studies in cats have uncovered the involvement of the cerebellar interposed nucleus in limb and paw placement during locomotion and skilled reaching (Armstrong and Edgley, 1984; Bracha et al., 1999). Extracellular recordings of neurons in the cat cerebellar interposed nucleus during locomotion on a moving belt demonstrated that the firing activity

of neurons in the cerebellar interposed nucleus is rhythmic during locomotion (Armstrong and Edgley, 1984). Muscimol inactivation of the cerebellar interposed nucleus in cats demonstrated that the cerebellar interposed nucleus is required for adequate limb flexing during locomotion and overcoming obstacles, and for accurate paw placement during skilled reaching (Bracha et al., 1999). Although these studies present the importance of the cerebellar interposed nucleus as an output structure for reaching and grasping precision, the circuit elements and functional relevance through which individual neuronal subpopulations within the cerebellar interposed nucleus contribute to specific movement has yet to be assessed.

Despite the advances of our understanding of the role of the interposed nucleus using advanced techniques, the consequences of optogenetic stimulation of cerebellar interposed nucleus on limb movement have mainly relied on indirect manipulations through Purkinje cells (Lee et al., 2015; Witter et al., 2013). A study using optogenetics, electroencephalography and electromyography, elegantly demonstrated that an indirect stimulation of cerebellar interposed nucleus led to synchronous activation the motor cortex and the biceps femoris (Witter et al., 2013). This light evoked motor response was further examined as a component of associative learning task (Lee et al., 2015). Optogenetic stimulus which serves as the unconditioned stimulus, was coupled to a tone which serves as the conditioned stimulus (Lee et al., 2015). Using this strategy, artificial disinhibition of the cerebellar interposed nucleus was identified as a potential teaching signal for motor activities (Lee et al., 2015). Thus, these data indicate that the intermediate cerebellum is able to control limb movements, but whether and how direct manipulation of neurons in the cerebellar interposed nucleus influences limb movements remain unknown.

Medial nucleus

The control of balance and posture, and oculomotor movement has been shown to be mediated by the cerebellar medial nucleus via projections to the vestibular, reticular, inferior olivary and the ventromedial thalamic nuclei (Batton et al., 1977; Noda et al., 1990; Teune et al., 2000). In addition to GABAergic and glutamatergic projection and local circuit neurons, the cerebellar medial nucleus contains glycinergic projection neurons (Bagnall et al., 2009). Within the medial nucleus, GABAergic neurons contribute to the feedback loop to the inferior olive and glutamatergic neurons project to thalamic regions, and both glutamatergic and glycinergic neurons provide innervations to the brainstem (Bagnall et al., 2009; Teune et al., 2000). A study utilizing mice that express GFP driven by the neuronal GlyT2 promoter and electrophysiological recordings demonstrated that glutamatergic neurons project contralaterally to both vestibular and reticular nuclei, whereas glycinergic neurons project only ipsilaterally to the same nuclei (Bagnall et al., 2009). The identification of the organization of ipsilateral inhibition and contralateral excitation provides mechanistic insight supporting the finding that inactivation of the medial nucleus unilaterally results in a tendency to fall toward only the ipsilateral side (Bagnall et al., 2009; Kurzan et al., 1993; Pelisson et al., 1998; Thach et al., 1992). Together, these studies indicate that the heterogeneous neuronal subpopulations within the cerebellar medial nucleus can potentially be organized according to neurotransmitter types, projection patterns and functions. However, the functional topographical map of each neuronal subtype and the identity of their targets remain undefined.

In summary, these studies have provided ample evidence that the cerebellar nuclei can control motor output. However, how the neurons in the cerebellar nuclei exert their activities

on relevant brain centers have not been defined. Additionally, the conditions and involvement of specific neuronal subtypes within the cerebellar nuclei to rhythmic and discrete movement have not been well characterized. Here, we focus on the investigating the connectivity and function of the cerebellar interposed nucleus. For instance, are there molecularly and functionally distinct neuronal subpopulations of neurons within the cerebellar interposed nucleus? What is the logic the organization of the efferent projections of neurons in the cerebellar interposed nucleus? Which pathways are accessed by neuronal subpopulations within the cerebellar interposed neurons for controlling specific elements of movement?

2.2 HYPOTHESIS

The cerebellar nuclei are situated in an ideal location to receive and compute a myriad of sensorimotor information and to broadcast processed output signals to diverse target regions within the brain. However, the logic and principles underlying how the seemingly heterogeneous mix of neurons within the cerebellar nuclei systematically coordinate motor activities are not clear. Moreover whether, different subpopulations of cerebellar nuclear neurons control distinct processes or distinct features of an individual process has not been examined. To address these questions, we set out to characterize the molecular signature of individual cerebellar nuclei using microarray and public expression databases, define the connectivity of subpopulations of neurons in the cerebellar nuclei using electrophysiological recordings and tract tracing, and examine the functional consequences of manipulating these neurons using chemogenetics and optogenetics. The following are major questions we aim to address:

- Are there molecular distinctions across the different cerebellar nuclei or neuronal subpopulations within each nucleus?
- What are the axonal projection patterns between different subpopulations of neurons in the cerebellar nuclei?
- Do neurons within the cerebellar nuclei directly influence movement, and if so do they modulate specific kinematic parameters in a context dependent manner?

Together, we aim to characterize the molecular expression of the cerebellar nuclei, connectivity patterns of the cerebellar interposed nucleus and the consequences of manipulating these pathways in skilled reaching and locomotion tasks behavior in order to

better understand the underlying mechanisms of the anatomical and functional diversity of cerebellar circuits in controlling motor behavior.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Male and female wild-type and genetically modified mice were used. Adult mice age P60 to P120 were used for all experiments. Only heterozygotes were used for each transgene allele. A list of Cre-driver mouse lines and reporter mouse lines were used for generation of experimental mice (Appendix A). All mice were housed under standard laboratory conditions with free access to food and water under a 12 hour light/dark cycle. All experimental procedures were approved by and conducted in accordance with the ethical guidelines of the animal care and use committees by the A*STAR Biological Resource Center / NTU / University of Pennsylvania IACUC committee.

2.3.2 Microarray

Tissue preparation

All tools were cleaned with 70% ethanol and RNaseZap® (Ambion). P60 wildtype mice brains were removed from Avertin, tribromoethanol, anaesthetized mice within 4 minutes, immediately flash freeze and stored in -80 °C. Tissues were cryosectioned at 18 µm thick and collected on polyethylene naphthalate membrane glass slides (Life Technologies, USA). Tissues were immediately stained after dehydration using desiccant pack. Slides with dried tissues were dipped in the respective solutions, 75% ethanol for 30 s, ddH₂O for 10 s, Cresyl violet and Eosin Y at 3:1 ratio for 10 s, 75% ethanol for 30 s, 95% ethanol for 30 s, 100% ethanol for 30 s, xylene for 30 s, fresh xylene for 60 s, and dehydrated in a box with desiccant packs. Dehydrated tissues were dissected for their respective cerebellar subnuclei using laser capture microdissection. Cells are collected on a Capsure® LCM Cap (ThermoFisher Scientific, USA) until cap is filled. Samples of each subnuclei were pooled

from 3 mice, stored in -80 °C and RNA was isolated within the same day of tissue collection. Arcturus® PicoPure® RNA Isolation Kit (Thermofisher Scientific, USA) was used. Thermoplastic film from Capsure® LCM Cap (Thermofisher Scientific, USA) with tissues were removed using sharp forceps and placed into 50 µL extraction buffer. After vortex, the mixture was incubated for 30 minutes at 42°C with occasional vortex. Column was preconditioned with 250µL Conditioning Buffer and incubated for 5 minutes at room temperature. While tissue mixture was centrifuged for 1 minute at 16 000 g, then 50 µL of 70% ethanol was added and mixed by pipetting. Tissue mixture was transferred into pre-conditioned column and centrifuged at 100 g for 2 minutes, followed by 16 000 g for 30 s. 100 µL of wash buffer was added, and centrifuged at 8000 g for 1 minute. Tissues were then treated with 40 µL of DNase1 and 160 µL RDD buffer (Qiagen RNase free DNase Kit; Qiagen, USA) for 15 minutes at room temperature. 40 µL of washing buffer 1 was added and centrifuged at 8000 g for 15 s. 100 µL of wash buffer 2 was added and centrifuged at 8000 g for 1 minute with lid closed. Column was transferred to new collection tube. 100 µL of wash buffer was added and centrifuged for 16 000 g for 2 minutes. Column was transferred to a new 0.5mL nuclease-free microcentrifuge tube. 11 µL of elution buffer was added and incubated for 1 minute at room temperature followed by centrifugation at 16 000 g for 1 minute to elute RNA. RNA samples were stored in -80 °C

Amplification, quantification and microarray

RNA samples were processed by A*STAR microarray facility and staff. The concentration and quality of RNA were tested using nanodrop and Agilent 2100 Bioanalyzer. Samples with a nucleic acid concentration ranging in between 100 pg/ul to 1 ng/ul and a RIN of >4 was chosen for amplification using Ovation Pico kit WTA System V2, leading to a

final output of 60 ng/ul of nucleic acid. The A_{260}/A_{280} ratio was tested to be in between 1.7 to 2.1, and 3 ul of final output sample was used for the microarray.

The Applied Biosystems™ GeneChip® Mouse Genome 430 2.0 Array (MOE430) includes over 45,000 probe sets representing over 34,000 well-substantiated mouse genes (ThermoFisher Scientific, USA). Our data files were normalized using Expression Console Software and its standard protocol, and utilized Transcriptome Analysis Console Software for further analysis including Principal component analysis, eBayes, and converting arbitrary units of fluorescence intensity as logarithms ($\log_2$) to be presented as signal values. To identify genes that are specifically expressed within the cerebellar nuclei, we excluded probes that are enriched with signal 7 and above in the cerebellar cortex and vestibular nucleus and excluded probes with signal below 9 in the cerebellar nuclei. These two parameters were determined by comparing our microarray signals to Allen Brain Atlas *in situ* hybridization database. We found that genes with a value <7 had low/no detectable expression, and values >9 had clear detectable expression; whereas probes with signal values ranging from >7 to <9 are not reliable indicator of expression.

2.3.3 Histological procedures

Mice were anaesthetized with avertin, tribromoethanol (Sigma-Aldrich, USA), 250 mg/Kg, intraperitoneally, then transcardially perfused with phosphate buffered saline (PBS) (1st Base, Singapore) followed by 4% paraformaldehyde (Sigma-Aldrich, USA) in phosphate buffer (4% PFA) and post-fixed for 2 hours at room temperature in 4% PFA. Brains were submerged in 30% sucrose, 0.1 M PBS (pH 7.4), for 36 hours at 4°C. Coronal sections were yielded at 40-80 μm using either a vibratome or cryosection machine. Cryosectioning

required additional preparation steps which included the casting of brain in optimal cutting temperature compound (Tissue-Tek, USA) and freezing in either dry ice or liquid nitrogen. Brain sections were collected in a cryoprotectant solution consisting of 25% glycerin (Sigma-Aldrich, USA), 30% ethylene glycol (Sigma-Aldrich, USA) and 45% DEPC treated PBS.

2.3.4 Immunohistochemistry

Immunohistochemistry was performed on 40 μ m cryosection and 100 μ m free-floating brain sections were treated with 0.2% TritonX100 (Sigma-Aldrich, USA) in PBS three times followed by incubation in primary antibodies diluted in blocking buffer, 3% horse serum in 0.2% Triton X100 for one hour at room temperature. Tissue sections were again washed three times with PBS (5 minutes each). Mice between P56-63 were used for characterization of the cerebellar nuclei. Primary antibodies, rat anti-GFP (1:1000; Naclai Tesque, Japan); rabbit anti- Glutamic acid decarboxylase 67 (GAD67) (1:1000; Invitrogen; PA5-21397); mouse anti-calbindin (CALB1) (1:750; Swant, Switzerland); rabbit anti-c-FOS (1:500; Santa Cruz, USA); rabbit anti-Transcription factor activator protein 2b (AP2b) (1:500; Santa Cruz, USA); rabbit anti-activating enhancer binding protein 2 alpha (Tfap2A) (1:500; Santa Cruz, USA), mouse α -tubulin (1:500; Santa Cruz, USA); rabbit anti-Calretinin (1:2000; abcam; ab702); mouse anti-NEUN (1:2000; Merck, USA); rabbit anti-vGluT1 (1:1000; SySy; #135303); guinea pig anti-vesicular glutamate transporter 2 (vGluT2) (1:2000; Merck; AB2251); rabbit anti-TBR1 (1:500; abcam; ab31940); mouse anti-mGluR2 (1:1500; Advanced Targeting Systems; AB-N32); mouse anti-SMI 32 (1:1000; Biolegend; #801701); guinea pig anti-Zebrin (1:1000; Frontier Institute) were used and incubated for 36 hours at 4°C. Samples were washed with 0.2% TritonX100 three times. Secondary antibodies Alexa488-, Alexa555-, and Alexa647-conjugated donkey anti-rabbit, -mouse, -rat, and -

guinea pig diluted in blocking buffer (1:1000; Molecular Probes, USA) were used accordingly to bind primary antibodies. Sections were washed with washing buffers before and after the addition of DAPI stain (1:1000; Life technologies, USA). Samples were mounted on a glass slide, in glycerol. Fluorescence signal was examined with a confocal laser scanning microscope (Zeiss, Germany).

The cerebellar nuclei were delineated for analysis after brain slices were stained with glutamatergic neuronal markers TBR1, Anti-neurofilament H non-phosphorylated (SMI32) and vGluT2, and nuclear marker, DAPI. We primarily used DAPI staining for locating large neuronal nuclei that are found only in the cerebellar nuclei and not the surrounding white matter (Heckroth 1994). We used these methods to delineate the distinct shapes of each subnuclei and cross-referenced our delineation with the mouse brain atlas (Paxinos and Franklin, 2007). 7-9 brain slices per mouse were used for data presented.

2.3.5 *In situ* hybridization

RNA probe synthesis

Total cerebellar RNA (Clontech #36660) was used to reverse transcribed into complementary DNA. DNA was used as a template for polymerase chain reaction. Primer sequence was designed against anti-sense strand of *Ucn3* gene and T7 RNA polymerase sequence (5'GCGCTAATACGACTCACTATAGGG 3') was added on the 5' end of the reverse primers to assist in initiation of transcription (Forward 5'CTTCAGCTGCCTCAACACAG 3', Reverse 5' GCGCTAATACGACTCACTATAGGGTGGTATCTGTACCGGGTGCT 3'). Transcribed RNA was labelled with 11-digoxigenin-UTP (Roche #11175025910).

Chromogenic in situ hybridisation

Tissue sections were fixed using cold 4% paraformaldehyde for 10 minutes and washed 3 times for 3 minutes each with fresh PBS. Tissues were permeabilized with Proteinase K (Promega #V3021) for 10 minutes. Tissues were fixed with 4% paraformaldehyde at room temperature for 5 minutes, washed and treated with acetylation solution containing triethanolamine (Sigma #108-24-7, Japan) and acetic anhydride (Sigma #411000, Japan). Tissues were washed before incubated with hybridization solution (50% formamide (Promega #H5052, Japan), 5X SSC (1st base #BUF-3050, Japan), 5X Denhardt's solution (Ficoll type 400 (Sigma #F9378, Japan), polyvinylpyrrolidone (Sigma #PVP40, Japan) and bovine serum albumin (Sigma #A7906, Japan), 250 µg/mL yeast RNA (Sigma #R6625-5G, Japan) and 500 µg/mL salmon sperm DNA (Promega #D1816, Japan)) at 63.5°C for 1 hour. RNA-labelled probes were diluted to 400-1000 ng/mL with hybridisation solution and denatured at 80°C for 5 minutes. Pre-hybridised sections were incubated with probes and coverslips on in a humidified box for 12 hours at 63.5°C.

After incubation with RNA-labeled probes, tissue sections were washed with 5X SSC pre-warmed at 63.5°C and coverslips were removed. Washing solution 0.2X SSC was used to wash the tissues twice for 30 minutes, each at 63.5°C. Tissue sections were then equilibrated to room temperature by incubating with buffer 1 containing 0.1M Tris pH7.5 and 0.15M NaCl, and blocked with 10% sheep serum diluted with buffer 1 for an hour at room temperature. After blocking, sections were incubated with anti-DIG-AP antibody (1:2000; Roche, Switzerland) 12 hours at 4°C. Sections were washed with buffer 1 thrice for 3 minutes each and buffer 3 containing 100mM Tris-HCL pH9.0, 150mM NaCl and 1mM MgCl for 5 minutes. BCIP/NBT substrate was added onto the slides and incubated for 6-24 hours till the

chromogenic signal appears. The indicator reaction was stopped with ice-cold methanol and sections were mounted with DakoMount (Agilent, USA).

2.3.6 Electrophysiology

Postnatal 27 to 33 days old mice were deeply anaesthetized prior to brain extraction. Coronal cerebellar sections were sliced using vibratome at room temperature, 200 μ m thick and incubated at 34°C for an hour in oxygenated standard extracellular solution (2.5 mM KCl, 126 mM NaCl, 1 mM NaH_2PO_4 , 24 mM NaHCO_3 , 2.5 mM CaCl_2 , 2 mM MgCl_2 , 10 mM glucose, 0.4 mM ascorbic acid, equilibrated with 95% O_2 /5% CO_2 to reach pH 7.4). Whole-cell patch clamp recordings were conducted at room temperature accordingly to previously described (Lee et al., 2010). For whole-cell recordings, electrodes (4-7 M Ω) were filled with an internal solution containing 10 mM HEPES, 5 mM EGTA, 4 mM Na_2ATP , 5 mM disodium phosphocreatine, 130 mM K-gluconate, 0.4 mM Na_3GTP , 10 mM KOH, 2.5 mM MgCl_2 , equilibrated to pH 7.3 with ~295 mOsm). Electrical responses were obtained using a Multiclamp 700B amplifier with Digidata 1440A interface (Molecular Devices, USA). Input resistance was measured from the slope of the current-voltage plot and membrane capacitance was calculated from the time constant determined by fitting the voltage response. Action potential half width was measured from the duration at half-amplitude between threshold and action potential peak. Cells were filled with Neurobiotin (0.5%, diluted in internal solution; Vectorlabs, USA) during recording by iontophoretic injection (Curti et al., 2012).

Brain slices were treated (as described in the section 2.4 *Immunohistochemistry*), an additional an hour incubation with AlexaFluor 633-conjugated Streptavidin (2 mg/ml; 1:250

dilution; Molecular Probes; S21375) was used to label the Neurobiotin filled cells. Cells were imaged under confocal laser scanning microscope (LSM710; Zeiss) and images were processed using ImageJ software. Optical z-section images were collected 0.8 μ m apart to reconstruct the entire cell body.

2.3.7 Tract tracing

Brain microinjection

Adult mouse was anaesthetized under 1% isoflurane in 1 L per minute O₂ flow. Surgical site was shaved and head-fixed on a stereotaxic frame (Taxic-600, World Precision Instruments, China). Buprenorphine (0.6 mg/kg) and lidocaine (5 mg/kg) were separately subcutaneously introduced. Ophthalmic ointment was applied to eyes during and after surgery. An incision was made along the midline using a scalpel to expose the cranium, followed by applying 10% hydrogen peroxide on a sterile Q-tip to remove tissues covering the cranium. The head was adjusted to level the bregma and lambda points horizontal.

Stereotaxic coordinates targeting region of interests were obtained from a stereotaxic atlas (Paxinos and Franklin, 2007): the IntA (from bregma: 5.88 mm posterior, 1.35 mm lateral, 2.50 mm ventral). A small burr hole was formed with a drill (Omidrill, WPI, China) using a 0.8 mm ball diameter drill bit at the coordinate of interest. Anterograde virus used in this study: rAAV2.*hSyn*::mCherry and rAAV2.*hSyn*::hChR2 (H134R)-mCherry (UNC Vector Core, USA). Injection solutions were administered into P56-63 mice at a rate of 2 nl/s using a NanoFil 10 μ l glass syringe with a 33 g beveled needle attached to a stereotaxic apparatus. Leakage was reduced by leaving the needle at the injection site for ten minutes prior to removal (Ruigrok and Apps, 2007). A slow pace (two minutes) was used to lower

and remove the needle from the site of injection. All mice were housed postoperatively three weeks before perfusion to allow for virus to be transduced throughout the entirety of the axonal arbor (Gao et al., 2016). For all surgical procedures mice were anesthetized with an induction concentration of 3% isoflurane and an oxygen rate of 2 liters/minute followed by maintenance concentration of 1-2% isoflurane and an oxygen rate of 1 liter/minute.

Retrograde analysis was performed by injecting Cholera toxin subunit B, Alexa fluor 555/647 conjugate (1% CTB555, 1% CTB647; Molecular Probes; C34776) into relevant brain regions of P56-63 mice (Conte et al., 2009). CTB was introduced into the M1 via pressure injection (as described above) at coordinates, from bregma: 0.5 mm anterior, 1.35 mm lateral, 0.55 mm ventral. All mice were housed postoperatively for 1 week before perfusion to allow for CTB to be transported throughout the entirety of the axonal arbor (Gao et al., 2016). After 1 week incubation, mice were perfused and stained.

Spinal cord microinjection

Spinal cord surgery to target the cervical and thoracic segments were conducted as previously described (Inquimbert et al., 2013; Keough et al., 2015). In brief, P60 mouse was deeply anaesthetized, and shaved at the area of surgery. An incision to expose the spine, the spine was fixed using the stereotaxic frame, along the vertebra segment, to stabilize the region. Cervical 6 to thoracic 1 segments were exposed by removing the small spinal muscles and ligament, followed by removal of the vertebra to expose the spinal cord. A puncture was made to the dura mater, and one microliter of Fluoro-Gold (FG) (Fluorochrome, USA) was bilaterally injected into the region (lateral from midline: 500 μ m, depth: 300 μ m) using a glass pipette attached to a NanoFil syringe. The vertebra was released, and the exposed

region was sutured. Postoperative care was given as earlier described. Mouse was incubated for 1 week followed by spinal cord dissection for histological analysis.

2.3.8 Diphtheria toxin receptor mediated ablation

Diphtheria toxin receptor (DTR) was expressed within the IntA of *Ucn3::Cre* mice using a Cre-dependent virus pAAV2-*EF1a::DF-DTR-GFP* (Cyagen biosciences, USA). The virus was introduced via pressure injection technique in P56-63 mice as described in section 2.7 *Tract tracing*.

Two weeks after virus incubation, three doses of diphtheria solution was administered to mice intra-muscularly at a concentration of 50 µg/kg, once every 48 hours on alternate hindlimb (Wu et al., 2008). For control, pAAV2-*EF1a::DF-DTR-GFP* was injected into *Ucn3::Cre* negative; reporter mice, and diphtheria toxin was administered as described above. *Ucn3::Cre*; reporter mice injected with pAAV2-*EF1a::DF-DTR-GFP*. Saline administered mice were included as controls. No difference in general health and motor ability was observed between groups and subsequently data from all control mice was analyzed together. Behavioral experiments begun a week after diphtheria toxin administration. Mice were sacrificed and perfused for histological analysis between P96 and 101.

2.3.9 Fiber implantation

Stainless steel screws (1.5 by 1.5 mm, #000-120 thread screws) were anchored at three respective coordinates 2.0 mm, 4.0 mm and 7.0 mm caudal to bregma and -2.0 mm, 2.0 mm and 2.0 mm lateral to midline. A small burr hole was drilled in the cranium using a drill with 0.8 mm ball diameter drill bit attached (Omidrill, WPI, China) at the coordinate 6.0 mm

caudal to bregma and -1.80 mm lateral to midline. A 2mm in length fiber optic ferrule (CFMLC12L02, Thorlabs, USA) was inserted using a probe holder connected to the stereotaxic arm at a rate of 2 mm/minute. Dental cement (Heraeus Kulzer, Germany) was applied, while ensuring contact with the ferrule and the anchors, and left to set for 5 minutes. The scalp was sutured, and the mouse was returned to a clean recovery cage under a heat lamp. Crushed food pellets soaked in water, and water containing meloxicam (5 mg/ml) and enrofloxacin (10 mg/kg) were provided to the mouse over the next three days.

2.3.10 Optogenetics

Photostimulation in vitro was conducted as previously described (Wang et al., 2007). In brief, slices were exposed to blue light (465-495 nm) from a mercury arc lamp. Simultaneously, cellular responses were measured in whole-cell patch clamp recordings. Data expressed as mean $\pm$ s.e.m. was analyzed with custom software to produce the synaptic input maps and averaged probability maps.

For in vivo photostimulation, light was delivered using a mono fiber-optic patch cord temporarily connected to the ferrule via a zirconia sleeve (Doric Lenses, Canada). A 473 nm diode laser (Shanghai Laser & Optics Century, China) and an arbitrary waveform generator (NI myDAQ; National Instruments, USA) were used to deliver pulses of laser (50-500 ms pulse width, 1-20 Hz, ~1-12 mW, fiber tip 0.1 mm dorsal to IntA). Laser strength was calibrated at the end of the ferrule tip using a power meter (Thor Labs) prior to implantation.

2.3.11 Open field

Activity in an open field was tested using a gray opaque Perspex box (60 X60 cm). Mice were allowed to freely move around for a total of 7 minutes. Distance traveled and velocity of movement were analyzed using EthoVision (Noldus, Netherlands).

2.3.12 Rotarod

To test motor function in the rotarod (TSE Bad Homburg, Germany). Mice were first habituated to the rotarod apparatus using a protocol which lasts for 5 minutes at 4 rpm protocol. Subsequently, mice were trained on an accelerating protocol from 4 to 40 rpm. Results were scored as latency to fall (in seconds) on a single day of testing.

2.3.13 Freely walking task

Foot print analysis

The methods for analysis of gait pattern have been previously described (Mendes et al., 2015). In brief, the footprints were detected optically with a walkway designed based on disrupted total internal reflection. The mice were habituated to the walkway prior to recording of walk gait. Paw prints were recorded using a camera. The length of each stride was measured on Photoshop CS6 (Adobe, USA).

Lateral profile analysis

Gait parameters of freely walking mice are previously described (Machado et al., 2015; Mendes et al., 2015). Briefly, each limb stride consists of two phases, a stance (duration in contact with the ground) and a swing (duration in the air). Each swing was further subdivided into a Swing-Flex and Swing- Extend phase based on flexion at the wrist joint observed as

the deflection from a perpendicular line drawn from the paw to platform. All paw trackings were grouped in the Swing-Flex phase, prior to crossing the perpendicular line, and the Swing-Extend phase thereafter. For the lateral gait analysis, male mice were habituated one week post-operation to the restricted walkway (3.2 cm x 30 cm x 70 cm) using a daily schedule of half an hour repeated over 3 days. For photostimulation experiments, randomized trials utilized a 473 nm laser at 1-10 mW at 1-20 Hz for 0.5-5 seconds (Azim et al., 2014b; Fink et al., 2014; Guo et al., 2015). Lateral motion paths of walking through a restricted walkway were captured within 50 cm window by the camera at 300 and 500 frames per second. The *x* and *y* coordinates of the forelimb were labelled for each video frames using MaxTRAQ 2D (Innovision Systems, Egypt) and the following parameters were extrapolated and analysed for stride, stance and swing duration, stride length, averaged vertical position across a stride and cadence (frequency of strides per second).

Paw movement in the skilled reaching task was tracked using MATLAB (MathWorks, USA) with a customized paw tracking program (Thanawalla A. R.). At each video frame, the positions of the nose, reaching forelimb and pellet was collected and analyzed for position, angles and errors.

2.3.14 Skilled reaching task

Skilled reaching task was conducted in a chamber as previously described (Baird et al., 2001; Klein and Dunnett, 2012). In brief, mice were food deprived for 20 hours. Mice were habituated to food pellets [Honey Stars, divided into five equal sized pieces (3.76 x 5.43 x 4.65 mm); Nestle, Switzerland] over 3 consecutive days. Subsequently, the mice were habituated to the chamber by placing food pellets along the surface of the central trough as

well as on the staircase steps for two days. Next, for training and actual tests, one pellet was placed on each side of the chamber, a total of 16 pellets. The mouse was placed in the start compartment, and allowed to reach for the food pellets. Each training session lasts for 15 minutes. All sessions were recorded for analysis using a camera (RX100IV, Sony, Japan).

Success rate for the staircase task is calculated as a percentage of total number of reaching attempts per step. Errors leading to an unsuccessful attempt were classified as reach, grasp and retrieving errors. Errors in relation to targeting the pellet were considered reaching errors. Reaching errors were recorded at the endpoint of each reach (before a grasping action or before the paw is retracted from the target). Grasping errors were recorded when mice target the pellet accurately but fail to grasp the pellet due to a failure to curl the paw into a grasp or inability to firmly grip the pellet. Retrieval errors were recorded when the mouse was able grasp the pellet, but when bringing the pellet to the mouth, the pellet drops or the mouse is unable to transfer the pellet to its mouth. All errors are calculated as a percentage of the total number of reaching movements made towards the pellet for each mouse. To control for possible differences in starting point of the body, we measured the nose position at the beginning of each reaching attempt, just prior to the reaching movement. If the mouse adjusts its body position during the course of the reach, the paw positions are not analysed as part the same movement. If the pellet is displaced from the well, analysis is concluded for the reach. The reaching endpoints were replotted using a density contour plot in MATLAB (MathWorks, USA).

2.3.15 Quantification of the distribution of recombined cell in double transgenic mice

To determine the number of recombined cells in the cerebellar nuclei, representative images of the cerebellar nuclei were collected from every two 40 µm coronal cerebellar sections, derived from *Ucn3::Cre; Rosa::lsl.GFP* and *Ucn3::Cre; Rosa::lsl. ChR2-eYFP*, using Zeiss LSM 710 confocal microscope (20 X objective). Recombined cells were manually counted using images co-localized with nucleus staining, DAPI. Software, ImageJ, was used to scan z-stack images while cell count was conducted. All cells which are sliced off at the bottom surface were excluded from the count to avoid over-estimation. The total cell count of each cerebellar nuclei was computed by multiplying the counts by two. The profile of each area of interest was traced at its largest cross section, identified from z-stack, and the area was computed by the program.

2.3.16 Quantification of nuclear protein expression

The IntA nucleus was imaged using the confocal microscope Z-stack option (20 X objective). To ensure that there were no biases in the intensity of all images, the laser settings were not changed. A counting frame on ImageJ was used to ensure systematic scanning of planes derived from the Z-stack imaging. The profile of each nucleus was traced at a plane in which it was the largest with reference to the internal standard, DAPI staining. The area and the mean gray value were computed and recorded. Due to the possibility of recording intensities originating from non-specific c-FOS staining, this described procedure was chosen over the conventional method of analyzing images derived from the consolidation of stacks into a single image.

To determine the average pixel intensity of the expression of mCherry and eYFP proteins at the projection sites, images of a fixed area in each site were collected on a Zeiss LSM 710 laser confocal microscope (40x objectives, numerical aperture 1.30, Plan-Apochromat), keeping individual pixel intensities in the linear range for images from control mice. The fluorescent pixel intensity of the protein of interest within each analyzed region were determined on cryosections at sub-saturating antibody concentrations and calculated using the histogram and color histogram function in NIH ImageJ (version 1.50g; NIH, USA). For analysis, pixel intensities were measured in an area of $45176 \mu\text{m}^2$ (40x objective) for each region- and protein- of interest and represented as an absolute number. The average pixel intensity per area measured at each brain region was used as threshold against the corresponding brain regions from negative control. For analysis of the colocalization, individual regions are measured by using the RG2B Colocalization macros in ImageJ (<https://imagej.nih.gov/ij/plugins/rg2bcolocalization.html>). Location of labeled terminals were mapped onto atlas landmarks (Paxinos and Franklin, 2007). Images were post-processed using Photoshop CS6.

2.3.17 Statistical analysis

Values are represented as mean $\pm$ s.e.m. unless otherwise noted. Comparisons were made by unpaired Mann-Whitney U test. The p values for comparisons of electrophysiological data and across more than two groups were calculated using ANOVA. Specifically, for the analysis of forelimb movement, comparable number of strides per animal of each parameter in control and ablated animals were analyzed to avoid disproportionate effect on the results. Similarly, for photostimulation experiments, equal number of strides from each animal during ON and OFF conditions were used for statistical

comparison. To achieve parity, samples from mice which did not provide sufficient stride trials were excluded. Strides were randomly sub-sampled from mice which provided a more than sufficient number of strides, by using the randomize function in Microsoft Excel. All statistical computations were performed using GraphPad Prism version 7.00 (GraphPad Software, USA).

