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CONTROL OF SKILLED MOVEMENT BY
THE CEREBELLAR INTERPOSED
ANTERIOR NUCLEUS

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2019

Control of Skilled Movement by the Cerebellar Interposed
Anterior Nucleus

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

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

14th January 2019

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from 2 papers published in the following peer-reviewed journals where I was the co-first author on the first paper: *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.* and third author on the second paper: *Zainolabidin, N., Kamath, S.P., Thanawalla, A.R., and Chen, A.I. (2017). Distinct Activities of Tfap2A and Tfap2B in the Specification of GABAergic Interneurons in the Developing Cerebellum. Frontiers in molecular neuroscience 10, 281-281.*

The contributions of the co-authors to the publications were as follows:

To,

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.

- A/Prof Chen provided the initial project direction, wrote and edited the manuscript drafts.
- I helped prepare the manuscript drafts with my colleagues Aloysius Low, Alaric Yip and my supervisor A/Prof Albert Chen.
- I co-designed the study with A/Prof Albert Chen and my co-authors Aloysius Low and Alaric Yip and performed all the laboratory work at the School of Biological Sciences, located at the Biopolis and at Lee Kong Chian School of medicine.
- Behavioral, surgical, optogenetics and ablation experiments for skilled reaching, general motor behavior and analysis of locomotor behavior was carried out by me.
- Surgical and molecular biology for neuronal characterisation and circuit mapping was done by Aloysius Low
- Behavioral, surgical, optogenetics experiments for gait analysis was performed by Alaric Yip and Kelly Wong

- Dr. Jinsook Kim and Prof. George Augustine, collaborated with us on electrophysiological recordings and provided valuable comments
- Dr. Martesa Tantra contributed to the original design of behavioral experiments

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The contributions of the co-authors are as follows:

- A/Prof. Chen provided the initial project direction, wrote and edited the manuscript drafts.
- Dr. Liyana Zainolabidin prepared the drafts of the manuscript with our supervisor A/Prof Albert Chen
- Dr. Liyana Zainolabidin performed all the molecular and functional analysis of TFAP2 genes in the cerebellum
- Dr. Sandhya Kamath assisted in performing all experiments
- I provided supplementary data describing the expression of TFAP2 in the deep cerebellum

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Abbreviations

deep cerebellar nuclei (DCN)

interposed anterior (IntA)

interposed posterior (IntP)

interposed dorsolateral (IntDL)

medial (Med)

medial dorsolateral (MedDL)

medial lateral part (MedL)

lateral (Lat)

lateral parvicellular (LatPPC)

Ucn3::Cre-recombined neurons in the IntA (IntA^{Ucn3})

molecular layer (ML)

Purkinje cell layer (PCL)

internal granular layer (IGL)

molecular layer interneurons (MLIs)

Large glutamatergic neurons in the DCN (GADn-L)

Glycinergic projection neurons in the DCN (Gly-F)

GABAergic/glycinergic inhibitory neurons in the DCN (Gly-I)

GABAergic projection neurons in the DCN (GAD-IO)

local GABAergic interneurons in the DCN (GAD-S)

local glutamatergic interneurons in the DCN (GADn-S)

interposed posterior parvicellular (IntPPC)

Inhibitory nucleocortical fibers (iNCs)

excitatory nucleocortical fibers (eNCs)

Gad2-cre recombined interneurons (GABApre)

Chx10+ propriospinal neurons (Chx10+ neurons)

deltoideus par acromialis (deltoid)

extensor carpi ulnaris (ECU)

triceps brachii (tricep)

diphtheria toxin (DT)

Electromyogram (EMG)

Rosa26::lsl-eGFP (*Rosa::eGFP*)

Rosa26::lsl-Channelrhodopsin (ChR2)-EYFP (*Rosa::ChR2*)

Neurofilament H (SMI32)

T-box, brain, 1 (TBR1)

Glutamate decarboxylase (GAD67)

Transcription factor activating enhancer binding protein 2 alpha (TFAP2A)

Neurobiotin (NB)

Light on at the initial stage of reach (Light-ONI)

Light on at the reach (Light-ONR).

Wildtype (WT)

magnocellular red nucleus (RMC)

superior colliculus (SC)

zona incerta (ZI)

ventral anterior-ventrolateral (VA-VL)

inferior olive dorsolateral part (IOD)

lateral reticular nucleus(LRt)

cholera toxin B subunit (CTB)

Lateral vestibular nucleus, Extensor (LVN-E)

Lateral vestibular nucleus, Co-activation (LVN-C)

Summary

The cerebellum is an important brain structure implicated in motor control. However, the underlying circuits and mechanisms for cerebellum-mediated control of voluntary movement have not been defined. As the output channels of the cerebellum, the deep cerebellar nuclei (DCN) represent an important element of cerebellar circuits for control of voluntary movements, but their precise role and the neuronal pathways mediating their effect in the context of voluntary movements have not been determined. By defining the contribution of the cerebellum, through the DCN, to voluntary movement, we hope to contribute to a systems level understanding of how voluntary movement is coordinated and executed by the brain.

In this thesis, we introduce a novel cre driver line, which recombines neurons in the DCN of the cerebellum. Within the cerebellum, the *Ucn3::Cre* mouse specifically permits recombination restricted to glutamatergic projection neurons of the interposed anterior nucleus of the DCN (IntA), a cerebellar structure implicated in motor behaviors. Manipulation of *Ucn3::Cre*-recombined neurons in the IntA (IntA^{Ucn3}) resulted in specific deficits in kinematic parameters of limb movement. Chemogenetic ablation of IntA^{Ucn3} neurons resulted in deficits in forelimb trajectories in locomotor and skilled reaching behavior without affecting general motor ability. Additionally, optogenetic stimulation of IntA^{Ucn3} neurons during skilled reaching suggests a specific role for IntA^{Ucn3} neurons in mediating the accuracy of reaching movements. To investigate the influence of IntA^{Ucn3} neurons on muscle activities, we performed simultaneous recording of extensor muscle activities during skilled reaching and found that optogenetic stimulation of IntA^{Ucn3} neurons reduced activity of synergistic extensor muscles. Finally, we present anatomical evidence

which suggests that, neurons in the IntA nucleus may influence movement through cerebello-thalamo-cortical or rubrospinal pathways.

Thus, using a novel genetic tool for the interrogation of the connectivity and function of the IntA nucleus in regulating limb movements, we identified the role of a subpopulation of neurons in the IntA in maintaining the precision of forelimb movement. We propose that the IntA nucleus represents a critical cerebellar region for the control of limb movements through its connectivity within a diverse motor network for voluntary movement.