2.4. RESULTS

2.4.1 Role of the cerebellar nuclei role in motor control

2.4.1.1 Molecular distinctions between cerebellar nuclei

In order to begin to uncover the molecular distinctions between neuronal subpopulations within the cerebellar nuclei, we conducted a DNA microarray analysis to define potential molecular signatures within each of the three cerebellar nuclei and the cerebellar cortex. Each of the cerebellar nuclear and cortical regions was isolated from freshly cryosectioned and Eosin Y and Cresyl violet stained tissues using laser capturing microdissection as previously described (Bidarimath et al., 2015; Clement-Ziza et al., 2009). This work flow and protocol enabled us to visualize and distinguish the five regions of interest including the cerebellar lateral nucleus, interposed nucleus, medial nucleus, ML and the vestibular nucleus, under a light microscope

To examine the molecular profiles of each cerebellar region and the vestibular nucleus, we analyzed the data using Principal component analysis. Virtually nothing is known about the molecular distinctions between the five brain regions we examined. An earlier study has reported a method using mouse lines and immunohistochemistry to delineate the cerebellar nuclei into 12 expression domains (Chung et al., 2009). However, this study did not assess the gene expression pattern of neighboring regions, such as the cerebellar cortex and vestibular nucleus (Chung et al., 2009). Furthermore, a complicated set of antibodies and transgenic mouse lines were required in order to identify one single domain (Chung et al., 2009). Our Principal component analysis revealed that genes expressed by the cerebellar nuclei are distinct from the cerebellar cortex and vestibular nucleus, and that genes expressed in the cerebellar nuclei are not equally distributed amongst each of the three subnuclear regions (Figure 5A).

Figure 5. Molecular profiling of the individual regions within the cerebellum. (A) Principal component analysis for the five cerebellar regions. (B, C) Summary of the number of genes that are differentially expressed. (C) Immunohistochemical staining of Complement component 1, q subcomponent-like (C1QL3). Fold change <-4 or 4 ; $P < 0.05$; False Discovery Rate < 0.05 ; eBayes. $N = 2-3$ replicates for each region; pooling of 3 mice for each replicate.

To determine the relative number of genes that might be differentially expressed within each of the profiled brain regions, we performed a comparison analysis of each brain region and plotted the results in a Venn diagram (Figure 5B-C). We determined the fold

change parameter by comparing signal values to Allen Brain Atlas *in situ* hybridization database, and characterized signal values with a fold change of 4 to have robust visually-detectable difference in RNA expression levels. Our analysis indicates that 2382 genes and 779 genes are differentially expressed by the cerebellar nuclei when compared to the ML and vestibular nuclei, respectively (Figure 5B). Out of which, 166 of these genes are differentially expressed across all three major brain regions. Therefore, our results indicate that the mouse cerebellar nuclei consist of differentially expressed genes when compared to its neighboring brain regions.

To compare the difference in levels of gene expression within the cerebellar nuclei, we conducted comparison analysis on the three major cerebellar nuclei. We found that there are 1956 genes that are differentially expressed among all three cerebellar nuclei, and only a scant number of genes had distinct levels of expression across the three cerebellar nuclei (Figure 5C). Thus, our data points out that there is molecular heterogeneity in the mouse cerebellar nuclei.

To identify genes that are specifically expressed within the cerebellar nuclei, we analyzed probes that are enriched in the cerebellar nuclei, and not in the cerebellar nuclei. In this analysis, genes expressed within the vestibular nuclei were not excluded because almost all genes are highly enriched within the cerebellar nuclei, except for 2 genes found in the cerebellar lateral nucleus, are also expressed in the vestibular nuclei. To begin the analysis, we qualitatively selected a range of signal values that reflects the presence of gene expression by comparing our microarray signals to Allen Brain Atlas *in situ* hybridization database. Using the derived signal values, our analysis revealed 2 probes which are exclusively expressed in the all three cerebellar nuclei including POU domain, class 3, transcription

factor 1 and hydroxysteroid (17-beta) dehydrogenase 14. A subsequent analysis was conducted by excluding vestibular nucleus from our comparison analysis. We identified 48 probes exclusively expressed by the cerebellar nuclei (Appendix B). Out of which, only protocadherin 11 X-linked showed differential expression within the cerebellar nuclei. Protocadherin 11 X-linked is positively expressed within the cerebellar lateral and interposed nucleus, but not the medial nucleus (Appendix B; last row). To validate the accuracy of our microarray data, we carried out immunohistochemical analysis of the expression of Complement component 1, q subcomponent-like (C1QL3) (Appendix B; Figure 5D). The protein expression pattern C1QL3 is consistent with our microarray data, Allen Brain Atlas *in situ* hybridization, and a study which describes C1QL3 expression within the cerebellar nuclei (Iijima et al., 2010) (Figure 5D).

Together, results from our microarray analysis indicate that while each of the three cerebellar nuclei does not appear to contain uniquely expressed genes though some genes may be expressed at differential levels within each the cerebellar nuclei. The microarray results did reveal a list of genes, including C1QL3, that are specifically by neurons in the cerebellar nuclei, but not by neurons in the cerebellar cortex or vestibular nucleus. We did however identify one gene that is selectively expressed by the lateral and interposed nuclei, but not medial nucleus. In short, our microarray analysis revealed a little molecular distinction between the cerebellar nuclei and raised the importance to take a more sophisticated strategy, such as single cell RNAseq, in order to define the molecular profiles of neuronal subpopulations in the cerebellar nuclei. Moreover, our results from our analysis underscores the major challenge of the lack of genetic tools for labeling, monitoring and manipulation of neuronal subpopulations within the cerebellar nuclei.

2.4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei

Characterization of the connectivity and function of neuronal subpopulation in the cerebellar nuclei requires the ability to reproducibly target and access neuronal subpopulations with precision. In order to achieve this precision, we set out to take advantage of the versatility of mouse genetics. To identify potentially useful mouse genetic tools, we first characterized the recombination pattern of *Parvalbumin (Pv)*, *GlyT2* and *PCP2* Cre-driver mouse lines that have been reported to occur in cerebellar neurons, within the cerebellar nuclei, or in neurons projecting into the cerebellar nuclei (Bagnall et al., 2009; Marzban and Hawkes, 2007; Neki et al., 1996; Nordquist et al., 1988). Additionally, we conducted a comprehensive screen through two public expression databases, Allen Brain Atlas and GENSAT Bacteria artificial chromosome (BAC) -Cre driver lines database (Gong et al., 2003; Madisen et al., 2012), to identify suitable transgenic Cre-driver mouse lines which have Cre-recombinase activity in the cerebellar nuclei.

Bacteria artificial chromosome transgenic lines are routinely used as reporter lines because the increased sensitivity from multiple copies of BAC transgenes permits visualization of gene expression that is not apparent from *in situ* hybridization or immunohistochemical analysis (Gong et al., 2003). However, promoters with a transcription unit larger than 100 kb are not compatible with BAC strategy because position effects result in genome integration and lead to a failure of Cre expression (Gong et al., 2003). To determine the recombination pattern of a few BAC Cre-mouse lines with reported recombination ability in the cerebellum, I assessed the recombination patterns of *GlyT2::Cre*, *Pv::Cre* and *Pcp2::Cre* in the cerebellar nuclei (Barski et al., 2000; Hippenmeyer et al., 2005; Ishihara et al., 2010). Adult double transgenic mice derived from each Cre-driver mouse line

crossed to a reporter mouse line (*Rosa.lsl.eGFP* and *Rosa.lsl.Chr2-eYFP*) were generated and their brains were isolated for immunohistochemical analysis. The expression of fluorescent reporters was amplified using anti-GFP antibody, which reacts with GFP and its variants including YFP, prior to analysis (Miyawaki et al., 1997; Palm and Wlodawer, 1999).

To determine whether the mouse lines described above might be useful for monitoring and manipulation of neuronal subpopulations in the cerebellar nuclei, we counted the number of recombined fluorescently labeled soma against neuronal subtype-specific markers or Nissl staining, and tabulated the counts into three broad categories: negative expression, 0% to 1% of total neuron type (-); sparse expression, 1% to 5% of total neuron type (+); uniform expression, 5% to 50% of total neuron type (++); high expression, 50% and more of total neuron type (+++) (Appendix C). Consistent with other studies, we observed a high number of recombined molecular layer interneurons and Purkinje cells in *Pv::Cre* that expresses Pv, a molecular marker specific for both cell types (Baimbridge et al., 1992; Celio, 1990) (Figure 6A, E). However, even though endogenous Pv is detected, no recombined cells were found within the cerebellar nuclei (Figure 6B-D, F-H).

To determine the recombination capability of *GlyT2::Cre* mice within the cerebellar nuclei, we assessed the expression of GFP along with GlyT2, a transmembrane protein localized at presynaptic terminals which recaptures glycine and glutamate decarboxylase 67 (GAD67), a GABA synthesizing enzyme localized within the soma, dendrites and axon (Chen and Hillman, 1993) (Figure 7A-H). We observed that GFP⁺ axon rarely co-labelled at a low incidence with GAD67⁺ labeling, indicating that *GlyT2::Cre* mice do not recombine in most GABAergic neurons within the cerebellar nuclei and Purkinje cells in the cerebellar cortex (Figure 7E-H). This observation suggests that GFP⁺ cells observed in the Purkinje

cells of the cerebellar cortex could be Bergmann glial (Figure 7A). Next, we found that there is very low number of GFP⁺ presynaptic terminals which colocalize with GlyT2 (Figure 7E-H). This suggests that either only a sparse number of GFP⁺ cells are glycinergic or that the GFP⁺ cells represent glutamatergic projection neurons (Uusisaari and De Schutter, 2011). Furthermore, the GFP⁺ neurons have a range of morphological sizes, 5 to 35 μ m in diameter, includes both the glycinergic and glutamatergic neuronal populations (Ankri et al., 2015; Uusisaari and Knopfel, 2011). Therefore, *GlyT2::Cre* mice do not appear to recombine specifically within glycinergic neurons in the cerebellar nuclei and a more extensive characterization needs to be done to determine the identity of these recombined neurons.

Figure 6. Recombination patterns and molecular expression of *Pv::Cre; Rosa::Isl.ChR2-eYFP*. (A-D) Recombined cells expressing eYFP (green). (E-H) Immunohistochemistry staining of eYFP expressing tissue with Pv (red). Enclosed in circles are locations with a soma, visibly marked by Pv antibody (red).

Figure 7. Recombination patterns and molecular expression of *GlyT2::Cre; Rosa::Isl.ChR2-eYFP*. (A-D) Recombined cells expressing eYFP (green). (E-H) Immunohistochemistry staining of eYFP (blue) expressing tissue with GAD67 (green) and GlyT2 (red). Magnification of cell bodies found in letter E (F-H), white squares. Respectively, arrows and arrow-heads point at magenta and cyan puncta. Enclosed in circles are locations with a soma.

The mouse line, *Pcp2::Cre*, utilizes a promoter for a gene known to be expressed only within the Purkinje cells of the cerebellum (Barski et al., 2000). We set out to determine the recombination pattern within the cerebellar nuclei of *Pcp2::Cre; Rosa.lsl.ChR2-eYFP* since this mouse line could serve as a tool for the manipulation of Purkinje cells to indirectly examine the function of neurons in the cerebellar nuclei. Hence, we stained brain slices of *Pcp2::Cre; Rosa.lsl.ChR2-eYFP* mice with anti-calbindin (CALB1) antibody, a Purkinje cell specific marker (Figure 8A-H), and anti-GFP antibody (Figure 8E). We observed that recombined cells strictly colocalizes with anti-CALB1 antibody labeling (Figure 8E). Within all three major cerebellar nuclei, there is a high level of colocalization between GFP and

CALB1 (Figure 8F-H). Colocalized signals form dotted frames around putative soma structures in the cerebellar nuclei (Figure 8F-H). These distinctive structures likely represent puncta originating from Purkinje cells, as it is well known that Purkinje cell axons project and terminates onto neurons in the cerebellar nuclei (Dezeuw and Berrebi, 1995). Thus, even though *Pcp2::Cre* does not recombine in neurons in the cerebellar nuclei, this mouse line might be useful for the analysis of Purkinje cell control of cerebellar nuclear neurons.

Using the second approach, we did a comprehensive screen through two public expression databases, Allen Brain Atlas and GENSAT BAC-Cre driver lines database (Gong et al., 2003; Madisen et al., 2012) to identify a mouse line with recombination capability specifically within the cerebellar nuclei, we identified and acquired a bacterial artificial chromosome transgenic mouse line, *Urocortin 3 (Ucn3)::Cre*, which mediates recombination in cerebellar nuclei neurons (GENSAT; Mutant Mouse Regional Center) (Madisen et al., 2012). We assessed the recombination ability of *Ucn3::Cre* mice by crossing them to four different reporter mice: *Rosa::lsl.eGFP*, *Rosa::lsl.ChR2-eYFP*, *Rosa::lsl.ChR-tdTomato* and *Rosa::lsl.Sun1-GFP* (Appendix C). We found recombined neurons located specifically within the interposed nucleus, and double transgenic mice derived from all four reporter mice showed consistent expression pattern (Figure 8A-F).

Next, we set out to quantify the number of recombined cells throughout the nine sub-regions of the cerebellar nuclei defined by histological distinctions (Paxinos and Franklin, 2007) (Figure 2). My quantification results revealed that recombined neurons are concentrated within the interposed anterior and sparsely distributed in other regions (Figure 12G). The interposed anterior contains approximately 7-fold more recombined cells than medial (Med) nucleus which is a region that contains the second highest number of

recombinant cells (Figure 9G). The remaining recombined cells, approximately less than 18%, are distributed among the medial dorsolateral protuberance (MedDL) nucleus, lateral (Lat) nucleus, posterior parvicellula (IntPPC) nucleus, interposed posterior part (IntP), lateral parvicellular part (LatPC) nucleus, medial lateral part (MedL) nucleus, and interposed dorsolateral hump (IntDL) nucleus (Figure 9G). Altogether, quantification of the distribution of *Ucn3::Cre* recombined neurons indicates that recombined cells are located specifically within the cerebellar nuclei, with more than 70% of total YFP⁺ neurons in the cerebellar nuclei found in the IntA, 15% in the medial nucleus and ~15% in the remaining five subnuclei (Figure 9G; Appendix C).

Figure 8. Recombination patterns and molecular expression of *Pcp2::Cre; Rosa::lsf.ChR2-eYFP*. (A-D) Recombined cells expressing eYFP (green). Enclosed in circles are locations with a soma. (E-H) Immunohistochemistry staining of eYFP (green) expressing tissue with CALB1 (green) and DAPI (blue). Zoomed in images of neurons derived from dotted circles in B to D, respectively, with arrow heads targeting yellow puncta framing each soma body (F-H).

Figure 9. Selective genetic targeting of neurons in the IntA. (A) GFP expression in the interposed nucleus of the cerebellar nuclei in coronal cerebellar slices derived from a *Ucn3::Cre; Rosa::Isl.eGFP* mouse (green). (B-C) Analysis of the expression of tdTomato in the cerebellar nuclei of a *Ucn3::Cre; Rosa::Isl.tdTomato* mouse (red, B) with colocalization of NeuN (C, green) and NISSL (C, blue), together with delineation of the different subnuclei within the DCN based on histological distinctions (Paxinos 2007). (D-F) Within each subdivision, YFP is detected primarily in the IntA of a *Ucn3::Cre; Rosa::Isl.ChR2-eYFP* mouse. (G) Quantification analysis of the distribution of YFP expressing neurons in each of the cerebellar subnuclei of *Ucn3::Cre; Rosa::Isl.ChR2* mice (Lat-lateral; LatPC-lateral parvicellular; IntDL-interposed dorsolateral; IntA-interposed anterior; IntP-

interposed posterior; IntPPC-interposed posterior parvicellular; MedDL-medial dorsolateral; Med-medial; MedL-medial lateral). Mean $\pm$ s.e.m. (n=2617, 2180, 4406 neurons). Age of mice used ranging from P56-63.

2.4.1.3 Recombination pattern of *Ucn3::Cre* in the developing mouse brain

A better understanding of the organization and function of the cerebellum requires information about the precise temporal and spatial expression pattern of genes and proteins throughout development, and the capability to identify and manipulate individual populations of neuronal cell types within the cerebellum. To determine the utility of the BAC transgenic mouse, *Ucn3::Cre* mouse line, as a genetic tool for precise targeting of specific neuronal cell types within the cerebellum, we characterized the recombination pattern in the developing mouse brain at three postnatal stages, P1, P7 and P23. We identified and mapped Cre-expressing neurons in mice generated from crossing *Ucn3::Cre* mouse line with a Cre-dependent fluorescent reporter mouse line (*Ucn3::Cre; Rosa::lsl.ChR2-eYFP*).

We observed recombined cells within the interposed nucleus of the cerebellar nuclei as early as postnatal day 1 (Appendix D). In addition to the cerebellum at P1, we also identified YFP⁺ Locus coeruleus neurons in the hindbrain (Appendix D). At P7, we found a similar number of YFP⁺ neurons in the cerebellum and hindbrain with one additional brain region, the triangular septal nucleus of the pallidum, with YFP⁺ recombined cells (Appendix D). At P23, the number of recombined brain regions increased to 6 regions, likely due to the significant increase in number of neurons throughout the brain between 1st and 3rd postnatal week. At P23, nine brain regions including the midbrain, hypothalamus, thalamus and striatum now also contain YFP⁺ recombined neurons (Appendix D). These results provide details of the temporal and spatial expression pattern of *Ucn3* gene in the brain. Moreover,

our characterization pinpoints the stages of neuronal development which could be examined using *Ucn3::Cre*-dependent labeling and/or genetic manipulation.

Figure 10. Identification of a distinct sub-population of glutamatergic neurons in the IntA. (A-C) YFP+ neurons in the IntA (green, A) express vGluT2 (red, B; colocalization C). (D) Percentage of YFP positive (45 ± 6) and YFP negative neurons (55 ± 4) within vGluT2+ neurons (G). (E-G) YFP+ neurons in the IntA (green, E) express SMI32 (red, F; colocalization G). (H-V) YFP+ neurons in the IntA (green, H, M, R) do not express GAD67 (red, I), Calretinin (red, N), or Tfap2A (red, S), but expresses SMI32 (blue, J, O and T) (merged, K, P, U; merged with DAPI, white, L, Q, V). Circles indicate cells with colocalization and arrow heads indicate cells without colocalization.

2.4.1.4 Molecular identity of *IntA^{Ucn3}* neurons in the cerebellar nuclei

The cerebellar nuclei contain three major classes of neurons: glutamatergic, GABAergic and glycinergic (Uusisaari and De Schutter, 2011). To define molecular distinctions of interposed anterior neurons targeted by *Ucn3::Cre* mouse (*IntA^{Ucn3}* neurons), we first assessed whether recombined neurons in *Ucn3::Cre; Rosa.lsl.ChR2-eYFP* express glutamatergic (vesicular glutamate transporter 2, vGluT2; anti-neurofilament H non-phosphorylated, SMI32; TBR1) or GABAergic molecular markers (GAD67; Calretinin; transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha, Tfap2A) (Chung et al., 2009; Leto et al., 2006; Zainolabidin et al., 2017). We found that *IntA^{Ucn3}* neurons express vGluT2, SMI32 and TBR1, but not GAD67, Calretinin or Tfap2A (Figure 10A-V; Appendix E). Even though TBR1 colocalizes with *IntA^{Ucn3}* neurons ($\text{TBR1+YFPON/YFPON} = 99\pm1\%$), the level of TBR1 expression is much lower in the interposed anterior compared nucleus to medial nucleus consistent with previously described (Chung et al., 2009) (Figure 11A-E; IGL= 38.13 ± 0.99 , lateral= 82.61 ± 2.37 , interposed= 97.34 ± 4.36 , medial= 160.10 ± 3.06 ; mean $\pm$ s.e.m.; n=132 cells). Also, we found that the trend of TBR1 expression to be uniform with our microarray analysis (Figure 11F; lateral= 2.47 ± 0.09 ; interposed= 2.917 ± 0.1765 ; medial= 5.13 ± 0.38 ; ML 3.06 ± 0.3579 ;

mean $\pm$ s.e.m). Together, our data indicates that IntA^{Ucn3} neurons make up approximately 45% of all vGluT2+ presumptive glutamatergic neurons in the IntA (Figure 10D).

Figure 11. Differential expression levels of TBR1. (A-D) The expression of TBR1 in the IGL (A), lateral (B), interposed (C), and medial nucleus (D) using immunohistochemical staining. (E) Analysis of the expression level of TBR1 in neurons within each of these regions. N=132 cells (F) The gene expression levels of TBR1 in ML and the respective cerebellar nuclei measured using microarray.

To compare the recombination pattern of *Ucn3::Cre* mice with the endogenous expression pattern of *Ucn3* in the cerebellum, we examined the expression of *Ucn3* mRNA expression in P60 wild type mouse using chromogenic *in situ* hybridization. Consistent with previous studies, we found *Ucn3* mRNA to be strongly expressed within the hypothalamus and the amygdala (Shemesh et al., 2016) (Figure 12D-F). However, we detected a very low

level of *Ucn3* expression in both the cerebellar nuclei and cerebellar cortex (Figure 12A-C). Likewise, our microarray result indicates that the level of *Ucn3* expression is low throughout the cerebellum (lateral= 3.31 ± 0.09 ; interposed= 3.26 ± 0.09 ; medial= 3.16 ± 0.06 ; ML 3.20 ± 0.15 ; mean $\pm$ s.e.m). Together, our results show that $\text{IntA}^{\text{Ucn3}}$ neurons do not express detectable *Ucn3* mRNA and that there is no specific expression of *Ucn3* within the interposed nucleus compared to the other two nuclei. Thus, even though the recombination capability of *Ucn3::Cre* mice will permit analysis of a highly specific neuronal subpopulation in the cerebellum, the recombination pattern is not recapitulated by the analysis of endogenous *Ucn3* expression pattern.

Figure 12. Expression of endogenous *Ucn3*. (A-F). Chromogenic *in situ* hybridization targeting *Ucn3* mRNA in wild type coronal brain slices at bregma -6.12 mm (A) and -0.34 mm (D). Zoom in on 4/5 Cb (B) and IntA

(C) from the red box in letter A. Magnified images of the hypothalamus (E) and amygdala (F) from letter D. P60 mouse brain.

2.4.1.4.1 Characterization of the activity level of cerebellar nuclei neurons using c-FOS

The majority of excitatory neurons within the cerebellar nuclei are larger in soma size and exhibits higher spontaneous activity than GABAergic neurons (Uusisaari and Knopfel, 2010). We examined the relative activity level of IntA neurons by measuring the intensity of c-FOS expression in homecaged mice (Carbo-Gas et al., 2014). We observed varying c-FOS expression within the nuclei of two subpopulations of cerebellar nuclear neurons (Figure 13A, C, E, G; high c-FOS versus 13B, D, F, H low c-FOS). A large majority of nuclei with less than $50\ \mu\text{m}^2$ have less prominent nucleolus, suggesting that these are glial nuclei, but not neuronal nuclei (Fix, 1975). The c-FOS expression levels in these putative glial cells were excluded from our analysis. To ensure that our c-FOS antibody and immunohistochemistry protocol are reliable, we stained a region of the brain where c-FOS expression has been well investigated, the dentate gyrus of the hippocampus (Dragunow et al., 1989). In the hippocampus, c-FOS antibody stained granule cells nuclei located at the dentate gyrus with varying intensities similar to previous studies (Dragunow et al., 1989) (Figure 13I-L).

Analysis of the frequency distribution of c-FOS expression level revealed that there might be two populations of neurons that can be differentiated into a population larger than $65.0\ \mu\text{m}^2$ and a population smaller than $65.0\ \mu\text{m}^2$ (x axis) (Figure 13M). Similar to previous report, we found that neurons in the cerebellar nuclei can be broadly divided into two populations: neurons with small nuclei, 38.9 to $50.0\ \mu\text{m}^2$, and neurons with large nuclei, 78.5 to $95.0\ \mu\text{m}^2$ (Heckroth, 1994). To examine the relationship between soma size and neuronal

Figure 13. Localized c-FOS immunoreaction within the nucleus of cells. (A-H) Images taken from the IntA. Expression of c-FOS (blue) with α -TUBULIN (red) and DAPI (green) staining. (A, C, E, G; white circles) Localized nuclear c-FOS staining and (B, D, F, H; white dotted circles) c-FOS negative cells were observed. (I-L) Images taken at the dentate gyrus of the hippocampus. Immunohistochemical reaction of c-FOS localized within the nuclei of granule cells. (M) The frequency distribution of nucleus size. The line (blue) segregates the two major putative cell populations. (N) Heat map rendering of nucleus area (bin widths of $5 \mu\text{m}^2$) plotted against c-FOS levels indicating two major putative cell populations. (O). A bar graph of comparing the neurons with large nuclei and small nuclei. All graphs have an x axis which starts from $50 \mu\text{m}^2$ ($n=3$ mice; 1000 samples from the IntA of each mouse).

activity, we generated a heatmap using the values of c-FOS mean gray value against the respective size of all nuclei (Figure 13N). The heatmap analysis shows two populations of neurons according to nuclei size and c-FOS expression (Figure 13N), and the difference is statistically significant [nuclei sizes; 50 to $< 65 \mu\text{m}^2$ (small nuclei) (n=3 mice; 750 neurons) and > 65 to $160 \mu\text{m}^2$ (large nuclei) (n=3; total neurons, 2250)] ($P < 0.0001$) (Figure 13O). Using cFOS as a proxy for neuronal activity, we found that neurons with smaller nuclei had a mean c-FOS expression value of 75.1 ($\text{SD} \pm 27.3$) (median, 70.5 ; first quartile, 55.1 ; third quartile, 92.1) whereas neurons with larger nuclei had a mean c-FOS expression value of 103.7 ($\text{SD} \pm 28.7$) (median, 101.1 ; first quartile, 83.6 ; third quartile, 123.0) (Figure 13O). Together, we found that large IntA neurons, presumably glutamatergic and include IntA^{Ucn3} neurons, express a higher level of c-FOS at basal level than smaller presumably inhibitory IntA neurons.

2.4.1.5 Electrophysiological properties of Ucn3::Cre neurons in the interposed nucleus

To determine the electrophysiological profile of the IntA^{Ucn3} neurons, patch-clamp recordings on YFP⁺ IntA^{Ucn3} neurons (YFP^{ON}) and YFP⁻ non-IntA^{Ucn3} neurons (YFP^{OFF}) were performed. Recorded neurons were intracellularly labeled using Neurobiotin, subsequently detected using streptavidin system with fluorescence visualization method. We further determined the molecular identity of intracellularly labeled neurons by checking for the expression of TBR1, a glutamatergic projection neuronal marker using immunohistochemical method. Patch-clamp recordings revealed that YFP^{ON} neurons have higher firing frequency than most YFP^{OFF} neurons (Figure 14A-B). YFP^{ON} and YFP^{OFF} neurons with similar firing rate are in range with firing rates of glutamatergic neurons in the cerebellar nuclei (Uusisaari et al., 2007) (Figure 14C).

Analysis of the diameter and area further subdivides these neurons into three groups (large YFP^{ON}, YFP^{ON}-L; large YFP^{OFF}, YFP^{OFF}-L; small YFP^{OFF}, YFP^{OFF}-S). YFP^{ON}-L and YFP^{OFF}-L neurons are ~four times larger in area and ~two times larger in diameter than YFP^{OFF}-S neurons (Figure 14D, Appendix F). While the distribution of soma sizes of non-GABAergic and GABAergic neurons is overlapping, soma size of greater than 300 μm^2 in area and 25 μm in diameter is indicative of non-GABAergic neurons in the cerebellar nuclei (Uusisaari et al., 2007). Analysis of the intrinsic electrical properties, including those previously described, revealed no significant differences between the properties of YFP^{ON}-L and YFP^{OFF}-L neurons (Uusisaari et al., 2007) (Figure 14E-I, Appendix F). However, there are significant differences between YFP^{ON}-L and YFP^{OFF}-S neurons, including input resistance, membrane capacitance, AP half width, and AP frequency (Figure 14E-I, Appendix F). The difference of AP frequency between YFP^{ON}-L and YFP^{OFF}-S is in line with our c-FOS analysis, a proxy test for neuronal activity (Figure 13N). Thus, of the six neuronal cell types described in the cerebellar nuclei (Uusisaari and Knopfel, 2011), we have determined that IntA^{Ucn3} neurons belong to a class of large glutamatergic neurons. Additionally, the presence of YFP^{OFF}-L neurons indicates that the IntA^{Ucn3} neurons comprise of a subpopulation of glutamatergic neurons in the IntA. Therefore, using *Ucn3::Cre* mice, we are able to selectively label a novel subset of glutamatergic neurons in the IntA that have distinct electrophysiological, morphological and molecular features.

Figure 14. Molecular and electrophysiological properties of *IntA^{Ucn3}* neurons. (A-B) *In vitro* whole-cell patch-clamp with Neurobiotin labeling (blue) in the IntA neuron soma of P30 *Ucn3::Cre; Rosa::Isl.ChR2* mice revealed the firing rate of a YFP+ (green) TBR1+ (red) and a YFP-TBR1- neuron. (C-I) Analysis of the action potential (AP) frequency (C), area (D), input resistance (E), action potential (AP) half width (F), membrane capacitance (G), slow afterhyperpolarization (AHP) peak (H), and frequency-current (F-I) plot (I) among YFPON-L, YFPOFF-S and YFPOFF-L neurons. Significant difference compared with YFPON-L (ANOVA with post hoc Tukey tests); mean $\pm$ s.e.m; n= 7-15 (YFPON-L), 6-9 (YFPOFF-L), 6-9 (YFPOFF-S) cells. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

2.4.1.6 Intra- and extra-cerebellar projections of Ucn3::Cre neurons in the interposed anterior nucleus

The diverse array of behavioral repertoire that associated with the cerebellum, which can be broadly classified as motor and non-motor functions, is likely mediated by sophisticated circuit elements. However, the anatomical connectivity and organizational principles are not well defined because of the lack of cerebellar nuclei-specific genetic tool. We proposed four possible configurations for IntA neuron to be linked with downstream brain regions (Figure 15A). Exclusive arrangement would involve distinct populations of IntA neurons projecting to separate target regions to establish separate communication channels that originate from one brain region. For overlapping-all connectivity, all IntA neurons send collaterals to the same brain regions, suggesting that IntA communicates through specific patterns of neuronal activity, not distinguished simply by specific anatomical projections. Overlapping-partial organization would comprise of sub-populations of IntA neurons targeting the different brain regions, with some overlapping targets. In the inclusive arrangement, a distinct sub-population of neurons targets a subset of regions targeted by the IntA neurons. The latter three configurations are suitable for simultaneous communication across diverse brain regions (Betley et al., 2013). Due to the lack of genetically tractable tools within the cerebellar nuclei, the anatomical organization of neural circuits formed by specific neuronal subpopulations within the cerebellar nuclei is not well studied. We examined which of these different circuit configuration is utilized by IntA neuron subpopulations by comparing IntA^{Ucn3} and non-IntA^{Ucn3} neurons.

Figure 15. Targeting and labeling IntA neurons to distinguish between two potentially distinct sub-populations. (A) Four possibilities for the pattern of axonal projections from IntA^{Ucn3} neurons (yellow) and non-IntA^{Ucn3} neurons (red). (B) Top: schematic of virus tracing strategy for axonal projections of IntA^{Ucn3} neurons (yellow) versus other IntA neurons (red). Bottom: Expression of mCherry in the IntA of a *Ucn3*::Cre; *Rosa*::*lsl*.ChR2-eYFP mouse after transduction with AAV2-*hSyn*::mCherry and AAV2-*hSyn*::ChR2-mCherry. (C-I) Analysis of the spread of AAV2-*hSyn*::ChR2-mCherry after injection into the cerebellar nuclei of a *Ucn3*::Cre; *Rosa*::*lsl*.ChR2 mouse. The six schematic images above contain red dots which represent mCherry+ neurons (C). The red box in each of the six schematic diagrams (C) is represented in images below showing mCherry (red), YFP (green) and SMI32 (blue) staining (D-I).

To define targets of $\text{IntA}^{\text{Ucn3}}$ neurons, we analyzed their axonal projections in *Ucn3::Cre; Rosa::lsf.ChR2-eYFP* mice. Even though *Ucn3::Cre* mice facilitate precise targeting of IntA neurons in the cerebellum (Figure 15B), axonal projections from other recombined regions such as amygdala and hypothalamus (Appendix D) could hinder our investigation (Shemesh et al., 2016). For this reason, we injected AAV2-*hSyn::mCherry* and AAV2-*hSyn::ChR2-mCherry* into the IntA of *Ucn3::Cre; Rosa::lsf.ChR2-eYFP* mice to distinguish between $\text{IntA}^{\text{Ucn3}}$ (YFP+mCherry+; yellow) and non- $\text{IntA}^{\text{Ucn3}}$ neuronal populations (mCherry+; red) (Figure 15B-I, Appendix G). We labeled approximately 92% of $\text{IntA}^{\text{Ucn3}}$ neurons with mCherry and found that $\text{IntA}^{\text{Ucn3}}$ neurons make up approximately half of all mCherry expressing neurons (Appendix G). Using this strategy, we were able to differentiate projections from $\text{IntA}^{\text{Ucn3}}$ neurons and those from non- $\text{IntA}^{\text{Ucn3}}$ neurons. However, one limitation of this double labeling strategy is that approximately 8% of $\text{IntA}^{\text{Ucn3}}$ neurons did not express mCherry, we are only able to analyze the projection patterns of the majority of $\text{IntA}^{\text{Ucn3}}$ neurons.

The cerebellar nuclei consist of nucleocortical neurons that send mossy fiber projections into the cerebellar cortex in a modular fashion (Ankri et al., 2015; Gao et al., 2016). To investigate the organization of IntA nucleocortical connectivity, we analyzed vGluT1 and vGluT2 expression in mossy fiber rosettes across all cortical lobules. In contrast to the general population of mossy fibers in the IGL that express both vGluT1 and vGluT2 (Hioki et al., 2003), glutamatergic nucleocortical mossy fibers only express vGluT2 (Gao et al., 2016). As expected, we observed that $\text{IntA}^{\text{Ucn3}}$ nucleocortical mossy fiber rosettes express vGluT2, but not vGluT1 (Figure 16A-J).

Figure 16. IntA neurons give rise to NC-MFs. (A) A coronal image of the cerebellum containing YFP positive mossy fibers derived from *Ucn3::Cre; Rosa::lsf.ChR2-eYFP* (green). (B) A magnified image of mossy fibers within the 4/5 Cb (C-F) YFP+ NC-MFs (green, C) within the IGL of the simple lobule (Sim) express mCherry (red, D) and vGluT2 (blue, E, vG2) (merge, F). (G-J) YFP+ NC-MFs (green, G) within the IGL of the simple lobule (Sim) express vGluT1 (red, H, vG1) and vGluT2 (blue, I, vG2) (merge, J).

Nucleocortical mossy fibers from IntA^{Ucn3} neurons are located primarily in the IGL of lobules IV-V and simple lobule in close proximity to Purkinje and Golgi cell somata (Figure

17A-H; Appendix H). Consistent with previous study, we observed a preferential targeting of nucleocortical mossy fibers to the Zebrin⁺ microzones and to the superficial region in the IGL (Gao et al., 2016) (Figure 17I-L). Remarkably, we found that IntA^{Ucn3} nucleocortical mossy fibers selectively target the anterior, but not the posterior forelimb region based on modules defined by olivo-cortico-nuclear connectivity (Voogd, 2003) (Figure 17M, N; Appendix H). These results indicate that IntA^{Ucn3} neurons comprise a subset of IntA neurons that projects to a restricted region of the cerebellar cortex distinct from other IntA neurons. Altogether, we introduce a genetic mouse tool to monitor and manipulate a subpopulation of IntA nucleocortical neurons that has been implicated in amplification of efference copy signals (Beitzel et al., 2017; Gao et al., 2016; Houck and Person, 2015).

Figure 17. IntA^{Ucn3} neurons give rise to nucleocortical mossy fibers restricted to the anterior forelimb associated region. (A-E) Analysis of YFP+ (green, A) and mCherry+ (red, B) mossy fibers that express vGluT2 (blue, C) (merge, D) in the cerebellar cortex of a *Ucn3::Cre; Rosa::Isl.ChR2* mouse injected with AAV2-*hSyn::mCherry*. Arrow heads indicate nucleocortical mossy fibers rosettes from IntA^{Ucn3} neurons, and open circles indicate vGluT2⁺ MF rosettes. (E) Distribution of IntA^{Ucn3} nucleocortical mossy fibers (circles = IntA^{Ucn3} nucleocortical mossy fiber rosettes; asterisk = vGluT2⁺ mossy fiber rosettes). (F-H) YFP⁺ mossy

fibers (green) are in close proximity to Pv^+ Purkinje cells (red, F) and $GAD67^+$ Golgi cell soma (red, G) and terminals (red, G) which surround presumptive granule cell dendrites (H). (I-L) Zebrin staining (I) shows $mCherry^+$ (red, J) and YFP^+ (green, K) NC-MFs are located in $Zebrin^-$ (K), but not in $Zebrin^+$ (L) microzone in the IGL of the simple lobule. (M) Distribution of non- $IntA^{Ucn3}$ and $IntA^{Ucn3}$ nucleocortical mossy fibers across coronal planes of the cerebellum at the indicated bregma points in forelimb and hindlimb associated regions (Voogd et al., 2003). (N) Quantification of the distribution of non- $IntA^{Ucn3}$ (red) and $IntA^{Ucn3}$ (yellow) nucleocortical mossy fibers as a percentage of total number of respective rosettes across all cerebellar lobules. Mean $\pm$ s.e.m. Age of mice: P56-63. Circles indicate cells with colocalization and arrow heads indicate cells without colocalization. Molecular layer (ML), Purkinje cell layer (PCL) and IGL. Scale bar=200 μ m (G), 150 μ m (K), 25 μ m (M), 10 μ m (O).

2.4.1.6.1 $IntA^{Ucn3}$ neurons send extensive projections to the VA-VL thalamus linked to the caudal forelimb area of M1

We next examined the extra-cerebellar regions targeted by the $IntA$ by comparing the projection patterns of $IntA^{Ucn3}$ and non- $IntA^{Ucn3}$ neurons with projections from all $IntA$ neurons. Labeled puncta in target regions colocalize with vGluT2, except in the dorsal nucleus of the inferior olivary complex (IOD), indicating that they represent glutamatergic synaptic terminals (Figure 18A-O, Figure 19A-I).

We quantified the distribution patterns of all $IntA$ axonal projections in six regions, and converted the incidence of puncta into a heat map coding for minimum and maximum percentage values, and organized the values from lowest to highest (Figure 19S, Appendix I). Even though both $IntA^{Ucn3}$ and non- $IntA^{Ucn3}$ neurons project to the same regions, puncta originating from each population are differentially distributed (Figure 19S, Appendix I). In the descending pathway, towards the brain stem, we found sparse $mCherry^+$ puncta in the IOD and LRt consistent with studies in the rat (Teune et al., 2000) (IOD, Figure 19-C; LRt,

Figure 18. Genetic and viral tracing reveal extracerebellar regions targeted by $\text{IntA}^{\text{Ucn3}}$ neurons. (A, D, G, J, M) Regions with mCherry+ puncta are magnified images denoted by red boxes in the schematics of coronal

sections to the left. (B, E, H, K, N) Further magnification of images denoted by red boxes in letters A, D, G, J, M. Few YFP and mCherry puncta colocalize in the inferior olive dorsal (IOD) (B) and lateral reticular nucleus (LRt) (K). Substantial YFP and mCherry puncta colocalize in the magnocellular part of the red nucleus (RMC) (N), with much less colocalization in, intermediate gray region of the superior colliculus (InG) (H), and zona incerta ventral (ZIV) (D). (C, F, I, L, O) Analysis of five regions that receive projections from IntA^{Ucn3} neurons. mCherry⁺YFP⁺ puncta within the IOD do not express vGluT2 (C). The majority of mCherry⁺ (red) and YFP⁺ (green) puncta from IntA^{Ucn3} neurons colocalize with vGluT2 (blue) within the ZIV (F), InG (I), LRt (L) and RMC (O).

Figure 19J-L; Appendix I). Less than approximately 15% of total mCherry puncta were found in each of these two regions (Appendix I). Furthermore, a scant ~25% of these mCherry⁺ puncta in the IOD and LRt belonged to IntA^{Ucn3} neurons (Figure 19S; Appendix I). These results suggest that IntA neurons sparsely project to the IOD and LRt, and that these are likely not the major targets of IntA^{Ucn3} neurons.

Next, we assessed the efferent targets in the ascending pathway, towards the midbrain and forebrain. We observed mCherry puncta in several regions on the contralateral hemisphere in the magnocellular part of the red nucleus (RMC, Figure 18M-O), intermediate gray region of the superior colliculus (InG, Figure 18G-I), ventral part of the zona incerta (ZIV, Figure 18D-F) and the VA-VL thalamus (VA-VL, Figure 19A-I). The red nucleus and the VA-VL are major targets of the interposed nucleus (Teune et al., 2000). Consistently, more than half of all mCherry⁺ puncta are found in the RMC and VA-VL (Figure 18M-O; Figure 19A-I; Appendix I). To investigate the potential distinction in distribution patterns between IntA^{Ucn3} (mCherry⁺/YFP⁺, yellow) and non-IntA^{Ucn3} (mCherry⁺/YFP⁻, red) populations, we segregated the quantified values of IntA^{Ucn3} (mCherry⁺/YFP⁺, yellow) and non-IntA^{Ucn3} (mCherry⁺/YFP⁻, red) terminations. As compared to non-IntA^{Ucn3}, we found a

Figure 19. Genetic and viral tracing reveal extracerebellar regions targeted by IntA^{Ucn3} neurons. (A) Top: Schematic of thalamic region with puncta from IntA^{Ucn3} neurons. Redbox denotes VA-VL subregion. Bottom: Puncta from virus traced IntA neurons expressing mCherry. (B-E) Puncta from IntA^{Ucn3} neurons that express both YFP (green, B) and mCherry (red, C) terminate outside the CB⁺ region (blue, D) in the anterior VA-VL

(merge, E). (F-I) YFP⁺ fibers (green, F) are found in a subregion of vGluT2L (red, G) and CB⁻ (blue, H) zone (merge, I). (J) Quantification of the area of vGluT2 puncta within CB⁺ and CB⁻ region of the VA-VL and YFP⁺ vGluT2 puncta (CB⁺ puncta: $5.98 \pm 0.27 \mu\text{m}^2$, CB⁻ puncta: $13.39 \pm 0.68 \mu\text{m}^2$, YFP⁺ puncta: $12.35 \pm 0.76 \mu\text{m}^2$, Kruskal-Wallis test $P < 0.0001$, mean $\pm$ s.e.m., $n = 201$). (K-R) Analysis of the size of vGluT2⁺ (red, L, P) and GAD67⁺ puncta (blue, M, Q) reveal two regions of the VA-VL thalamus. YFP⁺ (green, K) puncta are located within the CB⁻ region, but not the CB⁺ region (O) in a *Ucn3::Cre; Rosa::lsL.ChR2* mouse. YFP⁺ puncta express vGluT2, but not GAD67 (N). (S) Heat map of the relative distribution of traced puncta from IntA^{Ucn3} neurons (yellow, YFP⁺mCherry⁺) and other IntA neurons (red, YFP⁻mCherry⁺) to six major regions. Scale bar = 10 μm (A, C, E, G, I), 5 μm (B, G, I), 10 μm (E), 15 μm (C, K-H).

greater proportion of IntA^{Ucn3} puncta within the VA-VL than the other five targeted regions (Figure 19A-I; Appendix I). Our double genetic and viral tract tracing results show that IntA neurons differentially project to extra-cerebellar targets and that VA-VL is a major efferent target of IntA^{Ucn3} neurons.

To define the contribution of IntA^{Ucn3} neurons to the cerebello-thalamo-cortical pathway, we examined the axonal terminals within VA-VL thalamus in detail. First, we characterized the subregions of the VA-VL according to its molecular and morphological distinctions as previously described (Kuramoto et al., 2011) (Figure 19F-R). By means of immunohistochemistry, we identified three distinct subregions of the VAVL: a region containing a high density of large vGluT2 puncta, a region containing a low density of small vGluT2 puncta (Kuramoto et al., 2011) (Figure 19F-R), and a small zone within the large vGluT2 puncta subregion expressing calbindin expressing neurons (Kuramoto et al., 2011) (Figure 19F-R). Second, we assessed the spatial organization of IntA^{Ucn3} axonal terminals within the VA-VL. We found a high incidence of puncta from IntA^{Ucn3} neurons residing specifically in the lateral region of the vGluT2 large puncta and calbindin negative region

(Figure 19F-R). Third, we assessed the source of YFP⁺ puncta within the VAVL. Apart from the cerebellar nuclei, three major regions known to project into the VA-VL are substantia niagra, globus pallidus and reticular nucleus. We found that these three regions in *Ucn3::Cre; Rosa.lsl.ChR2-eYFP* brain do not contain recombined cell bodies (Figure 20A-M), indicating that the YFP⁺ puncta found within the VAVL are solely derived from the cerebellar nuclei.

Figure 20. Cre expressing puncta within the VA-VL is distinctively derived from IntA. (A, E, I) MAP2 expression (gray) as a counterstain on coronal sections of *Ucn3::Cre; Rosa.lsl.ChR2-eYFP*. (B-D, F-H, J-L) Magnified images derived from the regions demarcated within the red box in letters A, E, I. Respectively, they are targeting the substantia niagra, reticular part (SNR), globus pallidus (GP) and reticular nucleus (Rt). YFP expression (C, G, K) from the three regions are colocalized with the respective MAP2 (red) expression and counterstain DAPI (blue) (D, H, L). (M) A schematic of known VAVL afferents (YFP⁺, green; YFP⁻, gray).

Motor and sensory cortices receive projections from thalamic neurons derived from vGluT2L calbindin-negative zone (Kuramoto et al., 2011; Kuramoto et al., 2009). To examine this connection, we injected retrograde tracer, cholera toxin subunit B (CTB) conjugated with fluorophore-555, into the caudal forelimb area of the M1 region (Tennant et al., 2011) (Figure 21A, B). In the VA-VL ipsilateral to the injection site, we observed YFP⁺ puncta positioned close to retrogradely labeled CTB-555⁺ somata (Figure 21C-P) suggesting that IntA^{Ucn3} neurons communicate with the forelimb associated region of the motor cortex via neurons in the VA-VL (Tennant et al., 2011).

To examine the connectivity and collateral organization between the IntA and the spinal cord, we injected Fluoro-Gold (FG), a retrograde tracer, into the cervical 6-thoracic 1 segment of the spinal cord (Figure 22A-B), a region consisting of a large population of last-order interneurons associated with locomotion, reaching and grasping (Alstermark and Isa, 2012). Because IntA^{Ucn3} neurons give rise to nucleocortical projections in the forelimb associated region of the cerebellar cortex (Figure 17M, N), we examined whether these neurons targeted the spinal cord segment important for controlling forelimb movement. We examined the cerebellum and medulla oblongata for retrogradely FG labeled cell bodies. We did not find any FG labeled neurons in the cerebellar nuclei. We explored other brain regions that could serve as nodes to link the cerebellum and spinal cord to control forelimb movement. We identified FG labeled vestibulospinal neuronal cell bodies residing ventral to the cerebellar nuclei, specifically within the lateral vestibular nucleus, lateral and medial vestibular nucleus (Goldberg and Cullen, 2011; Murray et al., 2018) (Figure 22C-F). In addition, we observed FG labeled mossy fiber rosettes within the IGL of cerebellar vermis, lobules 1 Cb and 4/5 Cb (Figure 22G). Our results and previous studies have shown that mossy fiber collateral projections in 1 Cb, 2 Cb and 4/5 Cb belong to neurons located in the

Figure 21. $\text{IntA}^{\text{Ucn3}}$ neurons constitute a specific element of the cerebello-thalamo-cortical network. (A, B) Retrograde CTB injection site in the caudal forelimb area (CFA). (C, D) CTB^+ cell bodies (red) (C) overlap in the anterior VA-VL region containing fibers from $\text{IntA}^{\text{Ucn3}}$ neurons (green) (merged, D). (E-H) A magnified region from the white box in D, colocalization with DAPI in white (H). (I-P) Coronal planes of the thalamus containing retrogradely labeled neurons (I, K, M, O), colocalized with YFP expression derived from the double transgenic mouse (J, L, N, P) arranged from the anterior to the posterior. Age of mice: P56-63.

Figure 22. Vestibular neurons give rise to vestibulospinal projection and vestibulocerebellum mossy fiber in the cerebellar vermis. (A) Schematic of Fluoro-Gold (white) injection in the spinal cord. (B) Site of injection within the dorsal horn of the spinal cord. (C-E) Coronal sections of the cerebellum and vestibular nuclei, consisting of the cerebellar-VeCb, lateral-LVe, medial-MVe and superior-SuVe vestibular nuclei. (F) Higher magnification derived from red box in C, FG labeled vestibular neurons. (G) A zoom in of the cerebellar cortex from C, depicts FG labeled mossy fiber rosettes restricted within the cerebellar vermis.

cerebellar nuclei (Ruigrok et al., 2014) (Appendix H) and the vestibular nuclei (Lee et al., 2014).

We observed mossy fiber rosettes within the vermal region of the cerebellar cortex labeled with FG (Figure 22F, G). Spinocerebellar neurons with collateral axon terminals within the injection site may have contributed to this observation by transporting FG along its spinocerebellar axonal projections (Bras et al., 1988). In addition, no cerebellar nuclei neurons are found to be retrogradely labeled. Thus, the FG traced mossy fiber rosettes could be contributed by either vestibulocerebellar or ventral spinocerebellar tract neurons, or both

populations of neurons. Thus, our analysis uncovered cerebellar cortex 4/5 Cb as a potential neuroanatomical site for interaction and integration of IntA, vestibular nucleus and spinal cord efferent signals.

Together, my mapping analysis indicates that IntA^{Ucn3} neurons have a distinct intra- and extra-cerebellar connectivity pattern compared to non-IntA^{Ucn3} neurons. Furthermore, we identified specific downstream components of IntA^{Ucn3} neurons that may play an important role in the coordination of limb motor activities.

2.4.1.7 Ablation of IntA^{Ucn3} neurons perturbs the accuracy of skilled forelimb reaching

To evaluate the role of IntA^{Ucn3} neurons in the regulation of limb motor functions, we employed a chemogenetic strategy to selectively ablate these neurons (Figure 23A, B). We microinjected AAV2-*EF1a::double floxed-DTR-eGFP* into one hemisphere targeting the cerebellar nuclei of *Ucn3::Cre* reporter mice (Figure 23A). Two weeks of virus incubation, a subset of TBR1⁺ IntA neurons expressed DTR-eGFP (Figure 23C). We introduced diphtheria toxin via intraperitoneal injection three weeks after the initial injection of the virus (Figure 23B). After one week of diphtheria toxin treatment, we analyzed the cerebellar nuclei by staining for TBR1 expression and quantifying the number of TBR1 expressing neurons (Figure 23C-G). We observed a reduction of approximately a third population of TBR1⁺ neurons, indicating a close to total ablation of IntA^{Ucn3} neurons (Figure 23C-G).

We assessed the overall locomotion and motor coordination of IntA^{Ucn3}-ablated mice by subjecting them to an open field assay and a rotating rod test. We found that the distance

Figure 23. Ablation of $\text{IntA}^{\text{Ucn3}}$ neurons disrupts the accuracy of forelimb-skilled reaching. (A-B) Strategy for the neuron specific ablation of $\text{IntA}^{\text{Ucn3}}$ neurons (A), and the experiment timeline (B). (C-D) Cre-dependent expression of DTR-GFP (green; C) counterstained with DAPI (blue; D). (E-F) Cre-dependent ablation of $\text{IntA}^{\text{Ucn3}}$ neurons in Cre-negative mouse (E) versus Cre-positive mouse (F), counterstained with

TBR1 (red). (G) Quantification and analysis of the number of ablated TBR1 positive IntA neurons in control and ablated mice. (H-I) Analysis of open field distance (H) and speed (I), and rotating rod test (J). (K) Schematic of forelimb-skilled reaching set-up. (L) Success rate (as a percentage) in staircase skilled reaching of control and IntA^{Ucn3}-ablated mice (overall: 53±2, ablated: 39±3, two-way ANOVA, p=0.016). (M) Representations of the phases of reaching (insets). Error rate (as a percentage) of control and ablated mice (reach, control: 37 ± 13, ablated: 88 ± 5, p = 0.0025; grasp, control: 16 ± 8, ablated: 12 ± 5, p = 0.985; retrieve, control: 27 ± 11, ablated: 20 ± 6, p = 0.956). (N) A heatmap coding for reaching endpoints of ablated mice (white line runs through pellet position). (O) IntA^{Ucn3}-ablated mice approached the food pellet with a 25° deviation (from pellet) compared to control mice (control: 5.7 ± 10.1, ablated: -24 ± 7.23, P = 0.02). (P) Nose position in the staircase during the task is comparable between control and ablated mice (control: x: 0.49 ± 0.12, ablated x: 0.39 ± 0.12, P = 0.39; control, n = 10, ablated, n = 10 animals).

and speed traveled of ablated mice compared to control mice during the open field task were not significantly different between experimental groups (Figure 23H, I) (distance, control: 3472 ± 265.4 cm, ablated: 2841 ± 270.5 cm, P=0.34, speed, control: 8.34 ± 0.65 cm/s, ablated 6.89 ± 0.61 cm/s, P=0.34). Moreover, the rotating rod test of both groups of mice showed similar performance (Figure 23J) (control: 71.56 ± 4.43 s, ablated: 71.98 ± 5.45 s, P=0.40).

To investigate the function of IntA^{Ucn3} neurons in discrete forelimb movements, we trained IntA^{Ucn3}-ablated and control mice to perform a staircase reaching task (Klein and Dunnett, 2012) (Figure 23K). The staircase reaching task is designed for examination of the competence and kinematics of skilled reaching in mice, this task is similar to the single pellet reaching task (Klein and Dunnett, 2012). We found that ablated mice exhibited a higher rate of unsuccessful food pellet reaches compared to the control mice (Figure 23L). To further assess which segments of the skilled reach motion is influenced by the loss of IntA^{Ucn3} neurons, we deconstructed the skilled reaching movement into three phases; reach, grasp and

retrieve phases (Figure 23M inset). We analyzed the error rate for reaching for the food pellet, grasping of the food pellet, and retrieving the food pellet for consumption. We uncovered that the reaching phase was prominently disrupted in the ablated group compared to the control group (Figure 23M). The reaching error rate in ablated mice was more than two-folds more than the control (Figure 23M). In contrast, grasping and retrieving abilities of $\text{IntA}^{\text{Ucn3}}$ -ablated mice was not affected (Figure 23M).

We further study the reaching phase for the precise position of the paw during each motion, by plotting the endpoints of each reach attempt in a two-dimensional plane and the angle between the point vertically intersecting the pellet and the end position of the paw. Our analysis showed that control mice reach for the food pellet in a shortest possible trajectory targeting the upper most portion of the pellet with errors equal probability of under reach and over reach errors (Figure 23N). On the other hand, ablated mice over extended their forelimb before grasping the pellet followed by retrieving (Figure 23N). We quantified the angle of the paw at each endpoint from the pellet during the reach phase and discovered that control mice show near zero divergence from the pellet, ablated mice exhibited over-reach with approximately 25° divergence from the pellet (Figure 23O).

The staircase skilled reaching task permits measurement of forelimb reaching parameters with marginal constraints imposed on the mice. To avoid inaccuracy in our measurements, we analyzed the position of the mouse's nose during each reach. We found that both $\text{IntA}^{\text{Ucn3}}$ -ablated and control mice positioned themselves in a similar manner during the reaching task (Figure 23P; control: $x: 0.49 \pm 0.12$, ablated $x: 0.39 \pm 0.12$, $P = 0.39$). As a result of our analysis, the lack of $\text{IntA}^{\text{Ucn3}}$ neurons disrupts precision to reach for a target whereas other characteristics of prehension appears to be impervious. Additionally, the

ablation of $\text{IntA}^{\text{Ucn3}}$ neurons appears to cause the forelimb to miscalculate the trajectory towards the intended goal indicating that $\text{IntA}^{\text{Ucn3}}$ neurons are required for accurate forelimb positioning.

2.4.1.8 Consequences of the ablation of $\text{IntA}^{\text{Ucn3}}$ neurons on rhythmic forelimb movement

2.4.1.8.1 Elimination of $\text{IntA}^{\text{Ucn3}}$ neurons alters the timing of locomotion

To determine whether $\text{IntA}^{\text{Ucn3}}$ neurons also regulate forelimb positioning during rhythmic movement, we examined the footprint pattern of control and $\text{IntA}^{\text{Ucn3}}$ -ablated mice in a directed locomotor task, and analyzed the velocity and displacement of ipsilateral forelimb across each stride. We adapted and built a mouse gait apparatus that provides a means of quantifying the kinematic parameters of a freely walking mouse (Mendes et al., 2015) (Figure 24A, B). The locomotion paradigm is designed for individual mouse to walk through a straight and narrow corridor (Figure 24A). The spatial and temporal patterns of their footprints are recorded and analyzed using non-invasive detection together with a recording camera (Figure 24A).

The method of non-invasive detection is based on the total internal reflection of light within a transparent acrylic glass (Mendes et al., 2015) (Figure 24A). Light is introduced by LED surrounding the acrylic glass (Figure 24A). A disruption of total internal reflection by foot placement on the transparent acrylic glass leads to a scattering of light that is visible underneath surface of the acrylic glass, consequently serving as a touch sensor (Figure 24A). To visualize the mouse body, a light box is placed above the walkway casting a silhouette of the mouse as the animal walks down the corridor (Figure 24A). Finally, a mirror is placed at a 45° angle beneath the walkway in order to deflect the scattered light signals from the

Figure 24. Ablation of $\text{IntA}^{\text{Ucn3}}$ does not perturb the x-axis of strides in freely walking mice. (A) Schematic of set-up. (B) Image of paw print analysis. (C) Velocity of ipsilateral forelimb movement in control: 0.316 ± 0.017 mm/ms; ablated: 0.32 ± 0.019 mm/ms; $P = 0.8262$. (D) x-displacement of ipsilateral forelimb in control: 52.38 ± 1.30 mm; ablated: 49.55 ± 1.07 mm; $P = 0.0848$. A total of 31 strides from two mice were analyzed using Mann-Whitney U test. (E) A schematic of the DCN with optic fiber placement in *Ucn3::Cre*; *Rosa::Isl.ChR2-eYFP*. (F) A representative image of fiber tract targeting $\text{IntA}^{\text{Ucn3}}$ neurons (green; GFP) (G) Timeline of the experiment. (H, I) With or without stimulation (blue bar=light-ON; light-OFF: 35.1 ± 6.5 Hz, ON: 75.3 ± 6.5 Hz) and non-ChR2-expressing IntA neurons (J, K) with or without stimulation (light-OFF; 12.0 ± 2.2 Hz, light-ON: 12.3 ± 2.5 Hz). The graphs in I and K are plotted as normalized frequency (norm FR) in

order to better reflect changes in frequency. In these graphs, baseline frequency is normalized as 1. YFPON-L, n=15; YFPOFF-L, n=9; YFPOFF-S, n=9. (L) Swing velocity of ipsilateral forelimb during light off: 0.23 ± 0.02 mm/ms; light-on: 0.19 ± 0.02 mm/ms; $P=0.0069$. (M) x-displacement of ipsilateral forelimb during light-off: 48.26 ± 1.86 mm; light-on: 44.83 ± 1.28 mm; $P=0.0319$. (N) Schematic of the control; cerebellar nuclei with optic fiber placement in *Rosa::ls/ChR2-eYFP*. (O) Swing velocity of ipsilateral forelimb during light-off: 0.35 ± 0.04 mm/ms; light-on: 0.46 ± 0.09 mm/ms; $P = 0.56$. A total of 12 strides from two control mice, 42 ipsilateral from three photostimulated mice were collected for analysis using Wilcoxon matched-pairs signed rank test.

footprints and body outline which are captured by a video camera (Figure 24A, B). We analyzed the video recordings using Adobe Photoshop by plotting every footprint and measuring of the displacement and timing of consecutive footprints (Figure 24B). We focused our analysis on walk gait, defined as only one leg swinging at any one point. We found that $\text{IntA}^{\text{Ucn3}}$ -ablated mice exhibited no significant difference in displacement and time of each full step compared to control mice. Although not statistically significant, we observed that $\text{IntA}^{\text{Ucn3}}$ -ablated mice walked with faster (control: 0.316 ± 0.017 mm/ms; ablated: 0.32 ± 0.019 mm/ms; $P = 0.8262$) and shorter strides (control: 52.38 ± 1.30 mm; ablated: 49.55 ± 1.07 mm; $P = 0.0848$) (Figure 24C, D).

4.1.8.2 Photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons perturbs forelimb gait parameters

Optogenetics manipulation of channelrhodopsin and archaerhodopsin expressing Purkinje cells in the simple lobule respectively leads to delayed and short-latency forelimb movements (Lee et al., 2015; Witter et al., 2013). However, influences of the indirect activation and inactivation of Purkinje cells in the simple lobule on the activities of deep cerebellar neurons are likely not be specific to an individual cerebellar nucleus since Purkinje cells in the simple lobule send axonal projections into both the interposed and medial nuclei

(Lee et al., 2015; Witter et al., 2013). Due to the lack of genetic tools, consequences of direct manipulation of neurons in a specific cerebellar nucleus on limb movement has not been achieved.

Because we observed changes to forelimb gait parameters after the ablation of $\text{IntA}^{\text{Ucn3}}$ neurons, we set out to evaluate how transient and reversible manipulation of neurons in a specific cerebellar nucleus ($\text{IntA}^{\text{Ucn3}}$ neurons) might impact the forelimb movement. To express light-gated ion channels in IntA neurons, we generated transgenic mice carrying the *Ucn3::Cre* and *Rosa::lsf.ChR2-eYFP* alleles (Figure 24E-G). We first evaluated the photoresponse of ChR2-expressing $\text{IntA}^{\text{Ucn3}}$ neurons by conducting *ex vivo* cerebellar cell recording (Figure 24H-K). Light stimulation of ChR2-eYFP-expressing glutamatergic neurons led to more than 200% increase in firing frequency (light-off: 35.1 ± 6.5 Hz, light-on: 75.3 ± 6.5 Hz) (Figure 24H, I). In contrast, non-ChR2-expressing presumptive inhibitory neurons did not react to light stimulus (light-off: 12.0 ± 2.2 Hz, light-on: 12.3 ± 2.5 Hz; YFPON-L, n=15; YFPOFF-S, n=9) (Figure 24J-K).

To investigate the behavioral impact of photostimulating $\text{IntA}^{\text{Ucn3}}$ neurons, we implanted an optic fiber unilaterally in the cerebellar nuclei in order to introduce laser pulses onto ChR2-eYFP expressing $\text{IntA}^{\text{Ucn3}}$ neurons. We examined the consequence of photostimulation on gait kinematics of the ipsilateral forelimb using the freely walking task described earlier (Figure 24L-O). We found that the footprints of light stimulation of $\text{IntA}^{\text{Ucn3}}$ neurons during walk gait was significantly shorter in displacement and slower in speed when compared to trials without light stimulation (displacement, light-off: 48.26 ± 1.86 mm; light-on: 44.83 ± 1.28 mm; $P = 0.0319$; speed, light off: 0.23 ± 0.02 mm/ms; light-on: 0.19 ± 0.02 mm/ms; $P = 0.0069$) (Figure 24L, M). Moreover, when the mice were not moving, light-activation of

IntA^{Ucn3} neurons at did not ensue any form of forelimb movement, indicating that IntA^{Ucn3} neurons do not seem to control the initiation of forelimb movement (data not shown). We also did not observe any changes in forelimb movement in photostimulated trials of *Rosa::lsl.ChR2* control mice (light-off: 0.35 ± 0.04 mm/ms; light-on: 0.46 ± 0.09 mm/ms; $P = 0.56$) (Figure 24N, O). Taken together, we show that IntA neurons control the speed and displacement of rhythmic limb movement.

2.4.1.8.3 Photostimulation of IntA^{Ucn3} neurons disrupts the positioning and timing of forelimb movement during locomotion

The variations in walk gait parameters derived from analysis of the footprint of IntA^{Ucn3} ablated and light-stimulated mice prompted us to examine the forelimb trajectories of individual stride in more detail. To investigate the forelimb trajectory during a stride, we re-designed and built a transparent narrow corridor that limits the path of mouse movement to one direction. During each trial, we recorded the lateral profile of limb movement ipsilateral to the manipulated cerebellar hemisphere (Figure 25A, B). To examine the dynamics of the forelimb movement during locomotion, we divided each stride into two phases based on studies in the cat (Bracha et al., 1999). The first phase, stance, is defined as the period when the paw is in contact with the ground. The second phase termed as swing is defined as the segment of stride when the paw is in the air. We further categorized the swing phase into swing-flex and swing extend (Figure 25B).

We found that IntA^{Ucn3}-ablated mice spent approximately 15% (4.8 ms) less time in the swing-flex phase as compared to control mice (Figure 25F). Consequently, the forelimb of IntA^{Ucn3}-ablated mice reached its maximum height approximately 16% (16.6 ms) earlier than control mice (Figure 25G). Although less time was spent in the swing phase, IntA^{Ucn3}-ablated

Figure 25. Manipulation of $\text{IntA}^{\text{Ucn3}}$ perturbs specific phases of forelimb trajectory in freely walking

mice. (A) Schematic and dimensions of the locomotor behavioral paradigm. (B) Representative images of the various stride phases, including stance and swing phases, with the swing phase subdivided into swing-flex and swing-extend phases. (C) Two-dimensional traces of stride trajectory from a control (gray) and an $\text{IntA}^{\text{Ucn3}}$ -

ablated mouse (black). (D-E) Analysis of the x-displacement (Δx) over time (D) and y-displacement (Δy) over time (E). (F-I) Analysis of forelimb trajectory duration of swing-flex (F), maximum height reached (y max.) (G), Δy during swing-flex phase (H), and Δy during swing extend phase (I). (J) Two-dimensional traces of stride trajectory from a light-off (gray) and an $\text{IntA}^{\text{Ucn3}}$ -ablated mouse (cyan). (K-L) Analysis of the x-displacement (Δx) over time (K) and y-displacement (Δy) over time (L). (M-P) Analysis of forelimb trajectory duration of swing-flex (M), maximum height reached (y max.) (N), Δy during swing-flex phase (O), and Δy during swing extend phase (P).

mice required approximately 15% (60.9 ms) more time to complete a stride. This increment was due to the increase of approximately 23% (56.2 ms) more time spent in stance duration, and approximately 5.0 ms ($P = 0.220$) more time in swing phase (Appendix J). Notably, when measuring footsteps for duration, we observed a trend of decreased swing velocity but the trend is not statistically significant.