1. Hypothesis and Aims

The cerebellum has long been implicated in motor behavior and is known to contribute to motor circuits through neurons in the deep cerebellar nuclei. However, specifically how neurons in the DCN influence motor behavior has not been characterized. We hypothesize that DCN neurons, as a conduit for cerebellar input to cortical and spinal motor circuits, provides signals representing cerebellar information processing and is an important locus of motor function. Thus, defining the behavioral outcome of DCN manipulation is important to provide insight to how the cerebellum influences movement.

The broad aim of this study is to investigate how neurons in the DCN contribute to skilled movement, through their connectivity with cortical and subcortical brain regions. To achieve this, first, we sought to characterize a genetically tractable population of neurons restricted to the interposed anterior nucleus of the DCN. Our second aim was to define the motor deficits caused by interposed anterior neuronal manipulation. Moreover, we sought to define the specific kinematic contribution of the interposed anterior nucleus during skilled reaching. Finally, we have attempted to delineate the neuronal circuits through which the DCN influences extracerebellar motor circuits to modulate movement.

2. Introduction

The cerebellum is a structure of the central nervous system conventionally known for its critical role in motor control (Eccles, 1969; Ito, 2006; Manto et al., 2012). In particular, the cerebellum has been demonstrated to regulate voluntary adaptation of motor reflexes, motor learning and is implicated in skilled movements (De Zeeuw and Ten Brinke, 2015; Ito, 1982, 2000; Medina et al., 2000). The cerebellum is extensively connected with different regions of the brain and spinal cord, and receives diverse sensory input (Alstermark and Ekerot, 2015; Bostan et al., 2013; Jörntell, 2017). These inputs are also thought to carry copies of motor commands generated in the motor cortex, known as an efference copy (Azim et al., 2014a; Wolpert et al., 1998). The cerebellum is thought to use these signals to perform longer latency updates of motor commands as well as make rapid adjustments to motor responses, effectively fine-tuning movement through distinct connections with relevant brain and spinal cord regions (Azim and Alstermark, 2015; Zhou et al., 2014). However, due to the lack of genetic tools the ability to access cerebellar-mediated pathways selectively has precluded functional characterization of pathways arising from distinct populations of cerebellar neurons.

2.1 Organization and connectivity within the cerebellum

The cerebellum is divided along the dorso-ventral axis into the cerebellar cortex and the deep cerebellar nuclei (DCN) (Altman and Bayer, 1997) (Figure 1A). The cerebellar cortex consists of three layers, the molecular layer (ML) the Purkinje cell layer (PCL), and the internal granular layer (IGL) (Altman and Bayer, 1997; Eccles et al., 1967) (Figure 1B). The PCL contains a single layer of Purkinje cells, which are the sole output of the cerebellar cortex. Purkinje cells send axons to neurons of the deep cerebellum and provide GABAergic input to all neuronal subtypes of the DCN (Chan-Palay et al., 1979). Purkinje cells are

innervated by inhibitory molecular layer interneurons (MLIs), stellate, and basket cells; and excitatory parallel fibers originating from the internal granular layer (Altman and Bayer, 1997; Voogd and Ruigrok, 1997) (Figure 1B). The internal granular layer is the most dense cell layer, and consists of primarily excitatory granule cells and inhibitory Golgi cells (Altman and Bayer, 1997) (Figure 1B). Granule cells send parallel fibers up to the molecular layer and provide excitatory input to both Purkinje cells, MLIs as well as Golgi cells whose dendrites extend up to the molecular layer. In turn, Golgi cells provide feedback inhibition to mossy fibers and granule cells in the GCL (Hawkes, 1997; Voogd and Ruigrok, 1997) (Figure 1B). Although the cerebellar cortex comprises a complex microcircuitry performing computation within each of layer, the final output of the cerebellar cortex is channeled through the DCN before targeting extra-cerebellar regions (Chan-Palay, 1977; Uusisaari and De Schutter, 2011). Thus, defining the anatomical substrates through which the DCN operates is an important first step in defining the contribution of the cerebellum to behavior.

The cerebellar network is made up of multiple levels of convergence and divergence of neural signals. The cerebellar cortex and the DCN receive input from two major sources, the climbing fibers, originating in the inferior olive, and mossy fibers relaying information from the precerebellar nuclei (Glickstein, 1997; Montarolo et al., 1980; Ruigrok, 2011). Mossy fibers provide excitatory synapses to granule cells whose parallel fibers extend transversely across the molecular layer of the cerebellar cortex providing input to Purkinje cells and molecular layer interneurons (Altman and Bayer, 1997; Glickstein, 1997). Individual Purkinje cells have extensive dendritic trees which receive ~100,000 parallel fiber inputs (Apps and Garwicz, 2005). Purkinje cell inputs then converge onto DCN neurons in a ratio of 10-50 purkinje cell axons to 1 DCN neuron (Sultan et al., 2002; Uusisaari and De Schutter, 2011; Uusisaari and Knopfel, 2011; Zanjani et al., 1996) (Figure 1B). The DCN

also provides excitatory and inhibitory nucleo-cortical feedback projections to the cerebellar cortex (Ankri et al., 2015; Houck and Person, 2015) (Figure 2A). Thus, the DCN receives convergent signals from a large number of parallel fiber and Purkinje cells, further processes these signals and is presumed to play an important role in cerebellar computation (Uusisaari and De Schutter, 2011). However, due to the lack of cell specific tools, the role of identifiable neuronal subpopulations within the DCN independent of the cerebellar cortex has been difficult to elucidate.

Based on climbing fiber input, the cerebellum can be further subdivided sagittally into microzones (Sugihara and Shinoda, 2007). Each microzone consists of a 300 μm longitudinal zone, defined by a single climbing fiber input to its target Purkinje cells and the DCN neurons to which the Purkinje cells project (Apps and Hawkes, 2009; Sugihara et al., 2009). Each microzone has been proposed to be a functional unit of the cerebellum controlling various aspects of motor and non-motor behavior (Cerminara et al., 2015). Although the cerebellar cortex shows some somatotopic specificity, the convergence of Purkinje cells onto neurons in the DCN suggests that corresponding regions of the cerebellar nuclei may control distinct behaviors (Apps and Hawkes, 2009; Voogd, 2014). However, the organization and functional relevance of regions and neuronal subtypes within the deep cerebellum are still poorly understood.

Figure 1. Microcircuits of the cerebellar cortex. (A) Division of the cerebellum into the cerebellar cortex and the DCN, dotted lines indicate cerebellar nuclei. (B) Schematic showing the interaction between cell types of the cerebellar cortex, the primary inputs and output from the cerebellar cortex.