In addition, we found that the trend of kinematic parameters measured from photostimulated mice in our lateral profile limb movement analysis (Figure 25J-P) is similar to the previously analysed footprint measurements. Although not significantly different, we showed that each stride was slower (46.6 ms) and x displacement was shorter (1.69 mm) (Figure 24L, M). Interestingly, we found that acute optogenetic manipulation of $\text{IntA}^{\text{Ucn3}}$ neurons led to significant differences only in forelimb positioning, but not timing (Figure 25J-P). We observed close to 43% (1.95 mm) increase in maximum height reached in photostimulated mice compared to no stimulation (Figure 25N). Next, we assessed the changes in y displacement during flex-phase and extend-phase. We found that photostimulation led to an even larger increase of 63% (1.268 mm) than the extend-phase at 38% (1.406 mm) when compared to without stimulation (Figure 25O, P).

Taken together, analysis of the consequences of elimination and acute manipulation of IntA^{Ucn3} neurons on skilled reach and locomotion revealed that a specific genetically defined neuronal subpopulation within the IntA represents a key regulator of vertical positioning and timing of forelimb actions, and that these neurons are required for maintenance of the precision of discrete and rhythmic limb movements.

2.5. DISCUSSION

2.5.1 Accessibility to the cerebellar interposed nucleus with the *Ucn3::Cre* mouse line

The identification of transgenic mice with cell- or region-specific expression of Cre recombinase or reporters provides an opportunity to correlate gene expression with individual cell types. Moreover, the utility of such transgenic mice provides means to reproducibly analyze the function of the specific cell types within the brain, especially in brain regions containing heterogeneous subtypes. Our candidate screening of the recombination patterns of Cre mouse lines in the cerebellum led to the identification of *Ucn3::Cre* line as a powerful genetic tool to study neurons within the IntA nucleus. Due to concerns for BAC transgene integrity and fidelity, we characterised the expression of offsprings (F1 generation) derived from separately crossing *Ucn3::Cre* Tg(Ucn3-cre)KF31Gsat/Mmucd with four different reporter lines (*Rosa::lsl.eGFP*, *Rosa::lsl.ChR2-eYFP*, *Rosa::lsl.ChR-tdTomato* and *Rosa::lsl.Sun1-GFP*) (Appendix C). Our results show that the *Ucn3::Cre* F1 generation possesses a stable and reproducible Cre recombination pattern. Recombined cells were found in the same distinct regions even when crossed to different reporter lines.

Our results for the recombination pattern of *Ucn3::Cre* is consistent with images found in the Allen Brain Atlas and GENSAT BAC-Cre driver lines database (Gong et al., 2003; Madisen et al., 2012). However, a previous study reported that *Ucn3* expression may not present in the cerebellum (Lewis et al., 2001). Consistent with this, our chromogenetic ISH and microarray results showed that the endogenous expression of *Ucn3* is very low or not detectable compared to the ML and vestibular nucleus (Figure. 12A-F). However, additional analysis should be carried out before reaching a conclusion because previous studies using Northern blot analysis on whole cerebellar samples and chromogenic ISH technique may not be sufficiently sensitive. There remains a possibility that *Ucn3* is expressed in the cerebellar

nuclei because the BAC transgenic line contains a high copy number of BAC transgene and could reveal the presence of a low level of gene expression. One method to resolve this possibility is to employ real time-polymerase chain reaction (RT-PCR) using IntA nucleus samples from different developmental stages, as RT-PCR provides a more sensitive assay and samples from different developmental stages may be able to determine the possibility of Cre accumulation during development.

2.5.2 Functional significance of the intra-cerebellar connectivity of neurons in the IntA

Nucleocortical mossy fibers arising from the cerebellar nuclei to the cerebellar cortex are organized in an orderly manner (Umetani, 1990). Additionally, the cerebellar cortex is organized topographically (Voogd, 2003). Thus, the specificity of intra-cerebellar targets by neurons in the IntA reveals insights to organization and functional significance of these neurons. We observed that IntA^{Ucn3} neurons send nucleocortical mossy fibers primarily to lobules IV to V and the simple lobule, while other IntA neurons send additional nucleocortical mossy fibers to Crus1 and Crus2 (Figure 17M, N; Appendix H). Based on delineations by olivo-cortico-nuclear connectivity, these lobules represent the forelimb subdivision of the cerebellum (Voogd, 2003). Lobules IV–V and the simple lobule receive MFs from the forelimb and hindlimb through cuneocerebellar and spinocerebellar tracts, respectively, and limited MFs from vestibular and trigeminal nuclei (Altman and Bayer, 1997). In addition, there is a convergence of MFs in lobules I–V from LRt neurons that receive internal copies of motor signals from propriospinal neurons (Alstermark and Ekerot, 2013; Altman and Bayer, 1997; Azim et al., 2014b). Within this general region, lobules I–III receive MFs from the LRt that are carrying information from the hindlimb, whereas lobules IV and V receive MFs that are carrying information from the forelimb (Altman and Bayer,

1997). Thus, the specific targeting of nucleocortical mossy fibers IntA^{Ucn3} neurons indicates that these neurons play a role in internal closed-loop circuitry within the cerebellum to mediate forelimb movement.

Recently, internal feedback provided by cerebellar nucleocortical mossy fibers has been implicated in adaptive sensorimotor processing (Ankri et al., 2015; Houck and Person, 2015). In principle, corollary discharges generated by the cerebellar nuclei can significantly reduce the time required for signal integration and predictions to initiate new movements compared to sensory feedback from the peripheral system (Perrone and Krauzlis, 2008). In our study, we show that nucleocortical mossy fibers terminate in close proximity to Golgi cells somata and putative granule cells (Figure 17E-H). In line with a recent report, excitatory IntA neurons innervate Golgi cell and granule cell dendrites as part of a nucleocortical closed-loop circuitry of the cerebellum to relay corollary discharge of premotor signals and amplify this circuitry in controlling motor-related function (Gao et al., 2016). Altogether, the specific targeting of nucleocortical mossy fibers IntA^{Ucn3} neurons suggests that these neurons represent elements of an internal feedback circuit and relay signals that converge with forelimb movement-related information from the spinal cord to modify these sensory and motor signals.

2.5.3 Functional significance of the extra-cerebellar connectivity of neurons in the IntA

The cerebellar cortex has been proposed to consist of topographical units determined to be as small as 100 to 300 μm wide (Ito, 1997; Oscarsson, 1979). These units, termed as microzones, are determined by subdivisions of Purkinje cells receiving similar climbing fiber receptive fields (Apps and Hawkes, 2009). While the cerebellar cortex consists of 12 $\sim$ 1 mm spanning longitudinal zones and each zone can be further subdivided into multiple microzones, the cerebellar nuclei has been described to consist of 12 topographical units (Buisseret-Delmas and Angaut, 1993; Voogd and Bigaré, 1980). Thus, neuronal subpopulations in the cerebellar nuclei are likely to be involved in controlling different aspects of motor functions.

The cerebellum computes sensory information and internal motor commands to generate refined motor behavior, primarily through cerebello-thalamo-cortical and cerebello-rubro-spinal pathways (Franklin and Wolpert, 2011; Horne and Butler, 1995; Keifer and Houk, 1994). Our results indicate that IntA^{Ucn3} neurons are capable of influencing motor activities by targeting both the thalamus and red nucleus, but principally project to the cerebello-thalamo-cortical pathway. Although glutamatergic IntA^{Ucn3} and non-IntA^{Ucn3} neurons project into overlapping regions, the differential innervation patterns potentially modulate specific sets of movements.

Mapping the precise thalamic target regions by IntA^{Ucn3} neurons may provide insight to their function. For example, the neurons within the VA-VL are organized into CB⁻ and CB⁺ zones, and respectively project to layers II-V and layer I of cortical regions including the primary motor area, secondary motor area, primary somatosensory area, cingulate area,

frontal association area and limb areas (Kuramoto et al., 2009). Our cerebello-thalamo-cortical tract tracing results suggest that IntA^{Ucn3} neurons may influence the deep layers of cortical forelimb area. Additionally, motor cortical neurons in the caudal forelimb area have been shown to selectively engage in a short latency effector pathway for the control of trained forelimb reaching movement (Miri et al., 2017). Our tract tracing result shows indirect connection between the IntA to the caudal forelimb area via VA-VL provides a specific circuit element through which a subset of IntA neurons may differentially engage cortical activity pattern, and perhaps influence the activation of motor effector pathways in the motor cortex.

2.5.4 Role of the IntA in coordination of voluntary limb movement

Damage to the cerebellum often causes abnormalities in movement but not the loss of movement (Bastian, 2006). These abnormalities have characteristics of lack of coordination, decreased accuracy, inconsistency and tremor (Bastian, 2006). The cerebellum is thought to integrate internally directed copies of motor commands with ongoing peripheral feedback to provide online refinement throughout the movement (Bastian, 2006; Franklin and Wolpert, 2011). In addition to the role of providing feedback control, the cerebellum has been suggested to participate in planning of movement prior to the action (Bastian, 2006; Miall and Wolpert, 1996). Neurons in the cerebellar nuclei, as the major output neurons of the cerebellum, have been implicated in fine-tuning of forelimb movements in goal-oriented behavioral tasks in cat and monkey (Bracha et al., 1999; Kuper et al., 2011; Monzee and Smith, 2004). In order to investigate forelimb movement related pathways in the complex cerebellar circuits, mouse models serve as a powerful system due to the wide range of genetic tools available for manipulating specific cerebellar nuclei structures and neuronal subtypes.

The main challenge lies in targeting specific neuronal subpopulations within the cerebellar nuclei which consists of seemingly homogenous mix of neuronal populations. In addition, there is a lack of established assays to quantify the parameters of reaching and grasping in mice, such as position, direction and velocity. We based our analysis of the role of the cerebellar nuclei in controlling the kinematic parameters of skilled forelimb movement and locomotion on previously described assays (Azim et al., 2014a; Esposito et al., 2014; Fink et al., 2014; Klein and Dunnett, 2012). The identification of the *Ucn3::Cre* mouse line offers a means to assess the functional relevance of a genetically defined neuronal subpopulation in the cerebellar nuclei in behaviorally relevant contexts.

Electrophysiological studies conducted in monkeys showed that neuronal activities in the interposed and lateral nuclei influence ipsilateral motor behaviors (Harvey et al., 1979; Mackay, 1988). In human and cat, recordings and stimulation targeted at the interposed nucleus demonstrated its contribution in activation and inhibition of flexor and extensor limb muscle activities, respectively (Armstrong and Edgley, 1984; Nashold and Slaughter, 1969). In addition, a consequence of impaired interposed nucleus is the inability to compensate for muscle torques during reaching movement (Richter et al., 2004). Thus, a plausible role for $\text{IntA}^{\text{Ucn3}}$ neurons is to regulate positioning of forelimb movement in mice through coordination of the ipsilateral antagonistic muscles activities. We observed that ablation of $\text{IntA}^{\text{Ucn3}}$ neurons results in the early onset of the limb at its maximum vertical position of the stride, suggesting that $\text{IntA}^{\text{Ucn3}}$ neurons play a role in gating flexion movement (Appendix J, time to y max). In support of this notion, studies that analyzed stride parameters of Purkinje cell degeneration mice, a disease model with post-natal degeneration of cerebellar Purkinje cells, reported that while most kinematics were not affected, the vertical displacement of the forelimb is significantly increased (Machado et al., 2015; Mullen et al., 1976). Together,

Purkinje cells or cerebellar nuclei neurons influence the coordination of limb joint movements and alterations to these neurons cause discerning perturbation in vertical displacement, similar to patients with cerebellar damage (Bastian et al., 1996).

Signals encoding an array of kinematic parameter is discharged at multiple levels within the cerebellum (Ebner et al., 2011). Although the function of cerebellar output in ensuring appropriately timed movements is well accepted (Ivry et al., 2002), the role of the cerebellar nuclei in encoding temporal information and how effectors decode this signal are not well understood. Signals encoding error and prediction have been described mostly in the context of complex and simple spike firing of Purkinje cells (Ebner et al., 2011). However, information about how additional cerebellar neuronal subtypes are involved in well-timed motor events are lacking. Neuronal activities in the interposed and lateral nuclei of cat and monkey are related to speed and velocity of movement (Soechting et al., 1978; van Kan et al., 1993). Recordings performed in monkeys revealed that movement takes place after the onset of neuronal activity in the interposed and lateral nuclei, whereas neuronal activity in the medial nucleus follows the onset of movement (Bava et al., 1983; Thach, 1978).

We studied the control of onset and extent of forelimb positioning, and showed that $\text{IntA}^{\text{Ucn3}}$ neurons are capable of influencing the timing of limb movements. In addition, the dysmetria-like overreach performance by mice with $\text{IntA}^{\text{Ucn3}}$ neurons ablated suggests that the incapability of these mice to master the timing of movement necessary for precise forelimb targeting, supporting a proposed braking role of the IntA from studies in cats (Ekerot et al., 1997; Kitazawa, 1993). Consistent with the notion that the cerebellum controls and corrects ongoing motor activities, our study links a subpopulation of glutamatergic IntA

neurons and its distinctive intra- and extra-cerebellar circuitry with coordination of precise and effective movement.

2.6. FUTURE DIRECTIONS

2.6.1 Anatomical, functional and molecular diversity of neuronal subpopulations in the cerebellar nuclei for motor control

In our study, we have demonstrated the utility of *Ucn3::Cre* mouse line to delineate and manipulate neurons in the IntA. We characterized IntA^{Ucn3} neurons to be glutamatergic and primarily links to motor cortical regions via the VA-VL thalamic nucleus, but not directly to the spinal cord. Our chemogenetics ablation and optogenetics stimulation of IntA^{Ucn3} neurons resulted in the disruption of accurate prehension and locomotor kinematics. Together, our studies uncover a specific subpopulation of neurons within the cerebellar nuclei and its functional relevance, likely mediated by connectivity with ascending brain targets. Importantly, our results suggest the presence of additional specialized neuronal subpopulations in the IntA (non-IntA^{Ucn3}) and other cerebellar subnuclei that are required for fine-tuning movements via ascending and descending pathways. The following three major experiments are outlined to address unresolved questions.

Experiment 1. Define the identity of post-synaptic neurons connected to identifiable neuronal subpopulations within the cerebellar nuclei. The cerebellar nuclei target relevant motor centers to exert its role in motor control. Even though the projections made by the cerebellar nuclei have been mapped, the neuronal types receiving cerebellar inputs and the organization of neurons within the cerebellar nuclei are not well studied. Using Cre mouse lines driven by neurotransmitter-related promoters, monosynaptic retrograde rabies virus can be used to target neurotransmitter specific neurons at known cerebellar input regions to map the precise organization of projection neurons within the cerebellar nuclei and their post-synaptic neuronal types.

Experiment 2. Examine the functional significance of ascending versus descending pathways via specific optogenetic manipulation. Neurons within the cerebellar nuclei modulate a distinct kinematic parameter during movement. However, the mechanisms underlying the cerebellar influence on specific parameters remain unclear. We have shown how movement kinematics are disrupted as a consequence of manipulating ascending pathways in *Ucn3::Cre*. To compare our results to the function of descending pathway, a combination of gene inversion and double-flox technology can be used to target non-IntA^{Ucn3} neurons to express ChR2. In brief, inverted opsin gene flanked by double-flox sites (DIO) will only express the opsin in neurons that express Cre (Cre-ON). Non-inverted opsin gene flanked by double-flox sites (DO) will only express the opsin in neurons that do not express Cre (Cre-OFF). Next, we will examine the consequences of manipulating non-IntA^{Ucn3} neurons in skilled reaching and locomotor tasks.

Experiment 3. Characterize the molecular distinctions of neuronal subpopulations within the cerebellar nuclei. The specific circuit elements and function associated with IntA^{Ucn3} neurons versus non-IntA^{Ucn3} neurons indicate that neuronal subpopulations within the cerebellar nuclei are anatomically and functionally diverse, and may also be molecularly distinct. To profile the molecular signatures of neurons characterized by their projection pattern, we plan to retrogradely label cerebellar neurons at each cerebellar target region, isolate these neurons and perform single cell RNAseq. The identification of unique gene expression among neuronal subpopulations may provide the basis of developing novel genetic tools for future analysis. Furthermore, the data could reveal insights about the cerebellar nuclei molecular organization and axonal projection pattern.

CHAPTER 2:

NON-MOTOR CONTROL BY NEURONAL

SUBPOPULATIONS WITHIN THE DEEP CEREBELLUM

3. CHAPTER 2

3.1 INTRODUCTION

3.1.1 Role of the cerebellar nuclei in non-motor control

Over the past several decades, there is an accumulation of evidence supporting the functional role of the cerebellum beyond motor control and learning (Leiner et al., 1994; Schmahmann and Sherman, 1998; Stoodley and Schmahmann, 2018). For instance, the cerebellum has been implicated in cognitive, emotional and motivational processes (Buckner, 2013). However, the pathways mediating cerebellar influence on these non-motor functions are not well understood.

Lateral nucleus

There is growing evidence which suggests that excitatory cerebellar output neurons, particularly the lateral nucleus, contribute to non-motor functions (Ito, 2006; Locke et al., 2018; Schmahmann and Pandya, 1997). Attempts to define non-motor cerebellar-mediated pathways have taken a similar retrograde labeling approach by injecting HSV1 into the prefrontal cortex, a non-motor associated cerebral cortical region (Kelly and Strick, 2003; Middleton and Strick, 1997; Zemanick et al., 1991). Results from these studies demonstrated that the cerebellar lateral nucleus is indirectly linked to the prefrontal cortex via the mediodorsal thalamic nuclei, thus providing a neural substrate for the cerebellar influence of non-motor functions.

To investigate the connectivity between the cerebellar lateral nucleus and the prefrontal cortex, similar studies were conducted in rats (Angaut et al., 1985; Aumann et al., 1994;

Bauer et al., 2014; Haroian et al., 1981). Unlike the cerebellar-thalamo-cortical pathway discovered in primates, tract-tracing studies suggest that the rat cerebellar lateral nucleus is connected to the non-motor cerebral cortices through the ventroanterior-ventrolateral and ventromedial thalamic nuclei (Angaut et al., 1985; Aumann et al., 1994; Haroian et al., 1981). Additional support for this circuit in rats came from bilateral cerebellar lateral nucleus lesion studies which results in a ~30% reduction in basilar arbor and apical arbor in axonal projections in the prefrontal cortex arising from ventrolateral and ventromedial thalamocortical neurons (Bauer et al., 2014). Importantly, analysis of the behaviors of rats with bilateral electrolytic lesions of cerebellar lateral nuclei revealed a reduction in hedonic and purposive motivation (Bauer et al., 2014). Rats with lesion exhibited increased effort for obtaining each subsequent reward as well as an open field exploration task (Bauer et al., 2011). Together, these studies identified the cerebellar lateral nucleus as an important component of the cerebello-thalamo-cortical pathway which plays a critical role in non-motor function. However, the sources of relevant non-motor inputs required for the integration within the cerebellar lateral nucleus, and how these signals influence behaviour remain unresolved.

Interposed nucleus

A wide range of cognitive symptoms, affective and autonomic have been reported to be consequences of cerebellar lesions (Annoni et al., 2003; Damasio et al., 2000; Habas et al., 2009; Haines et al., 1997; Leggio et al., 2011; Schmahmann and Sherman, 1998; Schutter and Van Honk, 2005; Stoodley and Schmahmann, 2009). One clinical study reported that a patient with left cerebellar haemorrhage affecting parts of the cerebellar medial and interposed nucleus exhibited involuntary facial grimace, guttural “pathological laughter” and

flushing of the face whenever a movement was attempted with his left arm (O'Reilly et al., 2010). Similarly, a separate patient with unilateral cerebellar stroke which affected most of the cerebellar nuclei, except parts of the cerebellar lateral nucleus, experienced impaired autonomic reactions in reaction to negative situations (Haines et al., 1997). Studies in rats have shown that the cerebellar interposed nucleus is a region which consolidates memories for fear-conditioning (Sacchetti et al., 2002). In addition to the association between cerebellar interposed nucleus and emotional response, anatomical tract tracing studies have identified reciprocal connectivity between the cerebellum and the hypothalamus (Cavdar et al., 2001). Electrophysiological recordings in a subset of hypothalamic neurons had been reported to respond to cerebellar interposed nucleus and gastric vagal nerve stimulation (Zhu et al., 2006). Although these findings strongly suggest that the cerebellar interposed nucleus plays a role in emotional and visceral functions, there is currently very little direct evidence linking the interposed nucleus with regulation of non-motor processes.

Medial nucleus

The participation of the cerebellar medial nuclei in regulating non-motor related functions have emerged from numerous studies over the years (Dietrichs et al., 1994; Haines et al., 1997; Lisander and Martner, 1975a; Liu et al., 2000; Mahler et al., 1993; Martner, 1975; Min et al., 1989; Pu et al., 1995; Scalera, 1991; Wang et al., 1997). Similar to the cerebellar interposed nucleus, the cerebellar medial nucleus is bidirectionally interconnected to the hypothalamus implicating the cerebellar medial nucleus in motivational behaviors such as feeding (Dietrichs et al., 1994; Haines et al., 1997). Moreover, in non-human primates and cats, the cerebellar medial nucleus has been reported to project into the limbic system such as the hippocampus, septal nuclei, basolateral amygdalae and nucleus accumbens, to potentially

modulate emotional activities (Harper and Heath, 1973; Heath, 1976). A study supporting the role of the cerebellar medial nucleus in emotion revealed that stimulation of the nucleus in patients suffering uncontrollable violence-aggression using chronic implanted electrode led to a decrease in aggressive behavior (Heath, 1977). Consistent with this study, several fMRI studies in humans have reported that the cerebellar vermis increases in activity during emotion processing tasks (Baumann and Mattingley, 2012; Guell et al., 2018; Keren-Happuch et al., 2014; Stoodley and Schmahmann, 2009). Collectively, these studies provide ample evidence that the cerebellar medial nucleus is a key brain region involved in affective behavior. However, the mechanisms through which the cerebellar medial nucleus neuron influence affective behavior is still poorly understood.

3.1.1.1 The organization and connectivity of the cerebellar nuclei for control of feeding

Neurons in the cerebellar nuclei receives a myriad of information from internal and external sources and provide the cerebellum with the capability to influence behavior by linking the cerebellum with many brain regions (Bostan et al., 2013; Haines et al., 1997; Zhu et al., 2006). In the context of cerebellum's involvement in feeding behavior, several hypothalamic sub nuclei have been reported to target neurons in the granular, molecular and Purkinje cell layers of the cerebellar cortex (Dietrichs et al., 1985; Haines et al., 1997; Wang et al., 1994), and the cerebellar nuclei (Dietrichs et al., 1994; Haines et al., 1990). To identify sources of input into the cerebellum, one study employed retrograde labelling wheat germ agglutinin horseradish peroxidase injected in the cerebellar cortex of cats (Dietrichs, 1984). The origins of axonal projections in the cerebellar cortex were retrogradely traced to the lateral, posterior and dorsal hypothalamic areas, the supramammillary, tuberomammillary and lateral mammillary nuclei, the dorsomedial and ventromedial nuclei, and the

periventricular zone (Dietrichs, 1984). These direct hypothalamocerebellar connections were found in other species such as rat, tree shrew and non-human primate (Haines et al., 1997). While these studies provided the neural substrates relevant for visceral inputs to the cerebellum, the neuronal identity and the functional significance of these signals have yet to be explored. Moreover, whether/how neurons in the cerebellar nuclei can reciprocally influence these hypothalamic regions are not clear.

3.1.1.1.1 Direct hypothalamo-cerebellar pathways

Reciprocal connectivity between cerebellar and hypothalamic regions have been identified (Cavdar et al., 2018; Dietrichs et al., 1994; Dietrichs et al., 1985; Haines et al., 1997; Haines et al., 1990). Studies providing evidence for hypothalamocerebellar pathways were conducted in cats and non-human primates by injecting combinations of retrograde tracers, such as wheat germ agglutinin horse radish peroxidase, rhodamine B-isothiocyanate and Fluoro-Gold in the different regions of the cerebellar nuclei (Dietrichs et al., 1994; Haines et al., 1990) (Figure 26, magenta). Axonal projections into the cerebellar nuclei from the hypothalamus originate bilaterally from the lateral, posterior and dorsal hypothalamic regions, dorsomedial hypothalamic nucleus (DM) and tuberomammillary nucleus (Haines et al., 1990) (Figure 26, magenta). In a different study, two retrograde tracers were simultaneously used to target the cerebellar cortex and cerebellar nuclei in a non-overlapping manner (Dietrichs et al., 1994). Neurons within the hypothalamic regions were observed to be either projecting into just one of the two cerebellar regions or both the cerebellar cortical and nuclei regions, indicating the existence of at least three subtypes of hypothalamic neurons based on patterns of axonal collaterals (Dietrichs et al., 1994) (Figure 26, magenta). Together, it is becoming clear that subpopulations of hypothalamic neurons selectively target

different neuronal populations within different compartments of the cerebellum. However, the neurotransmitter identity of pre- and postsynaptic neuronal subtypes of these hypothalamocerebellar pathway have yet to be characterized.

Figure 26. A schematic of the hypothalamo-cerebellar and cerebello-hypothalamic pathways. Coronal plane consisting of hypothalamic nuclei that projects into the cerebellum (top, magenta), and hypothalamic nuclei targeted by the cerebellum (bottom, cyan) and those that do not (gray). Gray intersection indicates where the coronal planes are derived. Legend: arcuate nucleus, Arc; dorsal hypothalamic area, DA; dorsomedial hypothalamic nucleus, DM; lateral hypothalamic nucleus, LH; paraventricular nucleus, PV; posterior hypothalamic area, PH; tuberomammillary nucleus, TM, ventromedial hypothalamic nucleus, VM. (Cavdar et al., 2018; Dietrichs et al., 1994; Haines et al., 1990; Paxinos and Franklin, 2007).

3.1.1.1.2 Direct pathways from the cerebellar nuclei to the hypothalamus

Monosynaptic projections from the cerebellar nuclei to several distinct hypothalamic nuclei have been identified. Neuroanatomical anterograde tract tracing studies carried out in non-human primate have provided evidence that all cerebellar nuclei send axonal projections which terminate in the lateral, posterior and dorsal hypothalamic areas and in the DM and paraventricular nuclei (Haines et al., 1990) (Figure 26, cyan). While the anterogradely labeled axons in non-human primates were found to passage through the superior cerebellar peduncle before entering the hypothalamus (Haines et al., 1990), studies in rats have reported that the middle cerebellar peduncle is also involved in this pathway (Cavdar et al., 2018) (Figure 26, cyan). Notably, in comparison to non-human primate studies, it was observed that the rat cerebellohypothalamic pathway in rats consists of additional hypothalamic regions including the arcuate and ventromedial nuclei (Arc and VM) (Cavdar et al., 2018) (Figure 26, cyan). Although, these studies provide anatomical evidence for direct connectivity between the cerebellar nuclei to a variety of hypothalamic sub-regions, distinctions of target selectivity between different cerebellar sub-nuclei have not been defined.

3.1.1.1.3 Potential nodes linking the cerebellum to the hypothalamus

The axonal projections originating from hypothalamic regions are known to collateralize with pre-cerebellar regions such as the basal pontine nuclei and the LRt (Aas and Brodal, 1989; Allen and Hopkins, 1990) (Figure 27, respectively magenta and red). In cats and rats, hypothalamo-pontine collaterals are reported to arise from the dorsal, lateral, anterior and posterior regions of the hypothalamic areas (Aas and Brodal, 1989; Allen and Hopkins, 1990) (Figure 27, magenta). Two retrograde tracing studies in cats have reported that basal pontine nuclei neurons targeted by the hypothalamus send projections to cerebellar

cortical regions such as the paraflocculus, vermal areas, and the anterior lobe (Aas and Brodal, 1989; Brodal and Walberg, 1977; Pierce et al., 1977) (Figure 27, magenta). While these data support an indirect hypothalamo-ponto-cerebellar pathway, the functional relevance of this connectivity remains undefined.

Figure 27. Schematic of hypothetical indirect connections between the hypothalamus and the cerebellum.

Coronal plane consisting of all hypothalamic nuclei targeting the basal pontine (top, magenta) and lateral reticular nucleus, LRt (bottom, red) to target the cerebellum, and those that do not (gray). First order projections are labeled darker coloured dashed lines, and second order projections are labeled lighter colored dashed lines. Hypothalamic output neurons targeting intermediate regions which links to either the cerebellar cortex or nuclei (magenta and red pathways). Cerebello-zona incerta-arcuate nucleus pathway (cyan pathway). Gray intersection indicates where the coronal plane is derived. Legend: anterior hypothalamic area, AH; arcuate nucleus, Arc; dorsal hypothalamic area, DA; dorsomedial hypothalamic nucleus, DM; lateral hypothalamic nucleus, LH; posterior hypothalamic area, PH; tuberomammillary nucleus, TM; ventromedial hypothalamic nucleus, VM;

zona incerta, ZI (Aas and Brodal, 1989; Allen and Hopkins, 1990; Alstermark and Ekerot, 2013; Brodal and Walberg, 1977; Paxinos and Franklin, 2007; Pierce et al., 1977; Teune et al., 2000; Wang et al., 2015).

The lateral reticular nucleus (LRt), a brain region well known to give rise to extensive monosynaptic inputs into the cerebellum, may also serve as a bridge between the cerebellum and the hypothalamus (Dietrichs et al., 1985) (Figure 27, red). Even though the role of the LRt as a node for hypothalamic-related functions has not been assessed in detail, there are some evidence pointing to its involvement. A study in cats reported that neurons in the lateral, anterior, dorsal and posterior hypothalamic areas, tuberomammillary nucleus and DM send axons which terminate within the LRt (Dietrichs et al., 1985) (Figure 27, red). Both electrophysiological recording and tract tracing studies have shown that LRt consists of neurons that extensively collateralizes with all three major cerebellar nuclei, and provides tonic excitatory drive to the cerebellar nuclei (Alstermark and Ekerot, 2013) (Figure 27, red). Together, these studies provide the relevant neural substrates for indirect hypothalamocerebellar pathways.

The cerebellum is presumed to exert its influence on non-motor processes by targeting a large number of different brain regions including the hypothalamus (Teune et al., 2000; Wang et al., 2015). However, studies have largely focused on direct cerebello-hypothalamic pathways, and ignored indirect connections. A recent study identified a key structure within the subthalamic region to be important for feeding control (Zhang and van den Pol, 2017). Optogenetic manipulation of a subpopulation of neurons in the zona incerta (ZI) results in immediate binge-like feeding behavior and body weight increase (Zhang and van den Pol, 2017). The ZI is a region which receives extensive cerebellar input (Teune et al., 2000), and

has been reported to project into the Arc of the hypothalamus (Wang et al., 2015) (Figure 27, cyan). Despite these advances, specific details of the connectivity of these two pathways, and whether/how the cerebellum influences neuronal subpopulations in the ZI and the arcuate nucleus have not been examined.

3.1.1.2 Functional significance of the cerebellar nuclei in food intake

The regulation of energy balance through nutrient intake and energy expenditure is vital for survival in all living organisms. Homeostasis is mediated in brain centers including the hypothalamus (Aponte et al., 2011; Krashes et al., 2014), extended amygdala (Jennings et al., 2013) and brainstem (Carter et al., 2013). Although the cerebellum is not sensitive to nutritional and humoral signal, cerebellar output neurons are capable of innervating hypothalamic neurons which in turn can detect and respond to visceral related signals (Zhu and Wang, 2008). Moreover, functional imaging studies have associated the activation of the cerebellum with hunger, satiation, and appetite (Gautier et al., 1999; Pujol et al., 2018; Tataranni et al., 1999). Thus, it appears plausible that the cerebellum is essential for integration of somatic and visceral systems for the generation of controlled somatic-visceral response in food intake.

3.1.1.2.1 Functional connectivity of the cerebello-hypothalamic pathway

The cerebello-hypothalamic pathway comprises of the cerebellar interposed and medial nuclei and several regions of the hypothalamus including the lateral, ventral, DM and paraventricular hypothalamic nuclei (Wang et al., 1997; Wen et al., 2004; Zhu et al., 2006; Zhu et al., 2004). Studies in rats that focused on the functional connectivity between the

lateral hypothalamic neurons and neurons in the cerebellar nuclei have reported that lateral hypothalamic neurons are glucose sensitive and are inhibited by cerebellar nuclear neurons (Min et al., 1989). Glucose sensitive neurons maintain blood glucose homeostasis by detecting blood glucose level and activating downstream responses (Campfield and Smith, 2003). While less than 10% of all glucose-insensitive lateral hypothalamic neurons receive cerebellar medial nuclear inputs, these neurons are important for players in nutrient-seeking behaviour (Karádi et al., 1990; Parada et al., 1990). Thus, the functional connectivity between the cerebellum and the distinct hypothalamic neuronal subtypes suggests that the cerebellum influences hypothalamic glucose-sensitive and -insensitive neurons to mediate food intake and motivation to food seek. However, the identity of the cerebellar neuron subtype involved, the information they carry for signal integration and the downstream behavioral output these neurons regulate have yet to be examined.