2.2 Neuronal subtype diversity within the DCN

The DCN is made up of six major neuronal subtypes based on anatomical and electrophysiological distinctions (Uusisaari and Knopfel, 2011) (Figure 2A). Large glutamatergic neurons project towards extra-cerebellar regions and collateralize to form nucleocortical mossy fiber terminals in the cerebellar cortex (GADn-L). Glycinergic projection neurons, primarily found in the medial nucleus, project towards the brain stem and spinal cord (Gly-F). GABAergic/glycinergic inhibitory neurons provide nucleocortical input to Golgi cells in the IGL (Gly-I). Finally, there are GABAergic projection neurons which send inhibitory signals to the inferior olive (GAD-IO) as well as local GABAergic (GAD-S) and glutamatergic interneurons (GADn-S) (Uusisaari and Knopfel, 2011) (Figure 2A). While the neurotransmitter profile and local connectivity of these neurons have been characterized, the functional significance of each subtype of DCN neurons has not been elucidated.

Figure 2. Organization and neuronal subtypes within the DCN. (A) Schematic showing interactions of neurons of the DCN. Classification of neurons according to Uuisisaari and Knopfel, 2011: GADn-L (GAD negative large neurons), GADn-S (GAD negative small neurons), GAD-S (GAD positive small neurons), GAD-IO (inferior olive projecting GAD positive neurons), Gly-I (inhibitory glycinergic neurons), Gly-F (Fastigial nucleus projecting neurons), iNC (inhibitory nucleocortical fibers), eNC (excitatory nucleocortical fibers). (B) The DCN can be divided into 9 subnuclei, medial; MedL, MedDL, Med, interposed; IntA, IntPPC, IntDL, IntP, lateral; Lat, LatPC.

2.3 Extensive connectivity to extra-cerebellar regions by the DCN

The cerebellum is implicated in a diverse range of motor, non-motor and visceromotor behaviors (Ito, 2008; Schmahmann, 1998). This functional diversity is attributed, perhaps, to the cerebellum's extensive connectivity to many parts of the brain and spinal cord. The cerebellum is linked to a large number of extra-cerebellar target regions in humans, non-human primates and rodents (Kelly and Strick, 2003; Middleton and Strick, 1997; Schmahmann et al., 2004; Teune et al., 2000). Thus, the DCN, as the output nuclei of the cerebellum, represent an important locus for mediating cerebellar function. Moreover, the multitude of inputs received and the diversity of cell types within the DCN suggest that the DCN performs computational functions rather than simply serving as a relay station for the cerebellar cortex (Schmahmann and Pandya, 1997; Schmahmann et al., 2004; Uusisaari and De Schutter, 2011).

The DCN can be broadly divided into the lateral, interposed and medial nuclei based on histological distinctions (Paxinos and Franklin, 2007) (Figure 2B). These three major nuclei are further subdivided into 9 subnuclei: interposed anterior (IntA), interposed posterior parvicellular (IntPPC), interposed posterior (IntP), interposed dorsolateral (IntDL), medial (Med), medial dorsolateral (MedDL), medial lateral part (MedL), lateral (Lat), lateral parvicellular (LatPPC) (Paxinos and Franklin, 2007) (Figure 2B). Tract tracing experiments using horseradish peroxidase, electrophysiological mapping, and rabies virus tracing have revealed that axons originating from neurons within each nucleus connect to the thalamus, red nucleus, vestibular and limbic regions (Bagnall et al., 2009; Batton et al., 1977; Keifer and Houk, 1994; Sakai, 2013). Specificity in target regions by neurons in the interposed, lateral or medial nucleus suggests specific pathways may regulate distinct motor processes. For instance, the medial nucleus projects to the brain stem and vestibular nuclei, which are

involved in balance and control of head/trunk movement (Bagnall et al., 2009; Murray et al., 2018). The lateral and interposed nucleus both project to the premotor thalamus and red nucleus, which are two important centers for voluntary motor movement (Keifer and Houk, 1994; Tlamsa and Brumberg, 2010). Although many extra-cerebellar pathways have been identified, whether neurons in different cerebellar nuclei or neuronal subpopulations within each nucleus project to a unique set of targets has not been determined due to an inability to selectively label neurons in the DCN. Additionally, the functional significance of each pathway and how the DCN conveys integrated signals to cortical regions and the spinal cord to influence motor control has not been defined.

2.4 Cerebellar integration with cortical and spinal circuits for voluntary movement

In the mammalian nervous system, the control of voluntary movement is coordinated by the supplementary motor cortex, motor cortex, subcortical motor nuclei, basal ganglia, brain stem, cerebellum and spinal cord (Alstermark and Isa, 2012; Calabresi et al., 2014). Goals and intentions for movement arise in the supplementary motor area, and are communicated to the motor cortex to generate commands (Eccles, 1982; Gremel and Costa, 2013; Rouiller et al., 1999). These commands are sent to spinal neurons directly through the corticospinal tract and indirectly through reticulospinal pre-motor nuclei and the rubrospinal tracts (Alstermark and Isa, 2012). Superimposed on this system are modulatory mechanisms that exist within the spinal cord and cerebellum potentially influencing cortical commands to the periphery to facilitate efficient movement (Azim and Alstermark, 2015; Azim et al., 2014b; Fink et al., 2014). However, the cerebellar locus for this function has not been determined. Recent work delineating the role of neurons mediating cortical motor commands and modulatory mechanisms in the spinal cord lays the foundation to investigate neurons in

the cerebellum, which may influence motor commands to the spinal cord (Azim et al., 2014b; Fink et al., 2014; Ueno et al., 2018).

Voluntary movements are precise, regular and often require training (Rothwell, 2012). In order to achieve this precision, movements are required to be modulated on-line based on variable environmental conditions (Morasso, 1981). In the simplest form of modulation, movements can be adjusted based on sensory feedback obtained during the course of the movement (Wolpert et al., 1998). This may be in the form of visual, cutaneous and proprioceptive feedback (Azim et al., 2014a). However, sensory feedback is often delayed due to time lag involved in contraction of muscles or activation of proprioceptive sensory organs, and results in error prone movements and decays over time (Miall and Wolpert, 1996). Thus, with increased complexity of an action, particularly ones involving the recruitment of multiple joint segments, sensory feedback is likely to be inadequate to provide rapid, precise online modulation of movement (Azim and Alstermark, 2015; Wolpert et al., 1998). One way to solve the problem of feedback delays is to provide feedforward controls during the movement. This function has been attributed to the cerebellum and is thought to be achieved by altering motor commands to the periphery thus preemptively changing movement to obtain a goal (Barlow, 2002; Ito, 1982; Shidara et al., 1993). However, the neuronal substrates and the anatomical pathways linking the cerebellum to motor commands circuits has not been described. Furthermore, due to the lack of cell specific tools the behavioral outcomes of selectively manipulating output neurons of the cerebellum have been difficult to assess.