In addition to targeting of glucose sensitive and insensitive hypothalamic neurons by the cerebellum, cerebellar and visceral inputs have been shown to converge within in the hypothalamus (Zhang et al., 2003; Zhang et al., 2005; Zhu et al., 2006; Zhu et al., 2004) . In a study using rats, the cerebellar interposed nucleus and gastric vagal nerves were electrically stimulated and extracellular recordings were performed on neurons in the DM (Zhu et al., 2006). Not only did the results for this study revealed that the hypothalamic neurons responded to both stimuli, these neurons also responded to intravenous administrations of glucose and cholecystokinin sulphated octapeptide (Zhu et al., 2006). The function of the gastric vagal nerve is to provide negative-feedback signals from the gastrointestinal tract to the hypothalamus (Schwartz et al., 2000; Yuan and Barber, 1992, 1996). These signals consist of gut related mechanical, chemical and peptide feedback (Schwartz et al., 2000;

Yuan and Barber, 1996). Cholecystokinin is one of such gut-peptide known to influence the hypothalamus to elicit strong interim inhibition of food intake (Fink et al., 1998; Noble et al., 1999; Su et al., 2017). Taken together, these studies add to the several lines of evidence indicating that the cerebellum is a brain center actively participating in feeding regulation. However, the integration of gut-related feedback and cerebellar nuclei inputs, and the functional consequences of these integrated signals is still unclear.

3.1.1.2.2 Functional relevance of target regions of the cerebellum relating to food intake

The Arc is connected to the cerebellum via cerebello-hypothalamic fiber tracts found within the middle cerebellar peduncle (Cavdar et al., 2018). This finding was conducted by the application of biotinylated dextran amine tracer injections into the middle cerebellar peduncle in rats to anterogradely label cerebellar nuclei output fibers (Cavdar et al., 2018). This tract tracing study identified subregions of the hypothalamus, including the posterolateral and medial Arc, that receive cerebellar axonal projections.

The Arc consists of two distinct neuronal subpopulations distinguished by the expression of either agouti-related peptide (AgRP) or opiomelanocortin (POMC). These neuronal subpopulations in the Arc play complementary roles in influencing food seeking and food intake decisions (Morton et al., 2006). A study in mice showed that food deprivation leads to an elevated activity of AgRP neurons, and that these neurons positively regulate food intake behavior (Takahashi and Cone, 2005). Furthermore, optogenetics stimulation of AgRP neurons expressing channelrhodopsin in mice is sufficient to activate strong feeding response (Aponte et al., 2011). On the other hand, increased activity in

opiomelanocortin neurons is linked to inhibition of food intake activities (Tsuji and Bray, 1989; Yaswen et al., 1999). Genetic and pharmacological studies have provided evidence that these neurons halt food intake behavior by excreting α -melanocyte-stimulating hormone, a melanocortin receptor agonist (Tsuji and Bray, 1989; Yaswen et al., 1999). Agouti-related peptide neurons are capable of inhibiting opiomelanocortin neurons, and also block melanocortin receptors (Ollmann et al., 1997). Hence, agouti-related peptide neurons are recognized to be key modulators of the melanocortin pathway to increase food intake and reduce satiety (Cowley et al., 2001). Therefore, a better understanding of how the cerebellum mediate satiety and hunger responses will require characterization of the connectivity between neurons in the cerebellar nuclei and the AgRP and/or POMC neurons in the Arc.

Recently, a subpopulation of neurons found within the DM has been shown to have the ability to inhibit AgRP neurons in the Arc nucleus (Garfield et al., 2016). Interestingly, the DM is a brain region that has been reported to receive inputs from cerebellar interposed and medial nuclei (Zhu et al., 2006), thereby further implicating the cerebello-hypothalamic pathways in controlling food intake activities. Through optogenetic manipulation and electrophysiological recordings in mice, Arc-projecting leptin receptor-expressing neurons located in the ventral region of the DM were shown to have rapid inhibitory properties on agouti-related peptide Arc neurons (Garfield et al., 2016). This study demonstrated that leptin depolarizes DM neurons in acute slices, and that these neurons release GABA to inhibit agouti-related peptide Arc neurons (Garfield et al., 2016). Moreover, photostimulation of inhibitory DM neurons which express channelrhodopsin led to decreased food intake (Garfield et al., 2016). Therefore, an examination of the functional relevance of the

connection between the cerebellar nuclei and the ventral DM may provide insight to how the cerebellum integrates sensory information to dynamically modulate feeding behavior.

The ZI is subthalamic region known not only for its role in movement control, but also recently identified as a region that receives extensive cerebellar input and plays a role in feeding behavior (Amami et al., 2015; Novakova et al., 2011; Teune et al., 2000; Zahodne et al., 2011; Zhang and van den Pol, 2017). A study that utilized an array of experimental methods, including monosynaptic retrograde rabies virus tracing, optogenetics and electrophysiological recordings, demonstrated that GABAergic neurons in the ZI inhibit thalamic excitatory neurons in the paraventricular nucleus (PV) to evoke rapid binge-like eating behavior and body weight gain in mice (Garfield et al., 2016). Thus, the results from this elegant study indicate that cerebellar neurons could regulate food intake by modulating ZI neurons that increases appetite. However, whether cerebellar neurons communicate with ZI neurons in circuits controlling food intake have not been examined in detail.

In summary, these studies have provided ample evidence that the cerebellar interposed nucleus participates in feeding behaviors. Yet, the modulatory mechanisms employed by the cerebellar interposed nucleus to influence neurons in the hypothalamic regions have not been characterized. Key remaining questions that need to be addressed include the following: Are there distinct neuronal subpopulations in the cerebellar interposed nucleus that specifically regulate either motor or non-motor functions? How are cerebellar interposed neurons organized to provide selective inputs to the downstream brain targets? What is the pattern of

activity employed by neuronal subpopulations within the cerebellar interposed neurons to mediate specific visceral related behaviors?

3.2 HYPOTHESIS

The cerebellar nuclei are located in an ideal position to receive and integrate a range of sensorimotor information and to disseminate computed output signals to a multitude of target regions within the brain. However, the circuitries in which the cerebellar nuclei systematically regulate a diverse variety of non-motor functional roles are not clear. Moreover whether, distinct cerebellar nucleus control distinct processes are not well defined. To address these questions, we set out to characterize the connectivity of subpopulations of neurons in the cerebellar nuclei using tract tracing and fiber photometry, and examine the functional consequences of manipulating these neurons using chemogenetics. The following are major questions we aim to address:

- Do neurons within the cerebellar nuclei influence non-motor processes such as feeding-related behavior?
- What are the circuit elements that mediate these non-motor processes?

Together, we aim to characterize the connectivity patterns of the cerebellar interposed nucleus and the consequences of manipulating these pathways in feeding-related behavior in order to better understand the underlying mechanisms of the anatomical and functional diversity of cerebellar circuits in controlling non-motor behaviors.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Please find full description in 2.3.1. (Appendix A)

3.3.2 Histological procedures

Please find full description in 2.3.2.

3.3.3 Immunohistochemistry

Please find full description in 2.3.3.

3.3.4 Tract tracing

Brain microinjection

Please find full description in 2.3.1, the following adjustments to the protocol were made for this chapter. Stereotaxic coordinates targeting region of interests were obtained from a stereotaxic atlas (Paxinos and Franklin, 2007): the Arc (from bregma: -1.45 mm posterior, 0.25 mm lateral, -5.95 mm ventral).

3.3.5 Designer Receptors Exclusively Activated by Designer Drugs

To investigate the effects of chemo-genetic manipulation of IntA neurons on feeding behavior, 300 nl of either AAV2-*hSyn*::human M3 muscarinic receptor (hM3D) (Gq)-mCherry or AAV2-*hSyn*::human M4 muscarinic receptor hM4D(Gi)-mCherry virus (Addgene, USA) were introduced bilaterally via pressure injection into the IntA of 3 wildtype mice for each virus. Three mice were injected with saline to serve as controls. The

mice were housed postoperatively for six weeks before behavior tests were carried out to allow for adequate transduction of the viruses. Surgical procedures carried out follows that described in section 3.3.4 *Tract tracing*.

3.3.6 Fiber photometry

Fiber photometry experiments were carried out as previously described, and was conducted at Dr. Nicholas Betley laboratory in University of Pennsylvania (Su et al., 2017). In brief, a dual-wavelength fiber photometry rig was used (Lerner et al., 2015; Zalocusky et al., 2016). Excitation wavelengths 490 and 405 nm were used. Calcium-dependent fluorescence from GCaMP6s protein was excited by 490 nm, providing a measure of AgRP neuron activity. Calcium-independent fluorescence from GCaMP6s protein was excited using 405 nm, and serves as a control for movement and bleaching artifacts. The frequencies of 490 nm was modulated to 211 Hz and 405 nm was tuned to 566 Hz to prevent contamination from overhead lights at 120 Hz and harmonics and any possible cross-talk between 490 and 405 nm excitation wavelength. Excitation light were modulated by a real-time amplifier (RZ5P, Tucker-Davis Technology, USA) and passed through bandpass filters (MF469-35, 490 nm; FB405-10, 405 nm, Thorlabs, USA) prior to being collimated and combined by a 425 nm long-pass dichroic mirror (DMLP425, Thorlabs, USA). The combined excitation light was sent into a patch cord made of a 400 μ m core, 0.48 NA, low-fluorescence optical fiber (MFP_400/430/1100 0.48_1.5_FCM-MF, Doriclenses, Canada). The patch cord was connected to an implanted fiber constructed in a 2.5 mm diameter ferrule via an interconnector (ADAF2 for 2.5 mm, Thorlabs, USA). The GCaMP6s emission fluorescence signals were collected through the same patch cord, collimated, passed through a GFP emission filter (MF525-39, Thorlabs, USA), and focused onto a femtowatt photoreceiver

(Model 2151, gain set to AC LOW, Newport, USA) using a lens (62-561, Edmund Optics, USA). The emission lights were converted to electrical signals, sampled at 1017 Hz, and demodulated by the RZ5P real-time processor. The fiber photometry experiments were controlled by Synapse software (Tucker-Davis Technology, USA). Synchronized infra-red cameras (ELP-USB100W05MT-DL36, Ailipu Technology, China) controlled by Synapse were used to video record mice during fiber photometry experiments.

At the begin of fiber photometry experiments, baseline GCaMP6s fluorescence signals were set to similar levels by adjusting the output power of 490 and 405 nm LEDs. To attain maximum sensitivity of biological signals, the 490 nm signal was set to occupy 50% of the detection range of the photoreceiver (20-100 μ W at the tip of the fiber accounting for variations in GCaMP6s expressions and optical fiber positions over AgRP neurons). The 405 nm signal was set to occupy 5% of the detection range (2-10 μ W output power at the tip of the fiber) to avoid saturating the detector.

Fiber photometry recordings were performed in the mice home cage during the light cycle. Mice were either fasted for 24 h or fed *ab libitum*. Cages were opened and mice fiber were connected to fiber photometry patch fibers. To determine the effects of chow (maintenance food) on AgRP neuron activity, food-restricted mice were given chow pellets during fiber photometry recordings. Recordings were performed for 5 minutes prior to the introduction of any manipulation to attain a baseline activity. Subsequently, manipulations were introduced. Fiber photometry data were alighted to the time of manipulation. All mice with smaller than 20% $\Delta F/F$ resonse to chow were excluded from further experiments.

To investigate AgRP neuron responses to different caloric content of food to cerebellar nuclei excitation, food-restricted mice were given chow or peanut butter. Mice were habituated to peanut butter for two days prior to food-deprivation. Fiber photometry data were aligned to the introduction of food.

Demodulated data were exported from Synapse to MATLAB (MathWorks) using a standard script provided by Tucker-Davis Technology. The 490 and 405 nm signals were independently processed and normalized to baseline signals recorded during the first 5 minutes of each experiment, to determine $\Delta F/F = (F - F_{\text{baseline}})/F_{\text{baseline}}$ and F_{baseline} is the median of pre-stimulus signal. 405 nm, isosbestic, normalization was not introduced, nor data was presented with 490 nm excitation data aligned to isosbestic wavelength. In MATLAB (MathWorks, USA), data were down-sampled to 1 Hz for analysis.

3.3.7 Freely moving feeding assay

Food-intake recording was done to observe food-seeking behavior of mice following chemo-genetic manipulation of cerebellar interposed nucleus. A week prior to the experimental period, the mice were exposed to human contact and the feeding chamber for habituation. Each mouse was habituated twice on two separate days. Habituation involved scuffing the mice and placing them in the feeding chamber with food pellets for 1.5 h. The mice were subjected to 24 h food deprivation prior to each behavioral task. During each session, the mice were first injected with 0.1 mg/ml Clozapine-N-oxide (CNO) diluted in DMSO (0.07%) and 0.9% NaCl intraperitoneally at a concentration of 10 μ l per gram of body weight. The mice were left to rest for 20 minutes before commencement of recording to allow for CNO to metabolize and enter the blood brain barrier and for the mice to recover

from the injection (Gomez et al., 2017). Following the rest period, mice were placed into separate feeding chambers, each with 5 chow pellets evenly distributed across the chamber, for an hour. Each chow pellet weighed around 3 g. The sides of the feeding chamber were made from opaque white acrylic boards with a removal base. Chow intake was measured after each session. Crumbs of chow left in the feeding chamber was also collected and weighed to determine the food intake of each mouse more accurately. All sessions were recorded using an overhead webcam through OBS studio version 21.0.2 recording software for analysis.

3.3.8 Statistical analysis

Values are represented as mean $\pm$ s.e.m. unless otherwise noted. Comparisons were made by unpaired Mann-Whitney U test and Wilcoxon matched-paired sign rank test. Only non-parametric tests were used. All statistical computations were performed using GraphPad Prism version 7.00 (GraphPad Software, USA).

3.4 RESULTS

3.4.1 Behavioral impact of manipulating neurons in the interposed nucleus on food intake

To investigate the involvement of cerebellar neurons on non-motor behaviors, , we activated or inactivated neurons in the interposed nucleus using chemogenetic Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology (Roth, 2016). To begin, we performed bilateral injections of virus to express either human M3 muscarinic receptor (hM3D) (to activate) or human M4 muscarinic receptor (hM4D) (to inactivate) in the mouse cerebellar interposed nucleus, and injection of saline for control (Figure 28A-B). After surgery and habituation, the mice were allowed to recover from the surgery for six weeks before being subjected to a behavioural test (Figure 28A). Prior to each feeding task, all mice were food deprived for 24 hours followed by intraperitoneal injection of CNO, a pharmacologically inert metabolite of the atypical antipsychotic drug clozapine to mobilize intracellular calcium, or vehicle, and allowed to habituate for 20 minutes (Armbruster et al., 2007) (Figure 28C). During the feeding task, mice were placed into an arena where their behaviour would be recorded. Five food pellets are evenly spaced throughout the floor of the arena where mice could feed on the pellet freely for one hour without any disruptions. To assess consequences of manipulation of the cerebellar interposed nucleus on feeding behavior, we tested three groups of mice: sham, hM3D, and hM4D. Sham is an experimental control group that serves to ensure that the surgical procedure and the CNO used does not affect the normal behaviour of the mice.

To examine the consequences of manipulating cerebellar interposed nucleus on a non-motor behavior, we first analysed the food intake and the food contact latency in each experimental group. We found that CNO alone does not have a significant impact on feeding

Figure 28. Subpopulations of neurons in the cerebellar interposed nucleus neurons regulate food intake.

(A) Timeline for experiment. (B) Schematic for the site of injection, and the combination of viruses used in wild type and transgenic mice. (C) Experiment design and set-up for 1 h food intake trials. (D-I) Analysis of chow intake (D), latency to chow intake (E), movement speed (speed; F), time spent moving (mobility; G), area explored (exploration; H), and frequency of mice staying close to chow pellet (proximity to chow; I) (light gray

bars, saline I.P.; dark gray bars, CNO I.P. in sham mice.; red, CNO I.P. in hM3D injected mice; magenta, CNO I.P. in hM4D mice; n=3 mice in each group, 1-2 trials each mouse; Wilcoxon matched-paired signed rank test for D and E; Mann-Whitney test for F-I)

behaviour and feeding motivation (Figure 28A-B), as we observed no significant difference in chow intake and latency to chow intake between the control and CNO injection in the sham. Thus, any behavioural changes that we observed are mainly due to the effects of CNO on cerebellar interposed nucleus neurons that express hM3D or hM4D.

We observed that chemoactivation of cerebellar interposed nucleus neurons via hM3D led to a reduction in food intake (Figure 28D; Appendix K), suggesting these neurons negatively regulate feeding. We predicted that silencing of neurons in the cerebellar interposed nucleus with hM4d might result in an opposite effect. Interestingly, however, silencing of neurons in the cerebellar interposed nucleus did not lead to significant changes in food intake compared to vehicle injection (Figure 28D; Appendix K). Next, we examined the motivation of fasted mice to feed by measuring the time interval between the entry of chamber and their first biting attempt, also known as food contact latency (Figure 28E; Appendix K). We found that activation of neurons in the interposed nucleus with hM3D resulted in a tendency of increase in food contact latency, whereas silencing interposed nucleus did lead to changes in food contact latency (Figure 28E; Appendix K). Through the manipulation of the activities of neurons in the interposed nucleus, we learned that activation of cerebellar interposed nucleus leads to a reduction in food intake and increased food contact latency which could reflect an induction of satiety sensation in these mouse. Additionally, silencing of neurons in the cerebellar interposed nucleus does not lead to an

opposite effect which indicates that cerebellar interposed nucleus selectively sends out signals to suppress hunger sensation.

3.4.1.1 Consequences of activation and inactivation of neurons in the interposed nucleus on motor-related behaviors

To examine the role of the interposed nucleus on motor-related behaviour during feeding task, we analysed the speed of locomotion (speed), percentage of total time spent moving (mobility), percentage of total area explored within the arena (exploration), and the frequency events of being less than 2 mm away from chow pellet (proximity to chow) (Figure 28F-I; Appendix K). Activation of neurons in the interposed nucleus result in a reduction in traveling speed, mobility and exploration (Figure 28F-H; Appendix K). The decrease in chow intake is unlikely due to an inability of hM3D mice to access chow pellets, since chow pellets are within reaching range of the mice (Figure 28C, F-H; Appendix K). Chow pellets are readily accessible to the mice since pellets are located in every 140 cm² of the arena. The farthest distance a mouse could be from a chow pellet is only approximately 12 cm (Figure 28C; Appendix K). Moreover, we observed that activation of cerebellar interposed nucleus in hM3D mice results in a significant increase in frequency of the mice loitering near the food pellets without eating (Figure 28I; Appendix K). Thus, we speculate that the reduction in food intake is primarily due to the sense of satiation induced by the increase in neuronal activity within the interposed nucleus, and unlikely contributed by the reduction in traveling speed, mobility and exploration activity.

3.4.2 Identification of novel nodes linking the cerebellum and hypothalamus

3.4.2.1 Identification of direct and indirect targets that link the cerebellar nuclei to known feeding centers: trans-synaptic tracing

In order to identify direct and indirect pathways that might link the cerebellum with brain regions known to control food intake, we injected a virus that expresses Cre fused to WGA which recombines first order as well as second/third order neurons in the cerebellar nuclei of a Cre-reporter mouse, *ROSA::lsL.ChR2-eYFP* (Libbrecht et al., 2017; Yoshihara et al., 1999) (Figure. 29A). In brief, after expression of WGA-Cre fusion protein driven by the *eukaryotic translation elongation factor 1 alpha (EF1a)* promoter, WGA-Cre gets transported throughout the neuron and will be secreted. Nearby synaptically connected neurons will then take up the WGA-Cre, allowing multisynaptic labeling in an anterograde direction (Braz et al., 2002; Yoshihara et al., 1999). After replication of the virus and trans-synaptic transport of WGA-Cre fusion protein, histological analysis revealed neurons at the site of viral injection expressed mCherry (red) (Figure 29B-D). Our histological analysis showed that first order labeled neurons were confined to the cerebellar interposed nucleus.

We next assessed target regions of the labeled neurons, and found traced puncta originating from the interposed nucleus in extra-cerebellar regions including the VA-VL, RMC, IOD, LRt, ZI and InG (Figure 29E-P), which we and others have previously shown to be linked to the cerebellar nuclei (Houck and Person, 2015; Mitrofanis and deFonseka, 2001; Teune et al., 2000). The VAVL, RMC, IOD and LRt are regions linked to motor control (Alstermark and Ekerot, 2013; Biane et al., 2016; Gibson et al., 1985; Schweighofer et al., 2013), and contain solely traced cerebellar interposed nucleus puncta without any transfer of WGA-Cre resulting in recombination in the soma of neurons in these regions (Figure 29E-L). In contrast, we found recombined cell bodies in the ZIV and InG which presumably took up

Figure 29. Transsynaptic tracing reveals efficient uptake of WGA-Cre at the ZIV and InG. (A) Schematic for transsynaptic tracing strategy. (B) Injection site within the cerebellar interposed nucleus of *Rosa::Isl.ChR2-eYFP* using AAV2-*EF1a::mCherry-IRES-WGA-Cre* virus. (C) Confocal image derived from the red box in letter B. (D) Colocalization of mCherry (red), with Cre-mediated reporter expression (YFP, green), and nuclei stain (DAPI). (E-P) Images of the six regions receiving projections from IntA (E, G, I, K, M, O), with zoom-in images from the red box found in letters E, G, I, K, M, O, respectively presented on the right (F, H, J, L, N, P). Legend: Gigantocellular reticular nucleus, Gi; Gigantocellular reticular nucleus, ventral part, GiV; Inferior olive, dorsal nucleus, IOD; Inferior olive, principal nucleus, IOPr; Intermediate reticular nucleus, IRT; Intermediate white layer of the superior colliculus, InWh; Internal capsule, Ic; Lateral periaqueductal gray, LPAG; Medial terminal nucleus, MT; Medullary reticular nucleus, dorsal part, MdD; Medullary reticular nucleus, ventral part, MdV; Mesencephalic reticular formation, mRt; Optic nerve of the superior colliculus, Op; Parabrachial pigmented nucleus of the VTA, PBP; Peduncular part of lateral hypothalamus, PLH; Posterior

thalamic nuclear group, Po; Red nucleus, magnocellular part, RMC; Red nucleus, parvocellular part, RPC; Reticular nucleus (prethalamus), Rt; Subincertal nucleus, SubI; Submedius thalamic nucleus, Sub; Substantia nigra, reticular part, SNR; Superficial gray layer of the superior colliculus, SuG; Ventromedial thalamic nuclei, Vm.

WGA-Cre from axons originating from the interposed nucleus (Figure 29M-P). Thus, we conclude that second order WGA trans-synaptic labeling only occurred within the ZI and InG. The InG of the superior colliculus is involved in sensory integration and initiation of motor commands (Ito and Feldheim, 2018), and interestingly, the ZI was recently identified as a key brain region which mediates food intake (Zhang and van den Pol, 2017).

We further searched through brain sections for second order trans-synaptic labeling mediated by WGA-Cre, presumed to originate from neurons in the ZI and InG. We found bilateral labeling in numerous puncta and soma within the hypothalamus such as the dorsal medial hypothalamic area (DM) and Arc (Figure. 30A-C). Both the DM and Arc are brain regions that control food intake (Aponte et al., 2011; Yang et al., 2009). We also found several brain regions with trans-synaptic labeling including RSGc and DG (Figure. 30D-F). Consistent with the absence of trans-synaptic labeling of cell bodies in the VAVL, we found no second order trans-synaptically labeled neurons in the motor cortex including M1 and M2 (Figure 30G-I). These results are consistent with our previous work about cerebello-ZI connection, and a recent study using rabies virus-based trans-synaptic tracing technique, showing for the first time that the Arc receives input from the ZI, but not from the thalamus (Wang et al., 2015). In addition, the DM is known to provide direct inputs into the Arc and receives input from the cerebellar nuclei (Garfield et al., 2016; Haines et al., 1990).

Therefore, our tract tracing studies identified novel indirect pathways between the interposed

nucleus and Arc, via the ZI and/or the DM, that represent neural substrate for cerebellar influence of feeding behaviors.

Figure 30. Transsynaptic tracing reveals connectivity between the cerebellar interposed nucleus and the hypothalamus. (A-I) Confocal images of transsynaptic tracing in *Rosa::Isl.ChR2-eYFP* using AAV2-*EF1a::mCherry-IRES-WGA-Cre* virus within various brain regions. High concentrations of recombined cells were observed in DM (B, solid square box from A), Arc (C, solid square box from A), RSGc (E, solid square box from D) and DG (F, solid square box from D). No recombined cell bodies were found in the cerebral cortex

(G), zoom in of M2 (H) and M1 (I) respectively from the solid line and dotted line boxes. Legends: Arcuate hypothalamic nucleus, Arc; Caudate putamen (striatum), CPu; Dentate gyrus, DG; Dorsomedial hypothalamic nucleus, DM; Field CA3 of the hippocampus, CA3; Periventricular hypothalamic nucleus, Pe; Primary motor cortex, M1; Primary somatosensory cortex, forelimb region, S1FL; Primary somatosensory cortex, hindlimb region, S1HL; Retrosplenial dysgranular cortex, RSD; Retrosplenial granular cortex, c region, RSGc; Secondary motor cortex, M2; Ventromedial hypothalamic nucleus, VMH.

3.4.2.2 Defining the connectivity between the interposed nucleus and the arcuate nucleus of the hypothalamus: antero- and retrograde tracing

The role of IntA neurons in the modulation of satiation behaviours suggests that IntA neurons target brain regions related to feeding, such as Arc (Aponte et al., 2011). To characterize the connectivity between the IntA and Arc, we utilised an anterograde viral tracer to identify targets of IntA neurons and a retrograde tracer to back-label neurons projecting into the Arc. We also took advantage of the *Ucn3::Cre* mouse line which we have shown to recombine in the subpopulation of IntA neurons when crossed to a reporter mouse (Figure 9A-G). We utilised anterograde viral tracer AAV2-*hSyn::ChR2-mCherry* to label the projections of cerebellar neurons and retrograde tracer CTB 647 to label neurons that project into the Arc (Figure 31A-F). The viral anterograde tracer was unilaterally injected into the IntA of the *Rosa::Isl.ChR2-eYFP* mouse (Figure 31A-C) and retrograde tracer was injected in the contralateral site of the Arc 14 days after the viral injection (Figure 31D-F) and incubated for a week before histology analysis. We did not find retrogradely labeled CTB soma in the cerebellar nuclei, or anterogradely traced IntA puncta in the Arc. Thus, from these results we rule out the possibility of direct projection between the IntA and the Arc.

In search of the intermediate connection between the IntA and hypothalamus, we hypothesized that the ZI may represent a node linking the two regions as we and others have identified connections between the IntA and the hypothalamus (Teune et al., 2000; Wang et al., 2015) (Figure 18D-F; Figure 29M, N). In addition to the ZI, another potential node linking the cerebellum to the hypothalamus is the InG (Figure 18G-I; Figure 29O, P) and DM (Figure 30A, B) which we and others have shown to be connected to both the IntA and Arc (Teune et al., 2000; Wang et al., 2015). To begin our analysis, we generated coronal brain slices containing the ZI, DM and InG from brains injected with both antero- and retrograde tracers (Figure 31A-D; Figure 32A, G).

We found that neurons in the ZI were retrogradely labeled by tracer injected in the Arc (Figure 32A-C). To determine whether IntA neurons project into the ZI, we assessed the proximity of axon terminals from IntA neurons to retrogradely labeled somas in the ZI of *Ucn3::Cre* reporter mice. We found YFP⁺ axons (green) labeled by the mCherry anterograde tracer (red) project into the ZI, and that these axon terminals surround the soma of ZI neurons which project into the Arc (blue) (Figure 32C). The close proximity of the axonal projections from IntA neurons with the soma of neurons in the ZI that project to the Arc strongly suggests a di-synaptic connection between these two regions which very likely represents circuit elements for cerebellar output neurons to influence hypothalamic-related activities such as food intake.

Figure 31. Cerebellar interposed anterior nucleus does not contribute to direct cerebello-hypothalamic pathway. (A) Genetic and viral tracing strategy using *Rosa::Isl1*:ChR2-eYFP and AAV2-*hSyn*::mCherry. (B) Overview of a coronal section, visualized with DAPI counterstain (white), consisting of the site of virus injection (mcherry, red) within the IntA expressing fluorescence reporter (e-YFP, green). (C) Zoom in on the area within the red box, B. (D) Retrograde labeling strategy within the same mouse on post-surgery day 14. (E) CTB injection site (blue) within the Arc, with DAPI counterstain (white). (F) Zoom in on red box, E. (G-I) Absence of retrograde Arc labeling in the cerebellar nuclei (G), counterstained with DAPI (H, white; I, merged)

(J-L) Absence of anterograde labeling in the Arc (J, blue; zoom in on F), the lack of IntA puncta (K) detected in the Arc (merge).

Figure 32. Cerebellar interposed nucleus contributes to the cerebello-zona incerta-hypothalamic pathway. (A-C) Coronal plane consisting of ZI and DM. Zoom in on ZI from letter A red box, containing anterograde tracing (mCherry, red) and retrograde tracing (CTB, blue) (B). Additional zoom in on letter B, white box, with DAPI counterstain (white; merge), and colocalization with YFP (white circles represent red and green colocalized puncta) (C). (D-F) Retrograde labeling in DM (arrow head points at soma) (D), and absence of anterograde cerebellar nuclei puncta labeling (E; merged, F). (G-I) Coronal section containing InG (G) with anterograde puncta labeling (red) and lack of retrograde tracing (blue) (H) (I, merged with DAPI). Legend:

Dorsomedial Hypothalamus, DM; Lateral hypothalamus, LH; Ventromedial thalamic nuclei, VM; Zona incerta, dorsal part, ZID; Zona incerta, ventral part, ZIV.

3.4.3 Cerebellar output neurons are functionally linked to AgRP neurons in the arcuate nucleus

To further examine the connectivity between output neurons in the cerebellar nuclei and neurons in the Arc, we activated the cerebellar nuclei bilaterally using chemogenetic DREADD strategy and recorded calcium activity of AgRP neurons expressing GCaMP6s using fiber photometry (Cui et al., 2014; Roth, 2016) (Figure. 33A-B). We bilaterally injected a virus to express hM3Dq-mCherry (red) ubiquitously in the cerebellar nuclei of *AGRP::Cre* mice (AAV2.*hSyn::hM3D(Gq).mCherry*), and injected a virus to conditionally express GCaMP6s-YFP (green) in AGRP neurons in the Arc (AAV1.*Syn::flex.GCaMP6s.YFP*) (Figure. 33A-D). In order to record calcium-dependent fluorescence, an optical fiber is implanted to target the Arc (Figure. 33B, C).

To normalize calcium-dependent wavelength (490 nm, green) to non-calcium-dependent wavelength (405 nm, red), recording session were conducted while the mice were in their respective homecage. Physiological activity of AgRP neurons was monitored and recorded for at least five minutes prior to introduction of the stimulant (i.e. chow, injection of saline, injection of CNO, peanut butter chips). These recordings serve as baseline and control for movement and bleaching artifacts for individual trials (Lerner et al., 2015) (Figure. 33E-I).

We first sought to examine whether GCaMP6s was expressed in AgRP neurons and if the fiber implant is targeting GCaMP6s-expressing AgRP neurons (Figure. 33E). Additionally, we assessed whether the activity of AgRP neurons respond to saline injection (Figure. 33F). Consistent with previous reports (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015), we observed rapid and robust decrease in AgRP neuron calcium activity in fasted mice upon detection of chow, and silencing of AgRP neurons upon ingestion of chow, suggesting that GCaMP6s is functionally expressed within AgRP neurons and fiber implant accurately targets the Arc ($\Delta F/F = -0.5$) (Figure. 33E). Subsequent control experiments showed that intraperitoneal injection of saline does not lead to significant change in AgRP activity (Figure. 33F). These results indicate that CNO injection is capable of acutely activating hM3D-expressing neurons in the cerebellar nuclei.

To investigate the functional connectivity between the cerebellar nuclei neurons and AgRP neurons, we activated hM3D-expressing neurons in the cerebellar nuclei by injecting CNO intraperitoneally. Simultaneously, we recorded the activity of GCaMP6s expressing AgRP neurons using fiber photometry. The experiment was conducted in both sated and fasted mice (Figure. 33F, G). Although sated mice showed no substantial difference in the activity of AgRP neurons after hM3D activation (Figure. 33F), we detected substantial and gradual decrease in the activity of AgRP neurons in fasted mice ($\Delta F/F = -0.25$ at 30 minutes) (Figure. 33G).

To assess consequences of chemoactivating neurons in the cerebellar nuclei in presence of food on the activity of AgRP neurons, we introduced chow and peanut butter (Figure. 33H). We found that the activation of neurons in the cerebellar nuclei is capable of reducing AgRP neuronal activity, specifically the negative modulation was ~50% and ~30% of the

Figure 33. Activation of the cerebellar nuclei via hM3D decreases calcium activity of AgRP neurons in fasted state. (A) Timeline for experiment. (B) Schematic for the site of injection to ubiquitously express hM3D within the cerebellar nuclei, and site of injection to express GCaMP6s and implantation of photometry fiber to monitoring calcium dynamics in AgRP neurons. (C-D) Expression of GCaMP6s-YFP in the Arc and photometry fiber tract (C, green), and expression of hM3Dq-mCherry within the DCN (D, red). (E-I) Dual-wavelength fiber photometry setup used to record calcium-dependent fluorescence (excited at 490 nm, green) and calcium-independent fluorescence (excited at 405 nm, red). Average $\Delta F/F$ of GCaMP6s signals from each trial (n=3 mice/trial) are displayed. Baseline recording was conducted prior to introducing stimulants (vertical lines=contacting chow, =contacting peanut butter chips, =returning to cage after intraperitoneal injection of CNO).

maximum reduction in AgRP neurons activity accessible through feeding on chow and peanut butter respectively (Figure. 33H). Thus, these results indicate that the neurons in the cerebellar nuclei are functionally connected to AgRP neurons and, more importantly, these neurons are capable influencing the activities of AgRP neurons which dictate food intake based on nutritional state.

3.5 DISCUSSION

3.5.1 Multiple pathways linking the cerebellum to key feeding centers

The growing list of motor and non-motor processes linked to the cerebellum can be explained, in part, by the extensive connectivity with other brain regions and spinal cord mediated primarily through neurons in the cerebellar nuclei. In addition to the role of the cerebellum in motor coordination and sensorimotor integration, the involvement of the cerebellum in homeostasis, visceral and higher cognitive functions is emerging (Schmahmann and Sherman, 1997; Zhu and Wang, 2008). However, details of afferent inputs and efferent connectivity with extra-cerebellar regions relevant for a specific behavior has yet to be defined. Electrophysiological studies in mice have shown that the cerebellar nuclei are functionally connected with hypothalamic neurons indicating the cerebellum could influence hypothalamus-mediated behaviors (Zhu et al., 2006; Zhu and Wang, 2008).

Recently, the ZI, a subthalamic nucleus well-established to be directly connected to cerebellum has been reported to consist of GABAergic neurons responsible for binge-like eating (Zhang and van den Pol, 2017). The DM has also been reported to be important for food intake behavior, lesion studies done in rat demonstrated that DM lesion led to a reduction in food intake (Bernardis, 1970; Bernardis et al., 1963). In addition to DM, the Arc of the hypothalamus is sufficient to drive robust feeding behavior, and is an central to the regulation of energy homeostasis (Aponte et al., 2011; Belgardt et al., 2009). Our tract tracing analysis revealed that neurons in the interposed nucleus may influence food intake through these critical regions, including the ZI, and the DM and the Arc of the hypothalamus.

In rats, anterograde labeling from the middle cerebellar peduncle demonstrated direct connectivity between the cerebellum and the Arc (Cavdar et al., 2018). Monosynaptic retrograde rabies virus tracing in mice, showed that POMC and AgRP neurons in the Arc receive inputs from DM neurons, and POMC receives inputs from the ZI (Wang et al., 2015). Notably, AgRP neurons inhibits POMC neurons (Aponte et al., 2011). We showed through calcium recordings and anatomical examinations that excitatory cerebellar neurons in the interposed nucleus are capable of suppressing the activity of AgRP neurons. Recent study in mice showed that leptin sensitive GABAergic ventral DM neurons sends inhibitory modulatory signals to AgRP neurons (Garfield et al., 2016). Stimulation and electrophysiological recording in mice have demonstrated that neurons in the interposed nucleus are functionally connected to DM neurons sensitive to gastric vagal inputs, CCK and glucose (Zhu et al., 2006). Together, our results and work by others raise the possibility that neurons in the interposed nucleus participates in complex feeding regulation through multiple dedicated feeding circuit components, and highlight the importance of identifying the specific neuronal subtypes that receive projections from the glutamatergic projection neurons in the interposed nucleus.

3.5.2 Role of the cerebellar interposed nucleus in feeding related behaviors

The cerebellar nuclei receives and integrates a diverse array of motor and sensory afferent inputs originating from the cerebellar cortex, and contains the last order neurons within the cerebellum to relay processed inputs to relevant brain regions (Cerminara et al., 2015; Uusisaari and De Schutter, 2011). Conventionally, the cerebellar nuclei are known for their involvement in motor activities (Manto et al., 2012), leading to an assumption that perturbed feeding-related activity as a result of cerebellar dysfunction is due to deficits in mastication activity, control of movement required for chewing (Byrd and Luschei, 1980). In an ideal position to influence cerebellar output, the cerebellar nuclei has been proposed be involved in many more aspects of feeding-related behaviors such as nutritional homeostasis (Mahler et al., 1993), visceral control (Lisander and Martner, 1975a, b; Manchanda et al., 1972) and hedonic appetitive motivation (Bauer et al., 2011). Specifically, electrophysiological studies have provided evidence that the cerebellar interposed nucleus and the DM of the hypothalamus is functionally connected (Zhu et al., 2006). However, the functional relevance of the interposed nucleus on feeding-related activities via this pathway has yet to be determined.

Our study shows that activation of neurons in the cerebellar interposed nucleus results in a significant reduction in food intake providing direct support for the importance of the interposed nucleus in feeding control. Human functional imaging studies report significant differences in cerebellar network activities between healthy and obese patients (Park et al., 2016), which further supports the role of cerebellum in feeding regulation. While, fMRI studies in anorexic nervosa patients revealed lower cerebellar activity than bulimia nervosa patients when presented with images of food (Uher et al., 2004), anorexia patients reveal greater cerebellar activity than bulimia patients upon cognitive thoughts of eating food

(Brooks et al., 2011). Interestingly, in line with this notion, we observed that activation of cerebellar interposed nucleus in fasted mice increased time spent in proximity to food, but consumed ~75% less food compared to vehicle injected trials (Figure 28D, E). A mapping study of the human brain in response to unappetising food highlighted an increased cerebellar activity as one of a late phase temporal event (Pujol et al., 2018). Collectively, our data and previous studies point toward a possibility that the cerebellar interposed nucleus negatively modulates preconsummatory phase to influence the onset of food intake response.

The reduced food intake activity as a consequence of manipulating the cerebellar nuclei potentially recapitulates physiological responses of conflicting external sensory inputs such as nausea and dizziness (Oman and Cullen, 2014). Interference of gastrointestinal movements related to motion sickness and vomiting were found to be associated with cerebellar lesions (Bard et al., 1947). Cerebellar nuclei output neurons have been reported to target hindbrain vagal neuronal circuits critical for emetic reflexes via cerebellohypothalamic and cerebellovestibular pathways (Babic and Browning, 2014; Balaban et al., 2014; Suzuki et al., 2012), indicative of cerebellar neural substrates to modulate visceral functions such as nausea and vomiting. Although gut musculature was not monitored in our study, it has been shown that AgRP network dynamics is independent of stretching in the gut (Su et al., 2017). Thus, we propose that chemogenetics excitation of the cerebellar interposed nucleus possibly induces motion sickness effects, and provide integrated cues crucial for survival adaptation in feeding behavior, to maximize the nutritive value of food by delaying food intake when nauseous.

Even though our expression analysis indicates *Ucn3* is not expressed at a high level in the cerebellar nuclei (Figure 14), it remains possible that *Ucn3* plays an important role for

cerebellar nuclei mediated food intake related activities through corticotropin-releasing hormone (CRH) signaling (Sztainberg and Chen, 2012). Studies have reported that *Urocortin* (*Ucn*) expression is detected in neurons involved in the cerebello-vestibular pathway (Swinny et al., 2002), suggesting *Ucn*-expressing neurons are involved in mediating vestibular functions. Additionally, the cerebellum has been implicated in non-somatic functions via connections with subthalamic and hypothalamic targets (Zhu et al., 2006), thus the expression of *Ucn3* may provide means for cerebellar output neurons to exert autonomic modulations. We speculate that *Ucn3* in cerebellar nuclei neurons may employ CRH signaling to exert its activities, through interactions with *Ucn3* binding receptor, Corticotropin-releasing factor receptor type-2 (CRF-R2) (Lewis et al., 2001). CRF-R2 is expressed in many regions of the brain (Chalmers et al., 1995; Porcher et al., 2005; Tache and Bonaz, 2007; Van Pett et al., 2000; Wiley and Davenport, 2004), one of which, the hypothalamus, is a target of the cerebellum (Figure 30A-C) (Cavdar et al., 2018; Haines et al., 1990). Potentially, IntA neurons utilizes CRH signaling to influence neurons in the ventral medial hypothalamus to regulate energy homeostasis, feeding, and blood glucose levels (Figure 33) (Chen et al., 2013).

3.6 FUTURE DIRECTIONS

3.6.1 Interrogation of the role of the cerebellar nuclei in feeding behavior

In this thesis, we show that the cerebellum plays an important role in mediating feeding-related responses through the connectivity between the cerebellar interposed nucleus with ZI, DM and the Arc. Although we have identified the functional connectivity of cerebellar interposed neurons and AgRP neurons, which suggests that reduced food intake is not due to cerebellar influence on visceral control, it is still unclear if nutritional homeostasis or feeding-related higher cognition is mediated by the cerebellum. In addition, the neuronal identity of cerebellar targets remains unknown. Thus, the following three experiments are designed to define the circuit elements and role of cerebellar circuit in feeding behavior.

Experiment 1. Define the identity of neurons involved in the cerebello-

subthalamic/hypothalamic pathway. This thesis provided evidence that neurons in the cerebellar interposed nucleus is either directly or indirectly connected to feeding-associated brain regions. But, the precise connections between neuronal types in the cerebellar nuclei and target regions are yet to be identified. Utilizing Cre mouse lines driven by gene promoters such as those related to the expression of leptin and GAD67, neurons controlling food intake in the DM and ZI could be targeted respectively for monosynaptic retrograde rabies virus tracing to neurons in the cerebellar nuclei.

Experiment 2. Examine the cerebellar activity pattern and their targets during feeding-

related activities. The cerebellar nuclei target a range of brain centers to mediate diverse behavioral functions. The pattern of activities of neurons within the cerebellar nuclei may be crucial in contributing to the behavioral output. By employing deep-brain imaging of genetically encoded calcium indicators in the cerebellum and its target regions, endogenous

activity patterns of neurons during food intake in freely moving mice will be monitored and analyzed for association with distinct phases of feeding-related response.

Experiment 3. Determine the role of cerebello-subthalamic/hypothalamic pathway in feeding-related behavior. Our results reveal that decrease in food intake may be a result of activation of neurons in the cerebellar interposed nucleus. However, the functional relevance of the cerebellum in dictating food intake remains unclear. We plan to examine the effects of manipulating cerebellar neurons on feeding related behavior by placing mice through an array of behavioral paradigms such as appetitive operant conditioning task, open field exploration, caloric preference test, place preference test, motion sickness inducing chamber, metabolic chamber, and other post-mortem analysis to identify gut activities. Together, the results from these experiments could uncover the physiological relevance of cerebellar-mediated feeding response.

Experiment 4. Determine the neuronal subtypes involved in feeding-related behavior. We showed that activation of cerebellar interposed neurons resulted in a reduction of food intake, but inactivation did not lead to significant changes in food intake. The lack of opposing effect could potentially be due to either a floor or ceiling effect of the DREADD assay on specific DCN neuronal subpopulations responsible for food intake activities. In order to examine these effects, we plan to use optogenetics to manipulate different neuronal subpopulations using Cre mouse lines including *GlyT2::Cre*, *Ucn3::Cre*, *vGat::Cre* and *vGluT2::Cre*. A gradient of light stimulation intensities will be introduced to rule out ceiling effect, and photo-inhibition may identify floor effect in neuronal sub-populations that do not exhibit spontaneous firing.

4. REFERENCE

- Aas, J.E., and Brodal, P. (1989). Demonstration of a Mamillo-Ponto-Cerebellar Pathway. *The European journal of neuroscience* *1*, 61-74.
- Ahissar, E., and Kleinfeld, D. (2003). Closed-loop neuronal computations: focus on vibrissa somatosensation in rat. *Cereb Cortex* *13*, 53-62.
- Akintunde, A., and Buxton, D.F. (1992). Origins and collateralization of corticospinal, corticopontine, corticorubral and corticostriatal tracts: a multiple retrograde fluorescent tracing study. *Brain research* *586*, 208-218.
- Aksenov, D., Serdyukova, N., Irwin, K., and Bracha, V. (2004). GABA Neurotransmission in the Cerebellar Interposed Nuclei: Involvement in Classically Conditioned Eyeblinks and Neuronal Activity. *Journal of neurophysiology* *91*, 719-727.
- Allen, G.I., and Tsukahara, N. (1974). Cerebrocerebellar communication systems. *Physiological reviews* *54*, 957-1006.
- Allen, G.V., and Hopkins, D.A. (1990). Topography and synaptology of mamillary body projections to the mesencephalon and pons in the rat. *The Journal of comparative neurology* *301*, 214-231.
- Alstermark, B., and Ekerot, C.F. (2013). The lateral reticular nucleus: a precerebellar centre providing the cerebellum with overview and integration of motor functions at systems level. A new hypothesis. *The Journal of physiology* *591*, 5453-5458.
- Alstermark, B., and Isa, T. (2012). Circuits for skilled reaching and grasping. *Annu Rev Neurosci* *35*, 559-578.
- Altman, J., and Bayer, S.A. (1997). *Development of the cerebellar system : in relation to its evolution, structure, and functions* (Boca Raton: CRC Press).
- Amami, P., Dekker, I., Piacentini, S., Ferré, F., Romito, L.M., Franzini, A., Foncke, E.M.J., and Albanese, A. (2015). Impulse control behaviours in patients with Parkinson's disease after subthalamic deep brain stimulation: de novo cases and 3-year follow-up. *Journal of Neurology, Neurosurgery & Psychiatry* *86*, 562-564.
- Angaut, P., Cicirata, F., and Serapide, F. (1985). Topographic organization of the cerebellothalamic projections in the rat. An autoradiographic study. *Neuroscience* *15*, 389-401.
- Ankri, L., Husson, Z., Pietrajtis, K., Provaille, R., Lena, C., Yarom, Y., Dieudonne, S., and Uusisaari, M.Y. (2015). A novel inhibitory nucleo-cortical circuit controls cerebellar Golgi cell activity. *Elife* *4*.
- Annoni, J.M., Ptak, R., Caldara-Schnetzer, A.S., Khateb, A., and Pollermann, B.Z. (2003). Decoupling of autonomic and cognitive emotional reactions after cerebellar stroke. *Ann Neurol* *53*, 654-658.
- Aponte, Y., Atasoy, D., and Sternson, S.M. (2011). AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nature neuroscience* *14*, 351-355.
- Apps, R., and Garwicz, M. (2000). Precise matching of olivo-cortical divergence and cortico-nuclear convergence between somatotopically corresponding areas in the medial C1 and medial C3 zones of the paravermal cerebellum. *The European journal of neuroscience* *12*, 205-214.
- Apps, R., and Garwicz, M. (2005). Anatomical and physiological foundations of cerebellar information processing. *Nature reviews Neuroscience* *6*, 297-311.
- Apps, R., and Hawkes, R. (2009). Cerebellar cortical organization: a one-map hypothesis. *Nature reviews Neuroscience* *10*, 670-681.

- Armbruster, B.N., Li, X., Pausch, M.H., Herlitze, S., and Roth, B.L. (2007). Evolving the lock to fit the key to create a family of G protein-coupled receptors potentially activated by an inert ligand. *Proceedings of the National Academy of Sciences of the United States of America* 104, 5163-5168.
- Armstrong, D.M., and Edgley, S.A. (1984). Discharges of Purkinje cells in the paravermal part of the cerebellar anterior lobe during locomotion in the cat. *The Journal of physiology* 352, 403-424.
- Asanuma, C., Thach, W.T., and Jones, E.G. (1983). Distribution of Cerebellar Terminations and Their Relation to Other Afferent Terminations in the Ventral Lateral Thalamic Region of the Monkey. *Brain Res Rev* 5, 237-265.
- Aumann, T.D., Rawson, J.A., Finkelstein, D.I., and Horne, M.K. (1994). Projections from the Lateral and Interposed Cerebellar Nuclei to the Thalamus of the Rat - a Light and Electron-Microscopic Study Using Single and Double Anterograde Labeling. *Journal of Comparative Neurology* 349, 165-181.
- Azim, E., Fink, A.J., and Jessell, T.M. (2014a). Internal and External Feedback Circuits for Skilled Forelimb Movement. *Cold Spring Harb Symp Quant Biol* 79, 81-92.
- Azim, E., Jiang, J., Alstermark, B., and Jessell, T.M. (2014b). Skilled reaching relies on a V2a propriospinal internal copy circuit. *Nature* 508, 357-363.
- Babic, T., and Browning, K.N. (2014). The role of vagal neurocircuits in the regulation of nausea and vomiting. *Eur J Pharmacol* 722, 38-47.
- Bagnall, M.W., Zingg, B., Sakatos, A., Moghadam, S.H., Zeilhofer, H.U., and du Lac, S. (2009). Glycinergic projection neurons of the cerebellum. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29, 10104-10110.
- Baimbridge, K.G., Celio, M.R., and Rogers, J.H. (1992). Calcium-binding proteins in the nervous system. *Trends in neurosciences* 15, 303-308.
- Baird, A.L., Meldrum, A., and Dunnett, S.B. (2001). The staircase test of skilled reaching in mice. *Brain research bulletin* 54, 243-250.
- Balaban, C.D., Ogburn, S.W., Warshafsky, S.G., Ahmed, A., and Yates, B.J. (2014). Identification of Neural Networks That Contribute to Motion Sickness through Principal Components Analysis of Fos Labeling Induced by Galvanic Vestibular Stimulation. *PloS one* 9, e86730.
- Barbour, B. (1993). Synaptic currents evoked in purkinje cells by stimulating individual granule cells. *Neuron* 11, 759-769.
- Bard, P., and Woolsey, C.N. (1947). Delimitation of central nervous mechanisms involved in motion sickness. *Fed Proc* 6, 72.
- Barski, J.J., Dethleffsen, K., and Meyer, M. (2000). Cre recombinase expression in cerebellar Purkinje cells. *Genesis* 28, 93-98.
- Bastian, A.J. (2006). Learning to predict the future: the cerebellum adapts feedforward movement control. *Current opinion in neurobiology* 16, 645-649.
- Bastian, A.J., Martin, T.A., Keating, J.G., and Thach, W.T. (1996). Cerebellar ataxia: abnormal control of interaction torques across multiple joints. *Journal of neurophysiology* 76, 492-509.
- Batton, R.R., 3rd, Jayaraman, A., Ruggiero, D., and Carpenter, M.B. (1977). Fastigial efferent projections in the monkey: an autoradiographic study. *J Comp Neurol* 174, 281-305.
- Bauer, D.J., Kerr, A.L., and Swain, R.A. (2011). Cerebellar dentate nuclei lesions reduce motivation in appetitive operant conditioning and open field exploration. *Neurobiology of learning and memory* 95, 166-175.

- Bauer, D.J., Peterson, T.C., and Swain, R.A. (2014). Cerebellar dentate nuclei lesions alter prefrontal cortex dendritic spine morphology. *Brain research* 1544, 15-24.
- Baumann, O., and Mattingley, J.B. (2012). Functional topography of primary emotion processing in the human cerebellum. *NeuroImage* 61, 805-811.
- Bava, A., Grimm, R.J., and Rushmer, D.S. (1983). Fastigial unit activity during voluntary movement in primates. *Brain research* 288, 371-374.
- Beitzel, C.S., Houck, B.D., Lewis, S.M., and Person, A.L. (2017). Rubrocerebellar Feedback Loop Isolates the Interposed Nucleus as an Independent Processor of Corollary Discharge Information in Mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 37, 10085-10096.
- Belgardt, B.F., Okamura, T., and Bruning, J.C. (2009). Hormone and glucose signalling in POMC and AgRP neurons. *The Journal of physiology* 587, 5305-5314.
- Bengtsson, F., and Hesslow, G. (2006). Cerebellar control of the inferior olive. *The Cerebellum* 5, 7-14.
- Bernardis, L.L. (1970). Participation of the dorsomedial hypothalamic nucleus in the "feeding center" and water intake circuitry of the weanling rat. *Journal of Neuro-Visceral Relations* 31, 387-398.
- Bernardis, L.L., Box, B.M., and Stevenson, J.A.F. (1963). Growth Following Hypothalamic Lesions in the Weanling Rat1. *Endocrinology* 72, 684-692.
- Betley, J.N., Cao, Z.F.H., Ritola, K.D., and Sternson, S.M. (2013). Parallel, redundant circuit organization for homeostatic control of feeding behavior. *Cell* 155, 1337-1350.
- Betley, J.N., Xu, S., Cao, Z.F.H., Gong, R., Magnus, C.J., Yu, Y., and Sternson, S.M. (2015). Neurons for hunger and thirst transmit a negative-valence teaching signal. *Nature* 521, 180-185.
- Biane, J.S., Takashima, Y., Scanziani, M., Conner, J.M., and Tuszynski, M.H. (2016). Thalamocortical Projections onto Behaviorally Relevant Neurons Exhibit Plasticity during Adult Motor Learning. *Neuron* 89, 1173-1179.
- Bidarimath, M., Edwards, A.K., and Tayade, C. (2015). Laser capture microdissection for gene expression analysis. *Methods in molecular biology* 1219, 115-137.
- Bortoff, G.A., and Strick, P.L. (1993). Corticospinal terminations in two new-world primates: further evidence that corticomotoneuronal connections provide part of the neural substrate for manual dexterity. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 13, 5105-5118.
- Bostan, A.C., Dum, R.P., and Strick, P.L. (2013). Cerebellar networks with the cerebral cortex and basal ganglia. *Trends in Cogn Sci* 17, 241-254.
- Bracha, V., Kolb, F.P., Irwin, K.B., and Bloedel, J.R. (1999). Inactivation of interposed nuclei in the cat: classically conditioned withdrawal reflexes, voluntary limb movements and the action primitive hypothesis. *Experimental brain research* 126, 77-92.
- Bras, H., Cavallari, P., and Jankowska, E. (1988). Demonstration of initial axon collaterals of cells of origin of the ventral spinocerebellar tract in the cat. *The Journal of comparative neurology* 273, 584-592.
- Braz, J.M., Rico, B., and Basbaum, A.I. (2002). Transneuronal tracing of diverse CNS circuits by Cre-mediated induction of wheat germ agglutinin in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* 99, 15148-15153.
- Brodal, P., and Walberg, F. (1977). The pontine projection to the cerebellar anterior lobe. An experimental study in the cat with retrograde transport of horseradish peroxidase. *Experimental brain research* 29, 233-248.

- Brooks, S.J., O'Daly, O.G., Uher, R., Friederich, H.C., Giampietro, V., Brammer, M., Williams, S.C., Schiöth, H.B., Treasure, J., and Campbell, I.C. (2011). Differential neural responses to food images in women with bulimia versus anorexia nervosa. *PloS one* 6, e22259.
- Buckner, R.L. (2013). The cerebellum and cognitive function: 25 years of insight from anatomy and neuroimaging. *Neuron* 80, 807-815.
- Buisseret-Delmas, C., and Angaut, P. (1993). The cerebellar olivo-corticonuclear connections in the rat. *Progress in neurobiology* 40, 63-87.
- Byrd, K.E., and Luschei, E.S. (1980). Cerebellar ablation and mastication in the guinea pig (*Cavia porcellus*). *Brain research* 197, 577-581.
- Campfield, L.A., and Smith, F.J. (2003). Blood glucose dynamics and control of meal initiation: a pattern detection and recognition theory. *Physiological reviews* 83, 25-58.
- Carbo-Gas, M., Vazquez-Sanroman, D., Aguirre-Manzo, L., Coria-Avila, G.A., Manzo, J., Sanchis-Segura, C., and Miquel, M. (2014). Involving the cerebellum in cocaine-induced memory: pattern of cFos expression in mice trained to acquire conditioned preference for cocaine. *Addiction biology* 19, 61-76.
- Carter, M.E., Soden, M.E., Zweifel, L.S., and Palmiter, R.D. (2013). Genetic identification of a neural circuit that suppresses appetite. *Nature* 503, 111-114.
- Cavdar, S., Ozgur, M., Kuvvet, Y., and Bay, H.H. (2018). The Cerebello-Hypothalamic and Hypothalamo-Cerebellar Pathways via Superior and Middle Cerebellar Peduncle in the Rat. *Cerebellum*.
- Cavdar, S., San, T., Aker, R., Sehirli, U., and Onat, F. (2001). Cerebellar connections to the dorsomedial and posterior nuclei of the hypothalamus in the rat. *J Anat* 198, 37-45.
- Celio, M.R. (1990). Calbindin D-28k and parvalbumin in the rat nervous system. *Neuroscience* 35, 375-475.
- Cerminara, N.L., Lang, E.J., Sillitoe, R.V., and Apps, R. (2015). Redefining the cerebellar cortex as an assembly of non-uniform Purkinje cell microcircuits. *Nature reviews Neuroscience* 16, 79-93.
- Chadderton, P., Margrie, T.W., and Häusser, M. (2004). Integration of quanta in cerebellar granule cells during sensory processing. *Nature* 428, 856.
- Chalmers, D.T., Lovenberg, T.W., and De Souza, E.B. (1995). Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15, 6340-6350.
- Chan-Palay, V. (1977). Cerebellar dentate nucleus : organization, cytology and transmitters (Berlin ; New York: Springer-Verlag).
- Chen, P., Van Hove, C., Lindberg, D., and Li, C. (2013). Central urocortin 3 and type 2 corticotropin-releasing factor receptor in the regulation of energy homeostasis: critical involvement of the ventromedial hypothalamus. *Frontiers in Endocrinology* 3.
- Chen, S., and Hillman, D.E. (1993). Colocalization of neurotransmitters in the deep cerebellar nuclei. *Journal of Neurocytology* 22, 81-91.
- Chen, Y., Lin, Y.C., Kuo, T.W., and Knight, Z.A. (2015). Sensory detection of food rapidly modulates arcuate feeding circuits. *Cell* 160, 829-841.
- Chung, S.H., Marzban, H., and Hawkes, R. (2009). Compartmentation of the cerebellar nuclei of the mouse. *Neuroscience* 161, 123-138.

- Clement-Ziza, M., Gentien, D., Lyonnet, S., Thiery, J.P., Besmond, C., and Decraene, C. (2009). Evaluation of methods for amplification of picogram amounts of total RNA for whole genome expression profiling. *BMC genomics* 10, 246.
- Clower, D.M., West, R.A., Lynch, J.C., and Strick, P.L. (2001). The inferior parietal lobule is the target of output from the superior colliculus, hippocampus, and cerebellum. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21, 6283-6291.
- Conte, W.L., Kamishina, H., and Reep, R.L. (2009). Multiple neuroanatomical tract-tracing using fluorescent Alexa Fluor conjugates of cholera toxin subunit B in rats. *Nature protocols* 4, 1157-1166.
- Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdán, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411, 480.
- Cui, G., Jun, S.B., Jin, X., Luo, G., Pham, M.D., Lovinger, D.M., Vogel, S.S., and Costa, R.M. (2014). Deep brain optical measurements of cell type-specific neural activity in behaving mice. *Nature protocols* 9, 1213-1228.
- Damasio, A.R., Grabowski, T.J., Bechara, A., Damasio, H., Ponto, L.L.B., Parvizi, J., and Hichwa, R.D. (2000). Subcortical and cortical brain activity during the feeling of self-generated emotions. *Nature neuroscience* 3, 1049.
- De Zeeuw, C.I., and Berrebi, A.S. (1995). Postsynaptic targets of Purkinje cell terminals in the cerebellar and vestibular nuclei of the rat. *The European journal of neuroscience* 7, 2322-2333.
- Dezeeuw, C.I., and Berrebi, A.S. (1995). Postsynaptic Targets of Purkinje-Cell Terminals in the Cerebellar and Vestibular Nuclei of the Rat. *European Journal of Neuroscience* 7, 2322-2333.
- Dietrichs, E. (1984). Cerebellar autonomic function: direct hypothalamocerebellar pathway. *Science* 223, 591-593.
- Dietrichs, E., Haines, D.E., Roste, G.K., and Roste, L.S. (1994). Hypothalamocerebellar and cerebellohypothalamic projections--circuits for regulating nonsomatic cerebellar activity? *Histology and histopathology* 9, 603-614.
- Dietrichs, E., Walberg, F., and Haines, D.E. (1985). Cerebellar Nuclear Afferents from Feline Hypothalamus Demonstrated by Retrograde Transport after Implantation of Crystalline Wheat-Germ Agglutinin Horseradish Peroxidase Complex. *Neurosci Lett* 54, 129-133.
- Disterhoft, J.F., Quinn, K.J., Weiss, C., and Shipley, M.T. (1985). Accessory abducens nucleus and conditioned eye retraction/nictitating membrane extension in rabbit. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 5, 941-950.
- Dragunow, M., Abraham, W.C., Goulding, M., Mason, S.E., Robertson, H.A., and Faull, R.L. (1989). Long-term potentiation and the induction of c-fos mRNA and proteins in the dentate gyrus of unanesthetized rats. *Neurosci Lett* 101, 274-280.
- Dum, R.P., and Strick, P.L. (2003). An unfolded map of the cerebellar dentate nucleus and its projections to the cerebral cortex. *Journal of neurophysiology* 89, 634-639.
- Ebner, T.J., Hewitt, A.L., and Popa, L.S. (2011). What features of limb movements are encoded in the discharge of cerebellar neurons? *Cerebellum* 10, 683-693.
- Eccles, J.C., Ito, M., and Szentágothai, J.n. (1967). *The cerebellum as a neuronal machine* (Berlin, New York etc.: Springer-Verlag).

- Ekerot, C.F., Garwicz, M., and Jorntell, H. (1997). The control of forelimb movements by intermediate cerebellum. *Progress in brain research* 114, 423-429.
- Ellerman, J.M., Flament, D., Kim, S.G., Fu, Q.G., Merkle, H., Ebner, T.J., and Ugurbil, K. (1994). Spatial patterns of functional activation of the cerebellum investigated using high field (4 T) MRI. *NMR in biomedicine* 7, 63-68.
- Elsen, G., Juric-Sekhar, G., Daza, R.M., and Hevner, R. (2013). Development of Cerebellar Nuclei. In *Handbook of the Cerebellum and Cerebellar Disorders*, M. Manto, J. Schmahmann, F. Rossi, D. Gruol, and N. Koibuchi, eds. (Springer Netherlands), pp. 179-205.
- Esposito, M.S., Capelli, P., and Arber, S. (2014). Brainstem nucleus MdV mediates skilled forelimb motor tasks. *Nature* 508, 351-356.
- Fink, A.J., Croce, K.R., Huang, Z.J., Abbott, L.F., Jessell, T.M., and Azim, E. (2014). Presynaptic inhibition of spinal sensory feedback ensures smooth movement. *Nature* 509, 43-48.
- Fink, H., Rex, A., Voits, M., and Voigt, J.P. (1998). Major biological actions of CCK--a critical evaluation of research findings. *Experimental brain research* 123, 77-83.
- Fix, J.D. (1975). Dentate Nucleus of Ateles-Ater - Cytomorphometric Analysis. *Acta Anat* 93, 228-239.
- Franklin, D.W., and Wolpert, D.M. (2011). Computational mechanisms of sensorimotor control. *Neuron* 72, 425-442.
- Gao, Z., Proietti-Onori, M., Lin, Z., Ten Brinke, M.M., Boele, H.J., Potters, J.W., Ruigrok, T.J., Hoebeek, F.E., and De Zeeuw, C.I. (2016). Excitatory Cerebellar Nucleocortical Circuit Provides Internal Amplification during Associative Conditioning. *Neuron* 89, 645-657.
- Garfield, A.S., Shah, B.P., Burgess, C.R., Li, M.M., Li, C., Steger, J.S., Madara, J.C., Campbell, J.N., Kroeger, D., and Scammell, T.E. (2016). Dynamic GABAergic afferent modulation of AgRP neurons. *Nature neuroscience* 19, 1628-1635.
- Gautier, J.F., Chen, K., Uecker, A., Bandy, D., Frost, J., Salbe, A.D., Pratley, R.E., Lawson, M., Ravussin, E., and Reiman, E.M. (1999). Regions of the human brain affected during a liquid-meal taste perception in the fasting state: a positron emission tomography study. *The American journal of clinical nutrition* 70, 806-810.
- Gibson, A.R., Houk, J.C., and Kohlerman, N.J. (1985). Magnocellular red nucleus activity during different types of limb movement in the macaque monkey. *The Journal of physiology* 358, 527-549.
- Gilbert, P.F., and Thach, W.T. (1977). Purkinje cell activity during motor learning. *Brain research* 128, 309-328.
- Goldberg, J.M., and Cullen, K.E. (2011). Vestibular control of the head: possible functions of the vestibulocollic reflex. *Experimental brain research* 210, 331-345.
- Gomez, J.L., Bonaventura, J., Lesniak, W., Mathews, W.B., Sysa-Shah, P., Rodriguez, L.A., Ellis, R.J., Richie, C.T., Harvey, B.K., and Dannals, R.F. (2017). Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science* 357, 503-507.
- Gong, S., Zheng, C., Doughty, M.L., Losos, K., Didkovsky, N., Schambra, U.B., Nowak, N.J., Joyner, A., Leblanc, G., and Hatten, M.E. (2003). A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* 425, 917-925.
- Guell, X., Gabrieli, J.D.E., and Schmahmann, J.D. (2018). Triple representation of language, working memory, social and emotion processing in the cerebellum: convergent evidence from task and seed-based resting-state fMRI analyses in a single large cohort. *NeuroImage* 172, 437-449.