2.4.1 Motor commands are generated in the motor cortex

The ability of mammals to initiate movements to achieve goals has largely been attributed to the motor cortex sending commands to motor neurons in the spinal cord for execution of movement (Alstermark and Isa, 2012) (Figure 3A). Two recent studies demonstrate the role of the motor cortex in the initiation of skilled forelimb movements. Using a headfixed learned skilled reaching behavior paradigm and optogenetic silencing of cortical neurons, a study found that distinct neuronal subtypes in select regions of the motor and sensory cortex generate commands for voluntary movement (Guo et al., 2015). Optogenetic activation of cortical inhibitory interneurons prevented the initiation and execution of the reaching movement specifically at the initial stages of reaching (Guo et al., 2015). Inhibition of neurons in the motor cortex had a profound impact during the reaching movement, but once the initial stages were complete, cortical inhibition was unable to change the reaching movement (Guo et al., 2015). Furthermore, termination of inhibition resulted in a rebound generation of the full sequence of reaching movements (Guo et al., 2015). Together, these results indicate that the cortex generates the command for the sequence of actions required to perform a learned voluntary reaching movement (Guo et al., 2015).

The role of the motor cortex in controlling movement was examined in another study, which revealed that different pathways are engaged to influence muscle activity within different behavioral contexts (Miri et al., 2017). For skilled forelimb movement, inhibition of the motor cortex resulted in changes in forelimb muscle activity within 10 milliseconds, while in the context of locomotion, cortical inhibition of forelimb muscles took place only after 35 milliseconds (Miri et al., 2017). Furthermore, the population activity of cortical neurons was different for locomotion versus skilled forelimb movement, suggesting a potential mechanism through which the motor cortex recruits neuronal pathways for different

behaviors (Miri et al., 2017). Although the motor cortex is well established as a key brain region for generation of motor commands for movement, whether additional structures are involved in generating distinct patterns of population activity and whether short latency pathways may be modulated through inputs to the motor cortex remains unclear.

2.4.2 The role of spinal neurons in voluntary movement

In the spinal cord, axons of the corticospinal, rubrospinal and reticulospinal tracts converge onto motor neurons to control movement. However, in the past few decades, studies in cats have shown that information is first integrated at spinal interneurons before being actively transferred to motor neurons (Alstermark and Sasaki, 1986; Illert et al., 1981; Kitazawa et al., 1993) (Figure 3A-B). This disynaptic circuit organization to motor neuronal control enables integration of multiple descending motor pathways and sensory feedback from sensory receptors in the organ. Additionally, it allows for feedforward excitation or inhibition of motor neurons and could be a locus for modulatory input to spinal motor neurons (Alstermark and Isa, 2012).

In the context of skilled voluntary forelimb movements, two populations of interneurons play an important role in the integration of descending motor streams and feedback from sensory receptors in the forelimb, segmental interneurons from C5 to T1 and propriospinal neurons distributed in the cervical and upper thoracic segments of the spinal cord (Alstermark and Sasaki, 1986; Illert et al., 1981; Pivetta et al., 2014) (Figure 3B). Lower motor neurons innervating the forelimb are present from the cervical spinal segment C5 to thoracic spinal segment T1 (Jankowska, 1982) (Figure 3B). In spinal segments, molecularly distinct neurons relay cortical commands related to different aspects of behavior. A recent study demonstrated that Chx10⁺ interneurons in the spinal cord play an important role in

reaching movements, while vGluT3⁺ neurons control food release or transfer to the mouth (Ueno et al., 2018). Thus, while the cortex commands movement in fixed action patterns, spinal interneurons show more selective control over specific elements of a movement sequence. The identification of cell types in the spinal cord specific to behavior provides the groundwork to identify and characterize subcortical regions, such as the cerebellum, that regulate spinal neurons to refine and modulate voluntary skilled movements.

Figure 3. Coordination of motor signals for regulation of voluntary movement. (A) Schematic showing the communication of motor signals to the spinal cord for movement. (B) Schematic showing a broad classification of spinal interneurons involved in voluntary movement.

2.5 Role of the DCN in voluntary skilled movements

In the control of voluntary movements, the three nuclei differentially contribute to movement. Reversible inactivation studies using muscimol have revealed insights to the role of each nucleus of the DCN (Martin et al., 2000). Inactivation of both the interposed and dentate/lateral nuclei in cats subjected to an L shaped manipuladum behavioral paradigm revealed that the interposed and lateral nuclei are preferentially engaged during acquisition of motor skills. After an initial inactivation phase animals had to relearn the task, reflecting the tendency to incorporate the cerebellum in skilled motor tasks (Wang et al., 1998). Thus, neurons of the lateral/dentate and interposed nuclei have been shown to be important for skilled movement, but functional distinctions between each nucleus, within the same behavioral context were not defined in this study.

Analysis of the consequences of focal muscimol inactivation of each nucleus in cats on reaching behavior has shown that each nucleus controls different aspects of the reaching movements (Martin et al., 2000). Fastigial/Medial nucleus inactivation resulted in deficits in balance and head and trunk control during the reach, lateral/dentate nuclei inactivation resulted in loss of anticipatory response to the target, and interposed nucleus inactivation resulted in impaired forelimb control with additional distinctions in reaching between inactivation of the anterior versus posterior interposed nuclei (Martin et al., 2000). Thus, while the lateral and medial nuclei appear to have complementary roles in forelimb movement the interposed nucleus, in particular, appears to play a key role in the kinematic control of forelimb movement.

Additional insight to the involvement of the interposed nucleus in limb movement came from muscimol inactivation of the interposed in multiple behavioural contexts.

Inactivation of the interposed affected conditioning of the withdrawal reflexes, paw placement and precision during locomotion and hypermetria in forelimb reaching tasks indicating that the interposed nucleus plays an important role in the control of limb movements independent of context (Bracha et al., 1999). In addition, inactivation of the interposed in non-human primates also results in deficits in reaching (Mason et al., 1998). Thus, through these fundamental early studies, the interposed nucleus of DCN in particular is widely implicated in the control of movements, however the precise role of interposed signaling and how projection neurons from the interposed influence kinematics and contribute to motor circuits has not been elucidated.