- Guo, J.Z., Graves, A.R., Guo, W.W., Zheng, J., Lee, A., Rodriguez-Gonzalez, J., Li, N., Macklin, J.J., Phillips, J.W., and Mensh, B.D. (2015). Cortex commands the performance of skilled movement. *eLife* 4, e10774.
- Habas, C., Kamdar, N., Nguyen, D., Prater, K., Beckmann, C.F., Menon, V., and Greicius, M.D. (2009). Distinct cerebellar contributions to intrinsic connectivity networks. *J Neurosci* 29, 8586-8594.
- Haines, D.E., Dietrichs, E., Mihailoff, G.A., and McDonald, E.F. (1997). The cerebellar-hypothalamic axis: basic circuits and clinical observations. *Int Rev Neurobiol* 41, 83-107.
- Haines, D.E., May, P.J., and Dietrichs, E. (1990). Neuronal connections between the cerebellar nuclei and hypothalamus in *Macaca fascicularis*: cerebello-visceral circuits. *The Journal of comparative neurology* 299, 106-122.
- Haroian, A.J., Massopust, L.C., and Young, P.A. (1981). Cerebellothalamic projections in the rat: an autoradiographic and degeneration study. *The Journal of comparative neurology* 197, 217-236.
- Harper, J.W., and Heath, R.G. (1973). Anatomic connections of the fastigial nucleus to the rostral forebrain in the cat. *Exp Neurol* 39, 285-292.
- Harvey, R.J., Porter, R., and Rawson, J.A. (1979). Discharges of intracerebellar nuclear cells in monkeys. *The Journal of physiology* 297, 559-580.
- Hawkes, R., and Leclerc, N. (1987). Antigenic map of the rat cerebellar cortex: The distribution of parasagittal bands as revealed by monoclonal anti-purkinje cell antibody mabQ113. *Journal of Comparative Neurology* 256, 29-41.
- Heath, R.G. (1976). Brain function in epilepsy: midbrain, medullary, and cerebellar interaction with the rostral forebrain. *Journal of Neurology, Neurosurgery, and Psychiatry* 39, 1037-1051.
- Heath, R.G. (1977). Modulation of emotion with a brain pacemaker. Treatment for intractable psychiatric illness. *The Journal of nervous and mental disease* 165, 300-317.
- Heckroth, J.A. (1994). Quantitative Morphological Analysis of the Cerebellar Nuclei in Normal and Lurcher Mutant Mice .1. Morphology and Cell Number. *Journal of Comparative Neurology* 343, 173-182.
- Heiney, S.A., Wohl, M.P., Chettih, S.N., Ruffolo, L.I., and Medina, J.F. (2014). Cerebellar-dependent expression of motor learning during eyeblink conditioning in head-fixed mice. *J Neurosci* 34, 14845-14853.
- Hioki, H., Fujiyama, F., Taki, K., Tomioka, R., Furuta, T., Tamamaki, N., and Kaneko, T. (2003). Differential distribution of vesicular glutamate transporters in the rat cerebellar cortex. *Neuroscience* 117, 1-6.
- Hippenmeyer, S., Vrieseling, E., Sigrist, M., Portmann, T., Laengle, C., Ladle, D.R., and Arber, S. (2005). A developmental switch in the response of DRG neurons to ETS transcription factor signaling. *PLoS biology* 3, e159.
- Hoover, J.E., and Strick, P.L. (1999). The organization of cerebellar and basal ganglia outputs to primary motor cortex as revealed by retrograde transneuronal transport of herpes simplex virus type 1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19, 1446-1463.
- Horne, M.K., and Butler, E.G. (1995). The role of the cerebello-thalamo-cortical pathway in skilled movement. *Progress in neurobiology* 46, 199-213.
- Houck, B.D., and Person, A.L. (2015). Cerebellar premotor output neurons collateralize to innervate the cerebellar cortex. *The Journal of comparative neurology*.
- Iijima, T., Miura, E., Watanabe, M., and Yuzaki, M. (2010). Distinct expression of C1q-like family mRNAs in mouse brain and biochemical characterization of their encoded proteins, Vol 31.

- Inquimbert, P., Moll, M., Kohno, T., and Scholz, J. (2013). Stereotaxic injection of a viral vector for conditional gene manipulation in the mouse spinal cord. *Journal of visualized experiments : JoVE*, e50313.
- Ishihara, N., Armsen, W., Papadopoulos, T., Betz, H., and Eulenburg, V. (2010). Generation of a mouse line expressing Cre recombinase in glycinergic interneurons. *Genesis* 48, 437-445.
- Ito, M. (1997). Cerebellar Microcomplexes. In *Int Rev Neurobiol*, R.J. Bradley, R.A. Harris, P. Jenner, and J.D. Schmammann, eds. (Academic Press), pp. 475-487.
- Ito, M. (2001). Cerebellar long-term depression: characterization, signal transduction, and functional roles. *Physiological reviews* 81, 1143-1195.
- Ito, M. (2002). Historical review of the significance of the cerebellum and the role of Purkinje cells in motor learning. *Annals of the New York Academy of Sciences* 978, 273-288.
- Ito, M. (2006). Cerebellar circuitry as a neuronal machine. *Progress in brain research* 78, 272-303.
- Ito, M. (2013). Error detection and representation in the olivo-cerebellar system. *Frontiers in neural circuits* 7.
- Ito, S., and Feldheim, D.A. (2018). The Mouse Superior Colliculus: An Emerging Model for Studying Circuit Formation and Function. *Frontiers in neural circuits* 12, 10.
- Ivry, R.B., Spencer, R.M., Zelaznik, H.N., and Diedrichsen, J. (2002). The cerebellum and event timing. *Annals of the New York Academy of Sciences* 978, 302-317.
- Jennings, J.H., Rizzi, G., Stamatakis, A.M., Ung, R.L., and Stuber, G.D. (2013). The inhibitory circuit architecture of the lateral hypothalamus orchestrates feeding. *Science* 341, 1517-1521.
- Jörntell, H., and Ekerot, C.-F. (1999). Topographical organization of projections to cat motor cortex from nucleus interpositus anterior and forelimb skin. *The Journal of physiology* 514, 551-566.
- Karádi, Z., Oomura, Y., Nishino, H., Scott, T.R., Lénárd, L., and Aou, S. (1990). Complex attributes of lateral hypothalamic neurons in the regulation of feeding of alert rhesus monkeys. *Brain research bulletin* 25, 933-939.
- Keele, S.W., and Ivry, R. (1990). Does the cerebellum provide a common computation for diverse tasks? A timing hypothesis. *Annals of the New York Academy of Sciences* 608, 179-207; discussion 207-111.
- Keifer, J., and Houk, J.C. (1994). Motor function of the cerebellorubrospinal system. *Physiological reviews* 74, 509-542.
- Kelly, R.M., and Strick, P.L. (2003). Cerebellar loops with motor cortex and prefrontal cortex of a nonhuman primate. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23, 8432-8444.
- Keough, M.B., Jensen, S.K., and Yong, V.W. (2015). Experimental Demyelination and Remyelination of Murine Spinal Cord by Focal Injection of Lysolecithin. *Journal of visualized experiments : JoVE*, 52679.
- Keren-Happuch, E., Chen, S.-H.A., Ho, M.-H.R., and Desmond, J.E. (2014). A Meta-analysis of Cerebellar Contributions to Higher Cognition from PET and fMRI studies. *Human brain mapping* 35, 593-615.
- Kievit, J., and Kuypers, H.G. (1977). Organization of the thalamo-cortical connexions to the frontal lobe in the rhesus monkey. *Experimental brain research* 29, 299-322.
- Kitazawa, S. (1993). Quantitative evaluation of reaching movements in cats with and without cerebellar lesions using normalized integral of jerk. Role of the cerebellum and basal ganglia in voluntary movement.
- Klein, A., and Dunnett, S.B. (2012). Analysis of skilled forelimb movement in rats: the single pellet reaching test and staircase test. *Current protocols in neuroscience / editorial board, Jacqueline N Crawley [et al]* Chapter 8, Unit 8.28.

- Krashes, M.J., Shah, B.P., Madara, J.C., Olson, D.P., Strohlic, D.E., Garfield, A.S., Vong, L., Pei, H., Watabe-Uchida, M., and Uchida, N. (2014). An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. *Nature* *507*, 238-242.
- Kuper, M., Hermsdorfer, J., Brandauer, B., Thurling, M., Schoch, B., Theysohn, N., and Timmann, D. (2011). Lesions of the dentate and interposed nuclei are associated with impaired prehension in cerebellar patients. *Neurosci Lett* *499*, 132-136.
- Kuramoto, E., Fujiyama, F., Nakamura, K.C., Tanaka, Y., Hioki, H., and Kaneko, T. (2011). Complementary distribution of glutamatergic cerebellar and GABAergic basal ganglia afferents to the rat motor thalamic nuclei. *European Journal of Neuroscience* *33*, 95-109.
- Kuramoto, E., Furuta, T., Nakamura, K.C., Unzai, T., Hioki, H., and Kaneko, T. (2009). Two Types of Thalamocortical Projections from the Motor Thalamic Nuclei of the Rat: A Single Neuron-Tracing Study Using Viral Vectors. *Cereb Cortex* *19*, 2065-2077.
- Kurzan, R., Straube, A., and Buttner, U. (1993). The effect of muscimol micro-injections into the fastigial nucleus on the optokinetic response and the vestibulo-ocular reflex in the alert monkey. *Experimental brain research* *94*, 252-260.
- Lainé, J., and Axelrad, H. (1994). The candelabrum cell: A new interneuron in the cerebellar cortex. *Journal of Comparative Neurology* *339*, 159-173.
- Lee, K.H., Mathews, P.J., Reeves, A.M., Choe, K.Y., Jami, S.A., Serrano, R.E., and Otis, T.S. (2015). Circuit mechanisms underlying motor memory formation in the cerebellum. *Neuron* *86*, 529-540.
- Lee, R.X., Huang, J.J., Huang, C., Tsai, M.L., and Yen, C.T. (2014). Collateral projections from vestibular nuclear and inferior olivary neurons to lobules I/II and IX/X of the rat cerebellar vermis: a double retrograde labeling study. *The European journal of neuroscience* *40*, 2811-2821.
- Lee, S., Yoon, B.E., Berglund, K., Oh, S.J., Park, H., Shin, H.S., Augustine, G.J., and Lee, C.J. (2010). Channel-Mediated Tonic GABA Release from Glia. *Science* *330*, 790-796.
- Leergaard, T., and Bjaalie, J. (2007). Topography of the complete corticopontine projection: From experiments to principal maps. *Frontiers in neuroscience* *1*.
- Leggio, M.G., Chiricozzi, F.R., Clausi, S., Tedesco, A.M., and Molinari, M. (2011). The neuropsychological profile of cerebellar damage: The sequencing hypothesis. *Cortex; a journal devoted to the study of the nervous system and behavior* *47*, 137-144.
- Leiner, H.C., Leiner, A.L., and Dow, R.S. (1994). The underestimated cerebellum. *Human Brain Mapping* *2*, 244-254.
- Lerner, T.N., Shilyansky, C., Davidson, T.J., Evans, K.E., Beier, K.T., Zalocusky, K.A., Crow, A.K., Malenka, R.C., Luo, L., and Tomer, R. (2015). Intact-Brain Analyses Reveal Distinct Information Carried by SNc Dopamine Subcircuits. *Cell* *162*, 635-647.
- Leto, K., Carletti, B., Williams, I.M., Magrassi, L., and Rossi, F. (2006). Different types of cerebellar GABAergic interneurons originate from a common pool of multipotent progenitor cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* *26*, 11682-11694.
- Lewis, K., Li, C., Perrin, M.H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T.M., Gulyas, J., and Fischer, W. (2001). Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proceedings of the National Academy of Sciences of the United States of America* *98*, 7570-7575.

- Libbrecht, S., Van den Haute, C., Malinouskaya, L., Gijsbers, R., and Baekelandt, V. (2017). Evaluation of WGA-Cre-dependent topological transgene expression in the rodent brain. *Brain structure & function* 222, 717-733.
- Lisander, B., and Martner, J. (1975a). Effects on gastric motility from the cerebellar fastigial nucleus. *Acta physiologica Scandinavica* 94, 368-377.
- Lisander, B., and Martner, J. (1975b). Integrated Somatomotor, Cardiovascular and Gastrointestinal Adjustments Induced from the Cerebellar Fastigial Nucleus. *Acta physiologica Scandinavica* 94, 358-367.
- Liu, Y., Gao, J.-H., Liu, H.-L., and Fox, P.T. (2000). The temporal response of the brain after eating revealed by functional MRI. *Nature* 405, 1058.
- Locke, T.M., Soden, M.E., Miller, S.M., Hunker, A., Knakal, C., Licholai, J.A., Dhillon, K.S., Keene, C.D., Zweifel, L.S., and Carlson, E.S. (2018). Dopamine D₁ Receptor⁺ Positive Neurons in the Lateral Nucleus of the Cerebellum Contribute to Cognitive Behavior. *Biological psychiatry*.
- Low, A.Y.T., Thanawalla, A.R., Yip, A.K.K., Kim, J., Wong, K.L.L., Tantra, M., Augustine, G.J., and Chen, A.I. (2018). Precision of Discrete and Rhythmic Forelimb Movements Requires a Distinct Neuronal Subpopulation in the Interposed Anterior Nucleus. *Cell reports* 22, 2322-2333.
- Luo, Y., and Sugihara, I. (2014). Cerebellar afferents originating from the medullary reticular formation that are different from mossy, climbing or monoaminergic fibers in the rat. *Brain research* 1566, 31-46.
- Machado, A.S., Darmohray, D.M., Fayad, J., Marques, H.G., and Carey, M.R. (2015). A quantitative framework for whole-body coordination reveals specific deficits in freely walking ataxic mice. *Elife* 4.
- Mackay, W.A. (1988). Cerebellar Nuclear Activity in Relation to Simple Movements. *Experimental brain research* 71, 47-58.
- Madisen, L., Mao, T., Koch, H., Zhuo, J.M., Berenyi, A., Fujisawa, S., Hsu, Y.W., Garcia, A.J., 3rd, Gu, X., and Zanella, S. (2012). A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. *Nature neuroscience* 15, 793-802.
- Mahler, P., Guastavino, J.M., Jacquart, G., and Strazielle, C. (1993). An Unexpected Role of the Cerebellum - Involvement in Nutritional Organization. *Physiology & behavior* 54, 1063-1067.
- Manchanda, S.K., Tandon, O.P., and Aneja, I.S. (1972). Role of the cerebellum in the control of gastrointestinal motility. *Journal of neural transmission* 33, 195-209.
- Mandelblat-Cerf, Y., Ramesh, R.N., Burgess, C.R., Patella, P., Yang, Z.F., Lowell, B.B., and Andermann, M.L. (2015). Arcuate hypothalamic AgRP and putative POMC neurons show opposite changes in spiking across multiple timescales. *Elife* 4.
- Manto, M., Bower, J.M., Conforto, A.B., Delgado-Garcia, J.M., da Guarda, S.N., Gerwig, M., Habas, C., Hagura, N., Ivry, R.B., and Marien, P. (2012). Consensus paper: roles of the cerebellum in motor control-the diversity of ideas on cerebellar involvement in movement. *Cerebellum* 11, 457-487.
- Marr, D. (1969). A theory of cerebellar cortex. *The Journal of physiology* 202, 437-470.
- Martner, J. (1975). Influences on the Defecation and Micturition Reflexes by the Cerebellar Fastigial Nucleus. *Acta physiologica Scandinavica* 94, 95-104.
- Marzban, H., and Hawkes, R. (2007). Fibroblast growth factor promotes the development of deep cerebellar nuclear neurons in dissociated mouse cerebellar cultures. *Brain research* 1141, 25-36.
- McCormick, D.A., McGinley, M.J., and Salkoff, D.B. (2015). Brain state dependent activity in the cortex and thalamus. *Current opinion in neurobiology* 31, 133-140.

- McCurdy, M.L., Hansma, D.I., Houk, J.C., and Gibson, A.R. (1987). Selective projections from the cat red nucleus to digit motor neurons. *Journal of Comparative Neurology* 265, 367-379.
- Mendes, C.S., Bartos, I., Marka, Z., Akay, T., Marka, S., and Mann, R.S. (2015). Quantification of gait parameters in freely walking rodents. *Bmc Biol* 13.
- Miall, R.C., and Wolpert, D.M. (1996). Forward Models for Physiological Motor Control. *Neural Netw* 9, 1265-1279.
- Middleton, F.A., and Strick, P.L. (1997). Cerebellar Output Channels. In *Int Rev Neurobiol*, R.A.H.P.J. Ronald J. Bradley, and D.S. Jeremy, eds. (Academic Press), pp. 61-82.
- Mihailoff, G.A. (1993). Cerebellar nuclear projections from the basilar pontine nuclei and nucleus reticularis tegmenti pontis as demonstrated with PHA-L tracing in the rat. *The Journal of comparative neurology* 330, 130-146.
- Min, B.I., Oomura, Y., and Katafuchi, T. (1989). Responses of rat lateral hypothalamic neuronal activity to fastigial nucleus stimulation. *Journal of neurophysiology* 61, 1178-1184.
- Miri, A., Warriner, C.L., Seely, J.S., Elsayed, G.F., Cunningham, J.P., Churchland, M.M., and Jessell, T.M. (2017). Behaviorally Selective Engagement of Short-Latency Effector Pathways by Motor Cortex. *Neuron* 95, 683-696.e611.
- Mitrofanis, J., and deFonseka, R. (2001). Organisation of connections between the zona incerta and the interposed nucleus. *Anatomy and embryology* 204, 153-159.
- Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. (1997). Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature* 388, 882-887.
- Monzee, J., Drew, T., and Smith, A.M. (2004). Effects of muscimol inactivation of the cerebellar nuclei on precision grip. *Journal of neurophysiology* 91, 1240-1249.
- Monzee, J., and Smith, A.M. (2004). Responses of cerebellar interpositus neurons to predictable perturbations applied to an object held in a precision grip. *Journal of neurophysiology* 91, 1230-1239.
- Morton, G.J., Cummings, D.E., Baskin, D.G., Barsh, G.S., and Schwartz, M.W. (2006). Central nervous system control of food intake and body weight. *Nature* 443, 289-295.
- Mugnaini, E., Diño, M.R., and Jaarsma, D. (1997). Chapter 8 The unipolar brush cells of the mammalian cerebellum and cochlear nucleus: cytology and microcircuitry. In *Progress in brain research*, C.I. De Zeeuw, P. Strata, and J. Voogd, eds. (Elsevier), pp. 131-150.
- Mullen, R.J., Eicher, E.M., and Sidman, R.L. (1976). Purkinje cell degeneration, a new neurological mutation in the mouse. *Proceedings of the National Academy of Sciences of the United States of America* 73, 208-212.
- Murray, A.J., Croce, K., Belton, T., Akay, T., and Jessell, T.M. (2018). Balance Control Mediated by Vestibular Circuits Directing Limb Extension or Antagonist Muscle Co-activation. *Cell reports* 22, 1325-1338.
- Nashold, B.S., Jr., and Slaughter, D.G. (1969). Effects of stimulating or destroying the deep cerebellar regions in man. *Journal of neurosurgery* 31, 172-186.
- Neki, A., Ohishi, H., Kaneko, T., Shigemoto, R., Nakanishi, S., and Mizuno, N. (1996). Metabotropic glutamate receptors mGluR2 and mGluR5 are expressed in two non-overlapping populations of Golgi cells in the rat cerebellum. *Neuroscience* 75, 815-826.
- Nieto-bona, M.P., Garcia-segura, L.M., and Torres-alemán, I. (1997). Transynaptic modulation by insulin-like growth factor I of dendritic spines in Purkinje cells. *Int J Dev Neurosci* 15, 749-754.

- Noble, F., Wank, S.A., Crawley, J.N., Bradwejn, J., Seroogy, K.B., Hamon, M., and Roques, B.P. (1999). International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. *Pharmacological reviews* 51, 745-781.
- Noda, H., Sugita, S., and Ikeda, Y. (1990). Afferent and efferent connections of the oculomotor region of the fastigial nucleus in the macaque monkey. *The Journal of comparative neurology* 302, 330-348.
- Nordquist, D.T., Kozak, C.A., and Orr, H.T. (1988). cDNA cloning and characterization of three genes uniquely expressed in cerebellum by Purkinje neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 8, 4780-4789.
- Novakova, L., Haluzik, M., Jech, R., Urgosik, D., Ruzicka, F., and Ruzicka, E. (2011). Hormonal regulators of food intake and weight gain in Parkinson's disease after subthalamic nucleus stimulation. *Neuro endocrinology letters* 32, 437-441.
- O'Reilly, J.X., Beckmann, C.F., Tomassini, V., Ramnani, N., and Johansen-Berg, H. (2010). Distinct and overlapping functional zones in the cerebellum defined by resting state functional connectivity. *Cereb Cortex* 20, 953-965.
- O'Reilly, J.X., Mesulam, M.M., and Nobre, A.C. (2008). The cerebellum predicts the timing of perceptual events. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28, 2252-2260.
- Ojakangas, C.L., and Ebner, T.J. (1992). Purkinje cell complex and simple spike changes during a voluntary arm movement learning task in the monkey. *Journal of neurophysiology* 68, 2222-2236.
- Ojakangas, C.L., and Ebner, T.J. (1994). Purkinje cell complex spike activity during voluntary motor learning: relationship to kinematics. *Journal of neurophysiology* 72, 2617-2630.
- Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., and Barsh, G.S. (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 278, 135-138.
- Oman, C.M., and Cullen, K.E. (2014). Brainstem processing of vestibular sensory exafference: implications for motion sickness etiology. *Experimental brain research* 232, 2483-2492.
- Oscarsson, O. (1979). Functional units of the cerebellum sagittal zones and microzones. *TINS* 2, 143-145.
- Palm, G.J., and Wlodawer, A. (1999). [32] Spectral variants of green fluorescent protein. In *Methods in Enzymology* (Academic Press), pp. 378-394.
- Parada, M.A., Hernandez, L., Puig de Parada, M., Paez, X., and Hoebel, B.G. (1990). Dopamine in the lateral hypothalamus may be involved in the inhibition of locomotion related to food and water seeking. *Brain research bulletin* 25, 961-968.
- Park, B.-y., Seo, J., and Park, H. (2016). Functional brain networks associated with eating behaviors in obesity. *Scientific Reports* 6, 23891.
- Paxinos, G. and Franklin, K. (2007). *The Mouse Brain In Stereotaxic Coordinates* (Academic Press, San Diego).
- Pelisson, D., Goffart, L., and Guillaume, A. (1998). Contribution of the rostral fastigial nucleus to the control of orienting gaze shifts in the head-unrestrained cat. *Journal of neurophysiology* 80, 1180-1196.
- Perciavalle, V., Apps, R., Bracha, V., Delgado-García, J.M., Gibson, A.R., Leggio, M., Carrel, A.J., Cerminara, N., Coco, M., and Gruart, A. (2013). Consensus Paper: Current Views on the Role of Cerebellar Interpositus Nucleus in Movement Control and Emotion. *The Cerebellum* 12, 738-757.

- Perrone, J.A., and Krauzlis, R.J. (2008). Vector subtraction using visual and extraretinal motion signals: a new look at efference copy and corollary discharge theories. *Journal of vision* 8, 24.21-14.
- Pichitpornchai, C., Rawson, J.A., and Rees, S. (1994). Morphology of parallel fibres in the cerebellar cortex of the rat: an experimental light and electron microscopic study with biocytin. *The Journal of comparative neurology* 342, 206-220.
- Pierce, E.T., Hoddevik, G.H., and Walberg, F. (1977). The cerebellar projection from the raphe nuclei in the cat as studied with the method of retrograde transport of horseradish peroxidase. *Anatomy and embryology* 152, 73-87.
- Porcher, C., Juhem, A., Peinnequin, A., Sinniger, V., and Bonaz, B. (2005). Expression and effects of metabotropic CRF1 and CRF2 receptors in rat small intestine. *American journal of physiology Gastrointestinal and liver physiology* 288, G1091-1103.
- Pu, Y.-M., Wang, J.-J., Wang, T., and Yu, Q.-X. (1995). Cerebellar interpositus nucleus modulates neuronal activity of lateral hypothalamic area. *NeuroReport* 6, 985-988.
- Pujol, J., Blanco-Hinojo, L., Coronas, R., Esteba-Castillo, S., Rigla, M., Martinez-Vilavella, G., Deus, J., Novell, R., and Caixas, A. (2018). Mapping the sequence of brain events in response to disgusting food. *Hum Brain Mapp* 39, 369-380.
- Richter, S., Kaiser, O., Hein-Kropp, C., Dimitrova, A., Gizewski, E., Beck, A., Aurich, V., Ziegler, W., and Timmann, D. (2004). Preserved verb generation in patients with cerebellar atrophy. *Neuropsychologia* 42, 1235-1246.
- Roth, B.L. (2016). DREADDs for Neuroscientists. *Neuron* 89, 683-694.
- Ruigrok, T., Sillitoe, R.V., and Voogd, J. (2014). Cerebellum and Cerebellar Connections. In.
- Sacchetti, B., Baldi, E., Lorenzini, C.A., and Bucherelli, C. (2002). Cerebellar role in fear-conditioning consolidation. *Proceedings of the National Academy of Sciences* 99, 8406-8411.
- Sánchez-Campusano, R., Gruart, A., and Delgado-García, J.M. (2011). Dynamic Changes in the Cerebellar-Interpositus/Red-Nucleus-Motoneuron Pathway During Motor Learning. *The Cerebellum* 10, 702-710.
- Sánchez-Campusano, R., Gruart, A., Fernández-Mas, R., and Delgado-García, J. (2012). An agonist–antagonist cerebellar nuclear system controlling eyelid kinematics during motor learning. *Frontiers in neuroanatomy* 6.
- Scalera, G. (1991). Effects of Corticocerebellar Lesions on Taste Preferences, Body-Weight Gain, Food and Fluid Intake in the Rat. *J Physiol-Paris* 85, 214-222.
- Schmahmann, J.D., and Pandya, D.N. (1997). The cerebrocerebellar system. *Int Rev Neurobiol* 41, 31-60.
- Schmahmann, J.D., and Sherman, J.C. (1997). Cerebellar Cognitive Affective Syndrome. *41*, 433-440.
- Schmahmann, J.D., and Sherman, J.C. (1998). The cerebellar cognitive affective syndrome. *Brain : a journal of neurology* 121 (Pt 4), 561-579.
- Schutter, D.J.L.G., and Van Honk, J. (2005). The cerebellum on the rise in human emotion. *The Cerebellum* 4, 290-294.
- Schwartz, M.W., Woods, S.C., Porte, D., Jr., Seeley, R.J., and Baskin, D.G. (2000). Central nervous system control of food intake. *Nature* 404, 661-671.
- Schwarz, C., and Schmitz, Y. (1997). Projection from the cerebellar lateral nucleus to precerebellar nuclei in the mossy fiber pathway is glutamatergic: A study combining anterograde tracing with immunogold labeling in the rat. *Journal of Comparative Neurology* 381, 320-334.

- Schweighofer, N., Lang, E., and Kawato, M. (2013). Role of the olivo-cerebellar complex in motor learning and control. *Frontiers in neural circuits* 7.
- Shemesh, Y., Forkosh, O., Mahn, M., Anpilov, S., Sztainberg, Y., Manashirov, S., Shlapobersky, T., Elliott, E., Tabouy, L., and Ezra, G. (2016). Ucn3 and CRF-R2 in the medial amygdala regulate complex social dynamics. *Nature neuroscience* 19, 1489-1496.
- Shepherd, G.M. (2013). Corticostriatal connectivity and its role in disease. *Nature reviews Neuroscience* 14, 278-291.
- Shinoda, Y., Izawa, Y., Sugiuchi, Y., and Futami, T. (1997). Chapter 11 Functional significance of excitatory projections from the precerebellar nuclei to interpositus and dentate nucleus neurons for mediating motor, premotor and parietal cortical inputs. In *Progress in brain research*, C.I. De Zeeuw, P. Strata, and J. Voogd, eds. (Elsevier), pp. 193-207.
- Sillitoe, R.V., and Joyner, A.L. (2007). Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. *Annu Rev Cell Dev Biol* 23, 549-577.
- Soechting, J.F., Burton, J.E., and Onoda, N. (1978). Relationships between sensory input, motor output and unit activity in interpositus and red nuclei during intentional movement. *Brain research* 152, 65-79.
- Stoodley, C.J., and Schmahmann, J.D. (2009). Functional topography in the human cerebellum: A meta-analysis of neuroimaging studies. *NeuroImage* 44, 489-501.
- Stoodley, C.J., and Schmahmann, J.D. (2018). Chapter 4 - Functional topography of the human cerebellum. In *Handb Clin Neurol*, M. Manto, and T.A.G.M. Huisman, eds. (Elsevier), pp. 59-70.
- Strick, P.L., Dum, R.P., and Fiez, J.A. (2009). Cerebellum and nonmotor function. *Annu Rev Neurosci* 32, 413-434.
- Su, Z., Alhadeff, A.L., and Betley, J.N. (2017). Nutritive, Post-ingestive Signals Are the Primary Regulators of AgRP Neuron Activity. *Cell reports* 21, 2724-2736.
- Subramony, S.H. (1994). Degenerative ataxias. *Current Opinion in Neurology* 7, 316-322.
- Sugihara, I., and Shinoda, Y. (2007). Molecular, topographic, and functional organization of the cerebellar nuclei: analysis by three-dimensional mapping of the olivonuclear projection and aldolase C labeling. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 27, 9696-9710.
- Suzuki, L., Coulon, P., Sabel-Goedknecht, E.H., and Ruigrok, T.J. (2012). Organization of cerebral projections to identified cerebellar zones in the posterior cerebellum of the rat. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32, 10854-10869.
- Swinny, J.D., Kalicharan, D., Gramsbergen, A., and van der Want, J.J.L. (2002). The localisation of urocortin in the adult rat cerebellum: a light and electron microscopic study. *Neuroscience* 114, 891-903.
- Sztainberg, Y., and Chen, A. (2012). Chapter 15 - Neuropeptide Regulation of Stress-Induced Behavior: Insights from the CRF/Urocortin Family. In *Handbook of Neuroendocrinology*, G. Fink, D.W. Pfaff, and J.E. Levine, eds. (San Diego: Academic Press), pp. 355-375.
- Tache, Y., and Bonaz, B. (2007). Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. *J Clin Invest* 117, 33-40.
- Takahashi, K.A., and Cone, R.D. (2005). Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. *Endocrinology* 146, 1043-1047.

- Tataranni, P.A., Gautier, J.F., Chen, K.W., Uecker, A., Bandy, D., Salbe, A.D., Pratley, R.E., Lawson, M., Reiman, E.M., and Ravussin, E. (1999). Neuroanatomical correlates of hunger and satiation in humans using positron emission tomography. *Proceedings of the National Academy of Sciences of the United States of America* 96, 4569-4574.
- Tennant, K.A., Adkins, D.L., Donlan, N.A., Asay, A.L., Thomas, N., Kleim, J.A., and Jones, T.A. (2011). The Organization of the Forelimb Representation of the C57BL/6 Mouse Motor Cortex as Defined by Intracortical Microstimulation and Cytoarchitecture. *Cerebral Cortex* (New York, NY) 21, 865-876.
- Teune, T.M., van der Burg, J., de Zeeuw, C.I., Voogd, J., and Ruigrok, T.J. (1998). Single Purkinje cell can innervate multiple classes of projection neurons in the cerebellar nuclei of the rat: a light microscopic and ultrastructural triple-tracer study in the rat. *The Journal of comparative neurology* 392, 164-178.
- Teune, T.M., van der Burg, J., van der Moer, J., Voogd, J., and Ruigrok, T.J. (2000). Topography of cerebellar nuclear projections to the brain stem in the rat. *Progress in brain research* 124, 141-172.
- Thach, Goodkin H. P., a., and G., K.J. (1992). The Cerebellum and the Adaptive Coordination of Movement. *Annual Review of Neuroscience* 15, 403-442.
- Thach, W.T. (1978). Correlation of neural discharge with pattern and force of muscular activity, joint position, and direction of intended next movement in motor cortex and cerebellum. *Journal of neurophysiology* 41, 654-676.
- Tolbert, D.L., Bantli, H., and Bloedel, J.R. (1976). Anatomical and physiological evidence for a cerebellar nucleo-cortical projection in the cat. *Neuroscience* 1, 205-217.
- Tolbert, D.L., Bantli, H., and Bloedel, J.R. (1978). Multiple branching of cerebellar efferent projections in cats. *Experimental brain research* 31, 305-316.
- Tsujii, S., and Bray, G.A. (1989). Acetylation alters the feeding response to MSH and beta-endorphin. *Brain research bulletin* 23, 165-169.
- Uher, R., Murphy, T., Brammer, M.J., Dalgleish, T., Phillips, M.L., Ng, V.W., Andrew, C.M., Williams, S.C., Campbell, I.C., and Treasure, J. (2004). Medial prefrontal cortex activity associated with symptom provocation in eating disorders. *The American journal of psychiatry* 161, 1238-1246.
- Umetani, T. (1990). Topographic organization of the cerebellar nucleocortical projection in the albino rat: an autoradiographic orthograde study. *Brain research* 507, 216-224.
- Uusisaari, and De Schutter, E. (2011). The mysterious microcircuitry of the cerebellar nuclei. *The Journal of physiology* 589, 3441-3457.
- Uusisaari, and Knopfel, T. (2010). GlyT2+ neurons in the lateral cerebellar nucleus. *Cerebellum* 9, 42-55.
- Uusisaari, and Knopfel, T. (2011). Functional classification of neurons in the mouse lateral cerebellar nuclei. *Cerebellum* 10, 637-646.
- Uusisaari, and Knopfel, T. (2012). Diversity of neuronal elements and circuitry in the cerebellar nuclei. *Cerebellum* 11, 420-421.
- Uusisaari, Obata, K., and Knopfel, T. (2007). Morphological and electrophysiological properties of GABAergic and non-GABAergic cells in the deep cerebellar nuclei. *Journal of neurophysiology* 97, 901-911.
- Valle, M.S., Garifoli, A., Maci, T., and Perciavalle, V. (2001). Reticulocerebellar projections to the anterior and posterior lobes of the rat cerebellum. *Neurosci Lett* 314, 41-44.
- van Kan, P.L., Houk, J.C., and Gibson, A.R. (1993). Output organization of intermediate cerebellum of the monkey. *Journal of neurophysiology* 69, 57-73.

- Van Pett, K., Viau, V., Bittencourt, J.C., Chan, R.K., Li, H.Y., Arias, C., Prins, G.S., Perrin, M., Vale, W., and Sawchenko, P.E. (2000). Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *The Journal of comparative neurology* 428, 191-212.
- Vilis, T., and Hore, J. (1977). Effects of changes in mechanical state of limb on cerebellar intention tremor. *Journal of neurophysiology* 40, 1214-1224.
- Voogd, J. (2003). The human cerebellum. *J Chem Neuroanat* 26, 243-252.
- Voogd, J. (2004). *The rat nervous system*, 3rd edn (Amsterdam ; Boston: Elsevier Academic Press).
- Voogd, J. (2014). What we do not know about cerebellar systems neuroscience. *Front Syst Neurosci* 8, 227.
- Voogd, J., and Bigaré, F. (1980). Corticonuclear fibers in the cerebellum. *The Inferior Olivary Nucleus*, 207-234.
- Voogd, J., Bigare, F., Gerrits, N.M., and Marani, E. (1981). Structure and fiber connections of the cerebellum. *Progress in clinical and biological research* 59a, 259-268.
- Voogd, J., and Glickstein, M. (1998). The anatomy of the cerebellum. *Trends Cogn Sci* 2, 307-313.
- Wang, D., He, X., Zhao, Z., Feng, Q., Lin, R., Sun, Y., Ding, T., Xu, F., Luo, M., and Zhan, C. (2015). Whole-brain mapping of the direct inputs and axonal projections of POMC and AgRP neurons. *Frontiers in neuroanatomy* 9, 40.
- Wang, H., Peca, J., Matsuzaki, M., Matsuzaki, K., Noguchi, J., Qiu, L., Wang, D., Zhang, F., Boyden, E., and Deisseroth, K. (2007). High-speed mapping of synaptic connectivity using photostimulation in Channel rhodopsin-2 transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* 104, 8143-8148.
- Wang, J., Pu, Y., and Wang, T. (1997). Influences of cerebellar interpositus nucleus and fastigial nucleus on neuronal activity of lateral hypothalamic area. *Science in China Series C, Life sciences* 40, 176-183.
- Wang, T., Yu, Q.Y., and Wang, J.J. (1994). Effects of stimulating lateral hypothalamic area and ventromedial nucleus of hypothalamus on cerebellar cortical neuronal activity in the cat. *Chinese Journal of Physiological Sciences* 10, 17-25.
- Welsh, J.P., Lang, E.J., Sugihara, I., and Llinas, R. (1995). Dynamic organization of motor control within the olivocerebellar system. *Nature* 374, 453-457.
- Wen, Y.Q., Zhu, J.N., Zhang, Y.P., and Wang, J.J. (2004). Cerebellar interpositus nuclear inputs impinge on paraventricular neurons of the hypothalamus in rats. *Neurosci Lett* 370, 25-29.
- Wiley, K.E., and Davenport, A.P. (2004). CRF2 receptors are highly expressed in the human cardiovascular system and their cognate ligands urocortins 2 and 3 are potent vasodilators. *British journal of pharmacology* 143, 508-514.
- Witter, L., Canto, C.B., Hoogland, T.M., de Gruijl, J.R., and De Zeeuw, C.I. (2013). Strength and timing of motor responses mediated by rebound firing in the cerebellar nuclei after Purkinje cell activation. *Frontiers in neural circuits* 7, 133.
- Wu, H.S., Sugihara, I., and Shinoda, Y. (1999). Projection patterns of single mossy fibers originating from the lateral reticular nucleus in the rat cerebellar cortex and nuclei. *The Journal of comparative neurology* 411, 97-118.
- Wu, Q., Howell, M.P., and Palmiter, R.D. (2008). Ablation of neurons expressing agouti-related protein activates fos and gliosis in postsynaptic target regions. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28, 9218-9226.

- Yang, L., Scott, K.A., Hyun, J., Tamashiro, K.L., Tray, N., Moran, T.H., and Bi, S. (2009). Role of dorsomedial hypothalamic neuropeptide Y in modulating food intake and energy balance. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29, 179-190.
- Yaswen, L., Diehl, N., Brennan, M.B., and Hochgeschwender, U. (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nature medicine* 5, 1066-1070.
- Yoshihara, Y., Mizuno, T., Nakahira, M., Kawasaki, M., Watanabe, Y., Kagamiyama, H., Jishage, K., Ueda, O., Suzuki, H., and Tabuchi, K. (1999). A genetic approach to visualization of multisynaptic neural pathways using plant lectin transgene. *Neuron* 22, 33-41.
- Yuan, C.S., and Barber, W.D. (1992). Hypothalamic unitary responses to gastric vagal input from the proximal stomach. *Am J Physiol* 262, G74-80.
- Yuan, C.S., and Barber, W.D. (1996). Interactions of gastric vagal and peripheral nerves on single neurons of lateral hypothalamus in the cat. *Am J Physiol* 271, G858-865.
- Zahodne, L.B., Susatia, F., Bowers, D., Ong, T.L., Jacobson, C.E.t., Okun, M.S., Rodriguez, R.L., Malaty, I.A., Foote, K.D., and Fernandez, H.H. (2011). Binge eating in Parkinson's disease: prevalence, correlates and the contribution of deep brain stimulation. *The Journal of neuropsychiatry and clinical neurosciences* 23, 56-62.
- Zainolabidin, N., Kamath, S.P., Thanawalla, A.R., and Chen, A.I. (2017). Distinct Activities of Tfp2A and Tfp2B in the Specification of GABAergic Interneurons in the Developing Cerebellum. *Frontiers in Molecular Neuroscience* 10, 281.
- Zalocusky, K.A., Ramakrishnan, C., Lerner, T.N., Davidson, T.J., Knutson, B., and Deisseroth, K. (2016). Nucleus accumbens D2R cells signal prior outcomes and control risky decision-making. *Nature* 531, 642-646.
- Zemanick, M.C., Strick, P.L., and Dix, R.D. (1991). Direction of transneuronal transport of herpes simplex virus 1 in the primate motor system is strain-dependent. *Proceedings of the National Academy of Sciences of the United States of America* 88, 8048-8051.
- Zhang, X., and van den Pol, A.N. (2017). Rapid binge-like eating and body weight gain driven by zona incerta GABA neuron activation. *Science* 356, 853-859.
- Zhang, Y.P., Ma, C., Wen, Y.Q., and Wang, H.J. (2003). Convergence of gastric vagal and cerebellar fastigial nuclear inputs on glycemia-sensitive neurons of lateral hypothalamic area in the rat. *Neuroscience research* 45, 9-16.
- Zhang, Y.P., Zhu, J.N., Chen, K., Li, H.Z., and Wang, J.J. (2005). Neurons in the rat lateral hypothalamic area integrate information from the gastric vagal nerves and the cerebellar interpositus nucleus. *Neurosignals* 14, 234-243.
- Zhu, J.N., Li, H.Z., Ding, Y., and Wang, J.J. (2006). Cerebellar modulation of feeding-related neurons in rat dorsomedial hypothalamic nucleus. *Journal of Neuroscience Research* 84, 1597-1609.
- Zhu, J.N., and Wang, J.J. (2008). The cerebellum in feeding control: Possible function and mechanism. *Cell Mol Neurobiol* 28, 469-478.
- Zhu, J.N., Zhang, Y.P., Song, Y.N., and Wang, J.J. (2004). Cerebellar interpositus nuclear and gastric vagal afferent inputs reach and converge onto glycemia-sensitive neurons of the ventromedial hypothalamic nucleus in rats. *Neuroscience research* 48, 405-417.

APPENDIX

Appendix A. Cre and reporter mouse lines	
Cre mouse lines	
Mouse line	<i>GlyT2::Cre</i> [Tg(<i>Slc6a5-icre</i>)121Veu]
Description	A Cre line that drives cre-dependent expression in glycinergic neurons
Section title	4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei
Citation	Ishihara et al., 2010
Mouse line	<i>parvalbumin (Pv)::Cre</i> [(Pv-CRE#32)SA-09]
Description	A Cre line that drives cre-dependent expression in molecular interneurons and Purkinje cells
Section title	4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei
Citation	Hippenmeyer et al., 2005
Mouse line	<i>Purkinje cell protein 2 (Pcp2)::Cre</i> [Tg(<i>Pcp2-cre</i>)2Mpin.J]
Description	A Cre line that drives cre-dependent expression in Purkinje cells
Section title	4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei
Citation	Barski et al., 2000
Mouse line	<i>Urocortin 3 (Ucn3)::Cre</i> [Tg(<i>Ucn3-cre</i>)KF31Gsat/Mmucd]
Description	A Cre line that drives cre-dependent expression in Ucn3 ⁺ amygdala neurons
Section title	4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei
Citation	Harris et al., 2014; Shemesh et al., 2016
Mouse line	<i>AgRP::Cre</i> [<i>Agrptm1(cre)</i> Low]
Description	A Cre line that drives cre-dependent expression in AgRP ⁺ hypothalamic neurons
Section title	4.2.3 Cerebellar output neurons are functionally linked to AgRP neurons in the arcuate nucleus
Citation	Tong et al., 2008
Reporter mouse lines	
Mouse line	<i>Rosa::lsL.eGFP</i> [Gt(ROSA)26Sortm1Sor/J]
Description	A Cre-dependent line that expresses eGFP in Cre ⁺ cells
Section title	4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei
Citation	Soriano, 1999
Mouse line	<i>Rosa::lsL.Channelrhodopsin2 (ChR2)-eYFP</i> [Gt(ROSA)26Sortm32.1(CAG-COP4*H134R/eYFP)Hze]
Description	A Cre-dependent line that expresses ChR2-eYFP in Cre ⁺ cells
Section title	4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei, 4.1.3 Recombination pattern of <i>Ucn3::Cre</i> in the developing mouse brain, 4.1.4 Molecular identity of IntA ^{Ucn3} neurons in the cerebellar nuclei, 4.1.5 Electrophysiological properties of <i>Ucn3::Cre</i> neurons in the interposed nucleus, 4.1.6 Intra- and extra-cerebellar projections of <i>Ucn3::Cre</i> neurons in the interposed anterior nucleus, 4.1.7 Ablation of IntA ^{Ucn3} neurons perturbs the accuracy of skilled forelimb reaching, 4.1.8 Consequences of the ablation of IntA ^{Ucn3} neurons on rhythmic forelimb movement, 4.2.2 Identification of novel nodes linking the cerebellum and hypothalamus
Citation	Madisen et al., 2012
Mouse line	<i>Rosa::lsL.ChR2-tdTomato</i> [Gt(ROSA)26Sortm27.1(CAG-COP4*H134R/tdTomato)Hze]
Description	A Cre-dependent line that expresses ChR2-tdTomato in Cre ⁺ cells
Section title	4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei
Citation	Madisen et al., 2012
Mouse line	<i>Rosa::lsL.Sun1-GFP</i> [Rosa32Gt(ROSA)26Sortm5(CAG-Sun1/sfGFP)Nat]
Description	A Cre-dependent line that expresses SUN1 fusion protein at the inner nuclear membrane in Cre ⁺ cells
Section title	4.1.2 Identification and generation of a mouse line with recombination ability specific to the cerebellar nuclei
Citation	Kalish et al., 2018

Appendix B. List of genes that are distinctively expressed in the cerebellar nuclei within the cerebellum

Gene symbol	Molecular layer (signal)	Lateral nucleus (signal)	Interposed nucleus (signal)	Medial nucleus (signal)
<i>Acan</i>	6.1	10.14	10.11	10.14
<i>Agtr2</i>	4.57	9.25	9.98	9.75
<i>Anln</i>	6.92	9.53	10	10.16
<i>Arc</i>	5.66	9.8	9.78	9.52
<i>Arhgap24</i>	6.53	9.71	9.51	9.52
<i>Asah2</i>	5.81	9.14	9.26	9.24
<i>B3galt5</i>	6.8	9.35	9.13	9.45
<i>C1ql3</i>	6.95	12.72	12.58	13.3
<i>Cdk18</i>	6.65	9.1	9.3	9.64
<i>Chrna4</i>	6.03	9.48	9.57	10.17
<i>Col16a1</i>	6.29	9.58	9.04	9.67
<i>Cyp11a1</i>	6.38	9.45	9.09	9.27
<i>D7Ert443e</i>	6.66	9.49	9.29	9.33
<i>Daam2</i>	6.5	9.47	9.19	9.48
<i>Fam163a</i>	4.93	10.21	10.62	10.92
<i>Gal3st1</i>	6.88	9.14	9.33	9.54
<i>Gjc3</i>	6.13	9.15	9.14	9.06
<i>Gm16532</i>	6.6	10.01	10.29	9.91
<i>Gpc5</i>	6.73	10.16	10.34	10.51
<i>Grm5</i>	6.96	10.05	10.66	10.65
<i>Hapln1</i>	6.33	10.11	9.98	9.98
<i>Klhdc7a</i>	6.97	9.11	9.37	9.15
<i>Lpo</i>	5.64	9.85	10.32	9.88
<i>Lrrtm1</i>	6.01	9.34	9.78	9.11
<i>Myrf</i>	6.61	10.23	10.47	10.22
<i>Nrg1</i>	6.75	9.4	9.62	9.57
<i>Nxph4</i>	5.5	10.56	10.46	10.55
<i>Padi2</i>	6.96	9.06	9.13	9.24
<i>Pcsk2</i>	6.93	11.51	11.52	11.12
<i>Pkd2l1</i>	4.1	9.28	9.47	9.11
<i>Pls1</i>	6.44	9.01	9.33	9.59
<i>Pou3f2</i>	6.78	10.14	9.67	9.1
<i>Prr5l</i>	6.62	9.01	9.07	9.16
<i>Pter</i>	6.88	12.99	13.15	13.32
<i>Rab37</i>	6.71	11.08	10.89	11.33
<i>Rasgrf2</i>	6.03	9.64	9.98	9.77
<i>Rassf4</i>	5.81	10.5	10.17	9.99
<i>Sec14l5</i>	4.89	9.13	9.04	9.03
<i>Serpinb1a</i>	6.55	9.91	9.95	9.91
<i>Smim3</i>	6.38	9.41	9.29	9.82
<i>Sox2ot</i>	5.67	9.57	9.91	9.85
<i>Speer4a</i>	6.93	10.02	9.71	9.58
<i>Speer4b</i>	6.88	9.99	9.64	9.29
<i>Speer8-ps1</i>	6.7	9.63	9.39	9.19
<i>Sptssb</i>	6.89	9.41	9.85	9.8
<i>Tox2</i>	6.95	9.94	10.12	9.49
<i>Zdhhc22</i>	6	10.07	9.62	9.12
<i>Pcdh11x</i>	6.19	9.24	9.05	6.88

Criteria for selection: F test <0.005 ; signal value >9 in lateral nucleus, >9 in interposed nucleus, >9 in medial nucleus, <7 in molecular layer; except for Pcdh11x conditions were: >9 in lateral nucleus, >9 in interposed nucleus, <9 in medial nucleus, <7 in molecular layer.

Appendix C. Expression patterns of recombined cells in six individual double-transgenic mouse lines and a reporter mouse

Mouse lines	Cerebellar cortex					Deep cerebellar nuclei		
	MLI	Purkinje cells	Bergmann glial cells	Golgi cells	Granule cells	Lateral	Interposed	Medial
<i>Pv::Cre; Rosa32.lsl.ChR2-eYFP</i>	++	++	-	-	-	-	-	-
<i>GlyT2::Cre; Rosa26.lsl.eGFP</i>	++	-	+++	++	-	+	+	+
<i>Pcp2::Cre; Rosa32.lsl.ChR2-eYFP</i>	-	+++	-	-	-	-	-	-
<i>Ucn3::Cre; Rosa26.lsl.eGFP</i>	-	-	-	-	-	+	++	+
<i>Ucn3::Cre; Rosa32.lsl.ChR2-eYFP</i>	-	-	-	-	-	+	++	+
<i>Ucn3::Cre; Rosa32.lsl.ChR2-tdtomato</i>	-	-	-	-	-	+	++	+
<i>Ucn3::Cre; Rosa32.lsl.Sun1-GFP</i>	-	-	-	-	-	+	++	+
<i>Rosa32.lsl.ChR2-eYFP</i>	-	-	-	-	-	-	-	-

Mouse lines were scored after immunostaining for the number of recombined soma within the cerebellum.

Number of recombined cells were tabulated and represented accordingly to the following criteria: 0% to <1% of total neuronal type (-); sparse expression, 1% to <5% of total neuronal type (+); uniform expression, 5% to <50% of total neuronal type (++); high expression, 50% and more of total neuronal type (+++). Legend specific to this table: interposed nucleus (Int), lateral nucleus (Lat), medial nucleus (Med) and molecular layer interneuron (MLI).

Appendix D. Expression patterns of recombined cells at three different postnatal ages

Mouse postnatal age P1	P7	P23
Hindbrain: A6, Locus coeruleus	Hindbrain: Pontine nuclei; A6, Locus coeruleus	Hindbrain: Nucleus of the central acoustic tract; Pontine reticular nucleus, oral part; Ventral nucleus of the lateral lemniscus; Medioventral periolivary nucleus; A6, Locus coeruleus
Cerebellar nuclei: Interposed nucleus	Cerebellar nuclei: Interposed nucleus Pallidum: Triangular septal nucleus	Cerebellar nuclei: Interposed nucleus Pallidum: Triangular septal nucleus Midbrain: Central nucleus of the inferior colliculus; Red nucleus, magnocellular part; Sensory root of the trigeminal nerve; Sagulum nucleus; Precommisural nucleus Hypothalamus: Dorsal tuberomammillary nucleus; Periventricular hypothalamic nucleus Thalamus: Posterior thalamic nuclear group, triangular part; Paraventricular thalamic nucleus posterior part Striatum: Caudate putamen

Reporter proteins observed in P1, P7 and P23 of *Ucn3::Cre; Rosa::lsL*.ChR2-eYFP mice.

Appendix E. Analysis of the expression of glutamatergic molecular markers in YFP+ neurons in the cerebellar nuclei

A. Percentage distribution of the total recombined neurons across all subnuclei

	Lat	LatPC	IntDL	IntA	IntP	IntPPC	Med	MedDL	MedL
recombined neurons	5±1%	2±1%	1±0%	71±3%	3±1%	3±1%	11±3%	5±1%	1±1%

Coronal sections from 3 mice were analyzed (mean±s.e.m; n=1764, 1088, 648 cells).

B. The majority YFP+ cells are SMI32+ within each subnuclei

YFP+SMI32+/YFP+	100±0%	92±8%	100±0%	99±1%	99±1%	96±2%	100±0%	99±1%	97±3%
------------------------	--------	-------	--------	-------	-------	-------	--------	-------	-------

Coronal sections from 3 mice were analyzed (mean± s.e.m; n=1802, 1434, 2404 cells).

C. Percentage of SMI32+ cell population that are YFP+ within each subnuclei

SMI32+YFP+/SMI32+	3±0%	7±0%	2±0%	44±0%	4±0%	34±0%	9±0%	9±0%	4±0%
--------------------------	------	------	------	-------	------	-------	------	------	------

Coronal sections from 3 mice were analyzed (mean± s.e.m; n=1802, 1434, 2404 cells).

D. Percentage of TBR1+ cell populations that are YFP+ within each subnuclei

TBR1+YFP+/TBR1+	2±0%	12±0%	2±0%	30±0%	3±0%	18±0%	6±0%	6±0%	3±0%
------------------------	------	-------	------	-------	------	-------	------	------	------

Coronal sections from 3 mice were analyzed (mean± s.e.m; TBR1: n=2615, 2175, 4401 cells).

E. Overlapping TBR1+ and SMI32+ cell population within each subnuclei

SMI32+TBR1+/ SMI32+ and TBR1+	63±2%	40±16%	68±3%	66±5%	66±11%	60 ±8%	59±5%	57±2%	35±13%
--	-------	--------	-------	-------	--------	--------	-------	-------	--------

Coronal sections from 3 mice were analyzed (mean±s.e.m; n= 7 to 9 coronal slices; 2615, 2176, 4331 cells). Percentage values are rounded to nearest whole number.

Appendix F. Electrophysiological and morphological properties of neurons in the IntA of *Ucn3::Cre; Rosa::ChR2-eYFP* mice

	YFP^{ON}-L	YFP^{OFF}-L	YFP^{OFF}-S
input resistance, MΩ	166±20.2	195±34.1	645±127.7*
membrane capacitance, pF	161±16.2	160.8±19.9	45.1±9.7*
spontaneous AP frequency, Hz	35.4±5.7	26.3±3.5	8.1±1.4*
AP threshold, mV	-30.4±1.4	-33.5±2.4	-34.2±1.3
AP amplitude, mV	46±2.1	46.7±2.8	51.2±3.8
AP duration, ms	0.72±0.04	0.79±0.07	1.71±0.27*
slow AHP amplitude, mV	-14.3±0.6	-13.4±0.8	-17.4±1.4
diameter, μm	28.89±1.43	30.42±3.00	13.51±0.76*
area, μm²	344.2±37.15	430.4±46.71	91.86±6.91*

AP, action potential; AHP, afterhyperpolarization; *significant difference compared with YFP^{ON}-L (ANOVA with post hoc Tukey tests); mean±SEM; n numbers for electrophysiological properties=15 (YFP^{ON}-L), 9 (YFP^{OFF}-L), 9 (YFP^{OFF}-S) cells; n numbers for morphological properties=7 (YFP^{ON}-L), 6 (YFP^{OFF}-L), 6 (YFP^{OFF}-S) cells.

Appendix G. Analysis of the transduction efficiency of AAV2-*hSyn*::mCherry in *Ucn3*::Cre; *Rosa*::ChR2 mice

*A. Approximately 90% of all YFP+ cells are transduced by AAV2-*hSyn*::mCherry*

mouse	# of transduced YFP+ cells (YFP+mCherry+)	# of YFP+ cells	% of YFP+ cells that are transduced (YFP+mCherry+/YFP+)
1	135	147	92
2	135	149	91
3	30	34	88
mean±s.e.m			90±1

Coronal sections from 3 mice were analyzed (n=282, 284, 64 cells).

*B. Approximately 50% of all AAV2-*hSyn*::mCherry transduced cells are YFP*

mouse	# of transduced YFP+ cells (YFP+mCherry+)	# of transduced cells (all mCherry+)	% of Transduced cells that are YFP+ (YFP+mCherry+/mCherry+)
1	135	289	47
2	135	254	53
3	72	118	61
mean±s.e.m			54±4

Coronal sections from 3 mice were analyzed (n=289, 254, 118 cells). Percentage values are rounded to nearest whole number.

Appendix H. Analysis of the percentage distribution of nucleocortical mossy fibers in each cerebellar associated limb region

A. Percentage distribution of non-IntA^{Ucn3} NC-MF (mCherry+ only) across all cerebellar lobules with labeled mossy fibers

mouse	anterior HL (%)			anterior FL (%)									subtotal
	Sim	4/5Cb	subtotal	PFl	Crus1	Crus2	Sim	2Cb	3Cb	4/5Cb	6 Cb	PM	
1	1	0	1	7	5	0	18	0	1	22	7	6	66
2	1	5	6	1	2	1	0	2	11	45	0	0	62
3	0	0	0	0	4	0	27	0	3	0	12	0	47
mean	1	2	2	3	4	0	15	1	5	22	6	2	58
±s.e.m	±0	±1	±2	±2	±1	±0	±6	±0	±3	±11	±3	±2	±5
	posterior FL (%)							posterior HL (%)					subtotal
	PM	Cop	Crus1	Crus2	6Cb	10Cb	subtotal	PM	Cop	8Cb	9Cb	subtotal	
1	9	7	2	9	0	0	28	0	4	0	2	6	
2	1	25	0	0	0	0	25	0	0	0	6	6	
3	0	30	3	0	15	3	52	0	0	0	1	1	
mean	3	21	2	3	5	1	35	0	1	0	3	4	
±s.e.m	±2	±6	±1	±3	±4	±1	±7	±0	±1	±0	±1	±1	

B. Percentage distribution of IntA^{Ucn3} NC-MF (mCherry+/YFP+) across all cerebellar lobules with labeled mossy fibers

	anterior HL (%)			anterior FL (%)									subtotal
	Sim	4/5Cb	subtotal	PFl	Crus1	Crus2	Sim	2Cb	3Cb	4/5Cb	6 Cb	PM	
1	2	0	2	0	10	0	28	0	2	40	2	1	81
2	0	6	6	0	12	0	0	0	0	76	0	6	94
3	0	0	0	0	0	0	62	0	5	0	5	0	71
mean	1	2	3	0	7	0	30	0	2	39	2	2	82
±s.e.m	±0	±2	±1	±0	±3	±0	±15	±0	±1	±18	±1	±2	±5
	posterior FL (%)							posterior HL (%)					subtotal
	PM	Cop	Crus1	Crus2	6Cb	10Cb	subtotal	PM	Cop	8Cb	9Cb	subtotal	
1	3	0	0	2	0	0	5	2	4	0	7	12	
2	0	0	0	0	0	0	0	0	0	0	0	0	
3	0	10	5	0	5	5	24	0	0	0	5	5	
mean	1	3	2	1	2	2	10	1	1	0	4	6	
±s.e.m	±1	±3	±1	0	±1	±1	±6	±0	±1	±0	±2	±3	

Bregma points of anterior-HL: -4.96 to -5.68 mm, -FL: -5.80 to -6.64 mm, posterior-FL: -6.72 to -7.48 mm, -HL: -7.56 to -8.24 mm regions. Coronal sections from 3 mice were analyzed (n=30 slices, each mouse). Percentage values are rounded to nearest whole number.

Appendix I. Analysis of the distribution of labeled puncta across all cerebellar-cerebrum traced regions in *Ucn3::Cre; Rosa::ChR2* mice injected with AAV2-*hSyn::mCherry* at the IntA

		traced extra-cerebellar regions:					
		IOD	ZIV	InG	LRt	VA-VL	RMC
projections from IntA neurons (mCherry labeled)	mouse:						
	1	9%	14%	20%	25%	16%	16%
	2	5%	12%	14%	7%	38%	24%
	3	10%	10%	4%	9%	17%	50%
	mean±s.e.m	8±2%	12±1%	13±5%	14±6%	24±7%	30±10%
projections from Non-IntA ^{Ucn3} (mCherry+ only)	1	9%	14%	20%	26%	15%	16%
	2	4%	11%	14%	7%	39%	25%
	3	10%	10%	4%	8%	17%	51%
	mean±s.e.m	8±2%	12±1%	13±5%	14±6%	24±8%	31±10%
projections from IntA ^{Ucn3} (mCherry+/YFP+)	1	0%	10%	10%	26%	37%	17%
	2	1%	13%	8%	4%	54%	20%
	3	6%	7%	8%	5%	17%	58%
	mean±s.e.m	2±2%	10±2%	9±1%	12±7%	35±11%	32±13%

Coronal sections from 3 mice were analyzed (n=18 slices containing traced regions). Percentage values are rounded to nearest whole number.

Appendix J. Locomotion parameters of the ipsilateral forelimb in freely moving mice

	ablation			optogenetics		
	control	ablated	<i>P</i> value	light-OFF	light-ON	<i>P</i> value
<i>x</i>-displacement, mm	53.49±0.851	53.05±0.94	0.928	55.82±1.690	54.13±1.910	0.731
<i>y</i>-displacement, mm	2.98±0.070	3.14±0.094	0.250	3.132±0.124	4.490±0.237	<0.0001*
Δy (flex), mm	2.15±0.069	2.26±0.070	0.300	2.007±0.107	3.275±0.237	<0.0001*
Δy (extend), mm	3.29±0.140	3.30±0.090	0.730	3.749±0.139	5.155±0.256	<0.0001*
stride duration, ms	419.40±8.400	358.50±8.100	<0.0001*	329.40±21.83	376.00±28.06	0.193
stance duration, ms	244.60±6.800	188.40±5.400	<0.0001*	195.90±14.66	227.40±23.99	0.409
swing duration, ms	174.10±4.100	166.50±3.900	0.220	133.40±8.617	148.60±8.954	0.191
cadence, s⁻¹	2.45±0.050	2.89±0.060	<0.0001*	3.420±0.173	3.067±0.173	0.193
duration of swing-flex, ms	61.50±1.400	56.70±1.600	0.0189*	40.58±1.463	45.12±3.089	0.372
duration of swing-extend, ms	109.80±3.400	102.90±3.000	0.153	92.85±8.783	103.4±8.544	0.261
<i>y</i> max., mm	4.40±0.110	4.42±0.100	0.990	4.59±0.157	6.54±0.296	<0.0001*
time to <i>y</i> max., ms	105.50±3.000	88.90±3.000	0.0007*	84.05±5.722	78.45±4.912	0.745
trajectory, mm	55.16±0.900	57.09±0.900	0.131	53.94±1.795	55.71±1.924	0.439
trajectory speed, mm/ms	0.332±0.008	0.354±0.001	0.131	0.466±0.031	0.422±0.028	0.322

A total of nine mice were used for analysis. Cont.: N=3 mice, ablated: N=3 mice, n=25 to 30 trials per animal; optogenetics: N=3 mice, light-OFF: n=21 to 30 trials; light-ON: n=21 to 30 trials; mean±s.e.m; comparisons were made using non-parametric Mann-Whitney test; *, significant difference.

Appendix K. Behavioral consequences of manipulating the cerebellar interposed nucleus using DREADD

		sham		hM3D		hM4D	
		mean	±s.e.m	mean	±s.e.m	mean	±s.e.m
Chow intake (g)	Vehicle	0.48	0.05	0.70	0.06	0.63	0.03
	CNO	0.54	0.07	0.17	0.06	0.70	0.05
	P value	0.25		0.031*		0.063	
Latency to chow intake (s)	Vehicle	32.00	7.70	36.67	5.40	29.50	7.53
	CNO	28.00	2.83	199.80	57.34	12.00	1.77
	P value	>0.99		0.031*		0.125	
Speed (mm/s)	Vehicle	25.26	2.32	23.08	1.54	28.11	1.29
	CNO	24.84	2.89	14.80	3.00	25.27	1.73
	P value	0.79		0.047*		0.35	
Mobility (%)	Vehicle	93	1	86	2	93	1
	CNO	91	3	60	3	91	2
	P value	0.69		0.023*		0.3714	
Exploration (%)	Vehicle	76	1	78	1	76	3
	CNO	77	0	63	8	73	1
	P value	0.40		0.035*		0.33	
Proximity to chow (frequency)	Vehicle	26.83	9.54	82.33	22.08	23.75	7.19
	CNO	29.40	8.74	273.00	36.02	31.50	9.06
	P value	0.74		0.024*		0.76	

Wilcoxon matched-paired sign rank test was used for chow intake and latency to chow intake analysis. Mann Whitney test was used for speed, mobility, exploration and proximity to chow analysis. 9 mice were used, 3 mice each group; trials = 1-2 trials for each condition.