Although muscimol inactivation studies provide important groundwork for investigation of the role of the DCN in the goal-directed voluntary movement, this strategy results in a general inactivation of all neuronal subtypes within the DCN, and does not address the role of individual neuronal subtypes, as well their interactions with neurons in target brain regions. Thus, further investigation using strategies to examine identifiable and accessible neuronal subpopulations in the DCN is critical for a better understanding of the organization and function of the DCN.

2.6 Indirect manipulation of the interposed nucleus

The interposed nucleus has previously been implicated in movement kinematics through indirect manipulation of neurons in the interposed nucleus. Manipulation of the activities of DCN neurons through stimulation or inhibition of Purkinje neurons drives limb movements and results in changes in kinematics of eyelid movements (Heiney et al., 2014; Lee et al., 2015). Similarly, stimulation of molecular layer interneurons, which inhibit Purkinje cells, within the eyelid microzone of the cerebellum results in increased firing rate

of neurons in the interposed nucleus (Heiney et al., 2014). Varying the duration and intensity of stimulation is capable of driving changes in latency to eyelid closure and duration of closure (Heiney et al., 2014). Thus, changes in the activity of neurons in the interposed nucleus have been associated with changes in eyelid kinematics. Additionally, silencing of Purkinje neurons linked to the interposed nucleus results in limb movements, which are time-locked to the stimulus onset (Lee et al., 2015). Thus, manipulation of DCN neurons through the inhibition of Purkinje cell activity has been demonstrated to drive movement of multiple modalities. However, the impact of the direct manipulation of neurons in the interposed nucleus neurons on kinematics of limb movements has not been examined in detail. Moreover, whether stimulation of DCN neurons results in similar effects as the Purkinje cell-mediated disinhibition of DCN neurons has not been determined.

2.7 Defining the functional significance of DCN-mediated circuits

Recent studies have begun to systematically characterize the anatomical and functional distinctions between neuronal subtypes within the DCN using the combination of viral/ electrophysiological circuit mapping and behavioral assays. Studies have identified the presence and function of nucleocortical and nucleo-olivary neurons located within the deep cerebellar nuclei (Ankri et al., 2015; Gao et al., 2016; Houck and Person, 2015; Ruigrok, 1997). These feedback pathways are proposed to regulate granule cell input to neurons in the molecular and the Purkinje cell layers of the cerebellar cortex (Gao et al., 2016) (Figure 4A-C). Inhibitory nucleocortical cells (iNCs) of mixed GABA-glycine neurotransmitter subtype specifically inhibit Golgi cells in the internal granular layer of the cerebellar cortex (Ankri et al., 2015) (Figure 4A). Thus, iNCs have the potential to disinhibit a large number of granule cells and potentially excite Purkinje cells.

The role of nucleocortical pathways in the interposed nucleus was examined by a key study using the eye blink conditioning behavioral paradigm. Importantly, this study uncovered that excitatory nucleocortical axons (eNCs) preferentially excite parallel fiber-to-MLI synapses thus inhibiting Purkinje cells (Gao et al., 2016). Interestingly, this mechanism is specific to conditioned learned responses and could play a role in the amplification of learned responses by preferentially recruiting granule cells that target molecular layer interneurons. The activation of interneurons leads to inhibition of Purkinje cells via MLIs, and disinhibition of neurons in the DCN (Heiney et al., 2014; Witter et al., 2013) (Figure 4B). Thus, nucleocortical projections from the interposed nucleus appear to send feedback signals to the cerebellar cortex to inhibit or activate Purkinje activity, and may play an important role in selectively exciting parallel fiber connections in the molecular and Purkinje cell layer.

Similarly, nucleo-olivary circuits arising from GABAergic neurons in the DCN are known to provide feedback to the inferior olive and may inhibit excitatory input from climbing fibers (Najac and Raman, 2015). Climbing fibers represent an important source of error-related information for Purkinje cells, which in turn inhibit nucleo-olivary neurons (Kitazawa et al., 1998; Schweighofer et al., 2013) (Figure 4C). These circuits likely play an important role in classical conditioning as well as motor learning and adaptation (Hoogland et al., 2015; Medina et al., 2000). Thus, defining the origin and targets of specific circuits and their role in a given motor behavioral context may improve understanding of mechanisms of cerebellar processing beyond structure-function relationships. Although details about the nucleocortical and nucleo-olivary pathways as a component of internal feedback circuits is emerging, the functional relevance of extra-cerebellar DCN projections towards forebrain and midbrain nuclei is not clear. Moreover, the function of DCN glutamatergic projections

neurons in the modulation and refinement of movement within the context of goal-directed voluntary behavior has not been examined.

Figure 4. Functional relevance of identified DCN-mediated circuits. (A) Schematic showing the potential role of iNC fibers from Gly-I cells. (B) Schematic showing the role of eNC fibers from glutamatergic neurons of the DCN in learned behavior. (C) Schematic showing feedback inhibition of climbing fiber input.

2.8 Circuits and mechanisms underlying the modulation of skilled movement

Several regulatory mechanisms have been proposed to ensure smooth and precise movement. Spinal interneurons and cerebellar circuits are implicated in providing important modulatory input to ensure precise movement. Moreover, specialized neurons within the spinal cord represent important nodes, where cerebellar input from the DCN could converge to influence skilled movement (Azim et al., 2014a). Recently, two classes of spinal interneurons *Gad2*-cre recombined interneurons (GABApre) and Chx10+ neurons in the

spinal cord have been functionally characterized in the skilled reaching behavioral paradigm. GABApre neurons were found to use presynaptic inhibition to reduce excitation at sensory to motor neuron synapses and reduce the excitability of the postsynaptic motor neurons (Betley et al., 2009; Fink et al., 2014) (Figure 5A). The functional relevance of GABApre neurons was examined by analyzing consequences of optogenetic stimulation of GABApre neurons on electrophysiological properties of the postsynaptic neurons (Fink et al., 2014). Stimulation of these spinal neurons resulted in a loss of postsynaptic sensory neuron evoked currents (Fink et al., 2014). Analysis of skilled reach in GABApre ablated mice also revealed that there was a loss of gain control (Fink et al., 2014). Loss of GABApre neurons resulted in large oscillatory movements and a reduction in successful reaches when ablated mice reached for a target (Fink et al., 2014). Thus, a presynaptic inhibitory circuit mediated by GABApre neurons play an important role in controlling gain at sensory-to-motor neuron synapses.

A second proposed modulatory mechanism involves quick updating of motor commands that engage cerebellar circuits. The cerebellum is thought to modulate movement by processing internal copies of motor commands and determining necessary online changes to be made to movement based on the present sensory state (Kawato, 1999). One potential source of internal copy information received by the cerebellum is through propriospinal neurons in the cervical spinal cord known as Chx10+ propriospinal neurons (Azim et al., 2014b). Chx10+ neurons receive input from higher motor centers and, in turn, their axons branch into an ascending arm which project into the LRN known to be an important source of spinal mossy fiber input to the cerebellum (Alstermark and Ekerot, 2015) (Figure 5B). A recent study showed that optogenetic manipulation of the ascending arm of Chx10+ neurons resulted in an increase in the number of paw reversals in the skilled reaching paradigm and a reduction in reach success (Azim et al., 2014b). Together, these results demonstrate that

information from Chx10+ neurons is involved in the production of precise reaching movements, and could be a source of internal copy signals to the cerebellum. Thus, some modulatory mechanisms have been identified for circuits in the spinal cord to control movement, but the involvement of cerebellar circuits for generation of well timed, accurate skilled movement and the circuit mechanisms through which they may engage spinal neurons or the motor cortex is unclear. Defining the contribution of cerebellar neurons to behaviors previously linked to circuits in the spinal cord and motor cortex thus provides a framework in which to interpret the function of circuits arising from the cerebellum.

Figure 5. Modulation of skilled movement by specialized spinal interneurons(A) Schematic showing presynaptic inhibition of sensory neuron axons for gain control. (B) Schematic showing a potential circuit for efference copy signaling to the cerebellum.

Internal copies of motor commands are sent to the cerebellum from two possible sources: Chx10+ propriospinal neurons in the spinal cord as previously described, and the motor cortex through the pontine nuclei (Azim et al., 2014b; Glickstein and Doron, 2008; Huang et al., 2013). The cerebellum is thought to integrate these motor commands with incoming sensory information and its internal models to provide a prediction of the sensory consequences of the motor command (Tseng et al., 2007). Signals are then relayed to the ascending and descending motor systems to update motor commands and may ensure precise movement in variable environments (Wolpert et al., 1998). Thus, the cerebellum may enable feedforward alterations to motor commands and may be an important component of the central motor machinery involved in the generation of refined movement. Although a theoretical framework in which to interpret the function of the cerebellum in motor control has been described, empirical evidence linking specific circuits from output neurons in the cerebellar nuclei to control movement is lacking. Moreover, the nature of cerebellar influence on motor ability within specific behavioral contexts and the role of circuits arising from specific cerebellar output neurons are unknown.

This thesis aims to examine the role of projection neurons in the interposed anterior nucleus as well as identify and characterize the circuits through which these neurons exert their influence in the context of voluntary goal-directed skilled movement. Our identification of the *Ucn3::Cre* mouse line, which drives recombination in glutamatergic projection neurons of the IntA nucleus of the cerebellum, facilitates the labelling, monitoring and manipulation of a specific subpopulation of cerebellar output neurons for behavioral analysis. Through chemogenetic ablation via diphtheria toxin, we found that the IntA neurons affect specific kinematics of limb movement. Next, through optogenetic activation of these neurons, we confirmed that IntA neurons specifically control the extent of reaching movements. EMG

recordings of the ipsilateral forelimb revealed that the cerebellum regulates these effects, by influencing extensor muscle activities. Finally, in order to delineate the circuitry through which the cerebellum exerts this functional inhibition, we traced circuits from the IntA to the spinal cord and motor cortex, and identified potential sites of cerebellar convergence in the corticospinal and rubrospinal pathways.

Together, the results described in this thesis provide new insights into the organization and functional relevance of a genetically accessible neuronal subpopulation in the deep cerebellum and links the IntA nucleus to the direction of voluntary limb movements and muscle control. We introduce a genetic tool to study the cerebellar contributions within a wider motor network. Our findings are an important first step for future studies to delineate the physiological targets of the IntA nucleus that mediate skilled movements.

3. Materials and Methods

3.1 Animals and ethics statement

The mouse strains used in this study were, C57BL/6JInv (The Jackson Laboratory/JAX), *Urocortin3::Cre* (*Ucn3::Cre*; Mutant Mouse Regional Resource Center; Tg[Ucn3-cre]KF31Gsat/Mmucd) (Harris et al., 2014), *Rosa::eGFP* (B6;129-Gt[ROSA]26Sortm2Sho/J) (JAX), *Rosa::lox-stop-lox-ChR2-eYFP* (*Ai32*) (B6.Cg-Gt[ROSA]26Sortm32[CAG-COP4*H134R/EYFP]Hze/J) and *Rosa::lox-stop-lox-hChR2* (H134R)-tdTomato (*Ai27*) (B6.Cg-Gt[ROSA]26Sortm27.1[CAG-COP4*H134R/tdTomato]Hze/J) (JAX). Male and female mice aged P60-P120 were used for all experiments. All procedures and experiments were approved by NTU/A*STAR Biological Resource Center IACUC committees.

3.2 Fabrication of electromyogram electrodes

Electromyogram (EMG) electrodes to implant the deltoideus par acromialis (deltoid) triceps brachii, and extensor carpi ulnaris (ECU) were custom made using teflon coated stainless steel wire (#793200, A-M systems) according to previously established protocols (Akay et al., 2014; Pearson et al., 2005). 8 cm of Double stranded wire were lightly twisted together and a knot was made 2-3 cm from one end of the strands. The ends were soldered to two pins of an 8pin connector (pinnacle technology). ~2 mm on the other side of the knot, a small bared region (electrode to be inserted) ~1mm in length was made on each wire, ensuring that the two regions were ~1mm apart when the wires are twisted. The ends were then crimped into the barrel of a 27G needle. Three electrodes were soldered to each 8pin connector, such that three muscles could be implanted per mouse. A screw, soldered to the connector and fixed to the skull was used as a ground.

3.3 Surgical Procedures

For all cerebellar manipulations, craniotomies were made above the site of injection. Stereotaxic coordinates used in this study were obtained from a stereotaxic atlas (Paxinos and Franklin, 2007): the IntA (from lambda: 2.3 mm posterior, 1.45 mm lateral, 2.50 mm ventral). For all surgical procedures mice were anesthetized with an induction concentration of 3% isofluorane and a flow rate of 2 L/minute followed by maintenance concentration of 1-2% isofluorane and a flow rate of 1 L/minute.

Failed experimental approaches: Targeting the Interposed anterior using conventional burr holes proved difficult, due to the curvature of the skull over the cerebellum, position of the DCN and thickness of the dura. Thus, we switched to performing craniotomies ~2x2 mm starting under the lambda suture line, extending in the mediolateral direction through the midline. After removing the dura, the Interposed anterior was triangulated using the cerebellar folds as a guide.

3.3.1 Virus injection

Solutions were administered at a rate of 2-10 nl/s using a NanoFil 10 µl glass syringe with a 33G bevelled needle, or a 1mm outer diameter pulled glass capillary, attached to a stereotaxic apparatus. Leaks were prevented by leaving the needle at the injection site for 5 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 virus-injected mice were housed postoperatively for a minimum of three weeks before perfusion or behavior experiments to allow for virus to be transduced.

3.3.2 Optic fiber implantation

Mono fiber-optic cannulas with a zirconia ferrule housed optical fiber (200 μ m core diameter, fiber protrusion length 2.5 mm; Doric Lenses) were inserted into the cerebellum (from bregma: 5.88 mm posterior, 1.45 mm lateral, 2.40 mm ventral) using a stereotaxic cannula holder (Doric Lenses). The optic fiber was secured to the back of the skull using dental cement (Heraeus Kulzer, Germany) with two anchors (stainless steel screws, 1.5 by 1.5 mm, #000-120 thread screws)(Au - Ung and Au - Arenkiel, 2012). After one week of recovery, mice were tested in behavioral paradigms.

Failed experimental approaches: The protocol described in the above reference used etching with a needle on the skull to allow dental cement to adhere and fix the fibre, however, we were unable to use that technique to hold the fibre in place. Instead we had to drill holes in the skull to fit two additional screws fixed to the skull and used cement to adhere the fibre and the screws, thus holding optic fibres in place.

3.3.3 EMG electrode insertion

EMG electrodes were lead through an incision from the neck to the arm and forearm. At the muscle of interest, the electrode was implanted using the attached needle. A knot was made to secure the electrode in place, and any extra wire was cut off (Pearson et al., 2005). The connector was fixed to the skull using two anchor points (stainless steel, 1.5 by 1.5 mm, #000-120 thread screws) and dental cement.

Failed experimental approaches: The protocol described by Pearson and colleagues required the needle to be lead under the skin to reach the muscle of interest and the connector was then stitched to the skin. This method in my hands resulted in improper placement and

the connector was easily pulled off the skin. To circumvent these issues I had to modify the surgical technique to fix the EMG connector to skull, similar to optic fibre implantations, This results in a long surgery time, but stable implants over several weeks.

3.4 Ablation of neurons in the IntA nucleus

Diphtheria toxin receptor was expressed within the IntA of *Ucn3::Cre*; reporter mice using a Cre-dependent virus pAAV2-*EFla::Flex-DTR-GFP* (construct generously provided by Dr Ayumu Tashiro, Cyagen Biosciences, Santa Clara, CA). The virus was introduced via pressure injection in P56-63 mice as described above. After two weeks of virus incubation, three doses of diphtheria toxin (DT) solution were administered to mice intra-muscularly (50 µg/kg; once every 48 hours; alternate hindlimb for each dose) (Wu et al., 2008). For control, pAAV2-*EFla::Flex-DTR-GFP* was injected into wildtype (WT), and DT toxin was administered as above. *Ucn3::Cre*; reporter mice that had been injected with virus and subsequently administered saline were also included in these experiments. No difference in general health and motor ability was found between control groups and subsequently data from all control mice was analyzed together. Behavioral experiments commenced one week after toxin administration. Mice were sacrificed and perfused for histological analysis at P96-101.

Failed experimental approaches, histology for DTR ablated mice: To quantify the extent of ablation in DTR ablated mice, we initially wanted to quantify markers of cell death, using caspase 3 expression and tunnel positive cells. However, we were unable to determine the time point at which we could reliably quantify these markers. Thus, we unable to tell if our assay for cell death was unreliable or the cell death using DT was inefficient. Using

immunostaining for cell markers as previously described we were subsequently able to quantify DT ablation (Bagnall et al., 2009).

3.5 Photostimulation during behavioural recordings

Light was delivered using a mono fiber-optic patch cord temporarily connected to the ferrule via a zirconia sleeve (Doric Lenses). A 473 nm diode laser (Shanghai Laser) and an arbitrary waveform generator (NI myDAQ; National Instruments) were used to deliver pulses of light. Laser strength was calibrated at the end of the fiber tip using a power meter (Thor Labs) prior to implantation.

3.5.1 Photostimulation during skilled reach

Mice were photostimulated at two time points of the reach, during initiation and at reach using the break beam sensor. The trigger signal resulted in a shutter, which was connected to the laser, opening for 100 ms. Randomized trials were utilized for stimulation. Laser power was calibrated at 3-7 mW.

Failed experimental approaches: We first tried to optogenetically stimulate mice by manually timing a light-ON switch to specific limb movements, however this resulted in inconsistent Light-Onset time points thus our initial experiment, with one animal could not be used in this data, although the mouse showed a similar termination of reaching when photostimulated.

3.5.2 Photostimulation during locomotion

Random trials were utilized for stimulation trials, laser power was calibrated at 1-10 mW at 1 Hz. Post analysis trials were sorted into Light-ON at stance, Light-ON, swing-flex, Light-ON, swing-extend.

3.6 Electromyogram recording and analysis

EMG recordings were amplified and low pass filtered at 500 Hz, acquired and digitized using the acquisition system and Sirenia software from pinnacle technology (Whelan, 2003). Data was analysed using custom scripts written in MATLAB.

3.7 Motor behavioral analysis

3.7.1 Staircase task of skilled reaching

Skilled reaching task was conducted in the staircase chamber as previously described (Baird et al., 2001; Klein and Dunnett, 2012). Mice are trained to enter the chamber and reach downwards on each side to retrieve a food reward. The further mouse has to reach on lower steps, the greater the skill required. Mice were food deprived to 85% of their free feeding weight. At the beginning of testing, mice were introduced to the food pellet (Honey Stars, divided into five equal sized pieces (3.76 x 5.43 x 4.65 mm); Nestle) by placing pellets into each home cage on three consecutive days. Mice were then habituated to the chamber by placing food pellets along the surface of the central trough as well as on the staircase steps for an additional two days. Subsequently, along each step of the double staircase, one pellet was placed (eight on each side, total 16 pellets per test chamber) and the mouse was placed in the start compartment. Finally, during testing pellets were placed exclusively on step 6 and step 7 of the staircase apparatus, these two steps were chosen because they represent a difficulty level where mice are unable to retrieve the pellet with their mouth but still achieve a baseline success rate of 40-60%.

Furthermore, modifying the testing such that mice target just two steps, allowed us to perform more trials within one training session. Each training and testing session lasted for 15 minutes. All sessions were recorded for analysis (RX100IV, Sony). The number of pellets remaining on each step and dispersed were scored each day.

Success rate for the staircase task was 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 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 the 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 of the same movement. If the pellet is displaced from the well, analysis is concluded for the reach. The reaching endpoints were plotted using a density contour plot in MATLAB.

3.7.2 Single pellet reaching

To facilitate optogenetic manipulation of neurons within a reaching paradigm, we used a modified form of the single pellet reaching behavioural task (Azim et al., 2014b; Chen et al., 2014). Mice are made to reach through a narrow slit to obtain a reward at a fixed

position. The distance from the slit in either axis may be changed to increase the level of difficulty. A chamber made of clear acrylic was custom ordered (15 x 8.5 x 20 cm, length x breadth x height). The chamber consists of three vertical slits, 13 cm in length and 0.5 cm in width, through which mice could reach for the pellets. The slits were positioned on one side in the centre (for shaping) and on the other side at the left and right of the chamber (for testing). A reaching apparatus was also constructed as in Figure 13A, with adjustable target positions in the x , y and z planes.

	Distance from the slit (mm)
x	2.5
y	9
z	10

Table 1. Table showing position of pellet in relation to the skilled reaching chamber

Mice were food deprived to 85% of their free feeding weight and introduced to the food reward within the home cage (5TUT sucrose reward pellet, 5 mg test diet). Mice were then habituated to the chamber in three sessions for 10 minutes each. Shaping or determining the handedness of the mouse was then performed by placing the chamber with the centre slit down. Several pellets were placed at the base of the reaching apparatus and the number of reaches with each hand was counted. The mouse was considered to prefer a hand if more than 65% of reaches to the pellet were performed with that hand. For subsequent testing trials, the chamber was overturned and only the side of the preferred hand was used for the analysis.

In the testing phase of single pellet reaching task, one pellet was presented at a target location and each reach toward the pellet was considered to be one trial. Success rate and errors for the single pellet task was calculated as above. Reaching, grasping and retrieval

errors were calculated as a percentage of the total number of reach, grasp and retrieval movements made respectively.

Failed experimental approaches: The protocol for the single pellet reaching task, originally consisted of an 8cmX6cmX2cm (lXbXh) platform on which the pellet is placed in the divot. Using this setup, we found that majority of the mice were able to retrieve the pellet with their tongue, preventing analysis of limb movement. The stage for pellet positioning was thus replaced with the apparatus described above.

3.7.3 Open field analysis

To measure spontaneous movement in an open environment, activity in an open field was tested using a gray perspex box (60 x 60 cm). Mice were allowed to freely move around for a total of 7 minutes. Distance travelled and velocity of movement were analyzed using EthoVision (Noldus, Netherlands)(Seibenhener and Wooten, 2015).

3.7.4 Rotarod Analysis

To test motor function in the rotarod (TSE Bad Homburg, Germany), mice were first habituated to the rotarod apparatus using a 5 minute 4 rpm protocol. Mice were then trained on an accelerating protocol from 4 to 40 rpm, to test the ability of mice to adapt to increasing acceleration. Results were calculated as latency to fall (in seconds) on a single day of testing(Deacon, 2013; Shiotsuki et al., 2010).

3.7.5 Gait analyses of freely walking mice

Gait parameters of freely walking mice are measured as previously described (Machado et al., 2015; Mendes et al., 2015). Briefly, each limb stride consists of 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. For gait analysis from a lateral view, mice were habituated for a week post-surgery to the walkway (3.2 x 30 x 70 cm) using a daily schedule of 30 minutes over 3 days. After the initial habituation phase, mice were made to walk through the corridor and video recordings were made for kinematic analysis.

3.7.6 SHIRPA scoring

The SHIRPA protocol is a battery of tests developed in an effort to standardise behavioural phenotyping of genetic models (Hatcher et al., 2001; Rogers et al., 2001). In our study to test whether ablation had any effect of motor reflexes or health, we used the first stage of the SHIRPA protocol which consists of primary observations and scoring of characteristic behaviours. Behaviors were scored as follows:

- Palpebral closure: Degree of eye closure when the mouse is awake and alert, a score of scoring as follows, 0: Eyes closed, 1: Eyes half closed, 2: Eyes wide open
- Respiration rate: Qualitative description of how slow or fast the animal breathes, 0: Gasping, irregular, 1: Slow, Shallow, 2: Normal, 3: Hyperventilating
- Heart rate: Qualitative description of how slow or fast the animal's heart beats, 0: Slow, 1: Normal, 2: Fast
- Pinnae reflex: Ear retraction when are mice are probed on the ear with a von Frey hair, 0: none, 1: active retraction
- Corneal reflex: Eyelid closure when mice are probed on the eye with a von Frey hair, 0: none, 1: active single, 2: multiple repetitive
- Visual placing: Mice are held ~ 15 cm above a wire grid and slowly lowered, forelimb extension is observed. 0: none, 1: upon nose contact, 2: upon vibrissae contact, 3: before vibrissae contact (18mm), 4: early vigorous extension (25mm)

- Limb grasping: Mice are tested for their ability to grasp a wire grid, 0: absent, 1: present
- Grip strength: Strength of the grip is qualitatively assessed, 0: none, 1: slight grip, 2: moderate grip, 3: active grip
- Gait: Tests the fluidity of movement during walking within the home cage, 0: incapacity, 1: limited movement only, 2: fluid but abnormal, 3: normal
- Pelvic elevation: Elevation of the pelvis during locomotion, 0: flattened, 1: barely touches, 2: normal (3mm), 3: elevated (>3mm elevation)
- Tail elevation: Elevation of the tail during locomotion, 0: dragging, 1: horizontally extended, 2: elevated/ straight tail (rigid, elevated), 3: other
- Trunk curl: Curvature of the body when mice are held ~15 cm above a surface, trunk curl: 0: absent, 1: present

3.8 Integrated setup for EMG recording and optogenetic stimulation

To facilitate temporally specific optogenetic stimulation during the reach and to provide a time stamp to the EMG recording, an infrared break beam motion sensor is placed across the height of the slit (Figure 14A) (IR Break Beam Sensor – 3 mm LEDs, Adafruit). With each reach, the beam generates a 5 V TTL signal using an Arduino board. This signal is then used (1) to trigger the laser via a shutter *during the reach*, (2) to provide an time reference to the EMG recording, and (3) to increment a counter visible through the video recording which links videos to the EMG.

3.9 Forelimb kinematic analysis

3.9.1 Kinematic analysis of skilled reach

Paw movement in the staircase task was tracked using the DeepLabCut software (Mathis et al., 2018). Paw markings were then annotated using MATLAB (MathWorks) with a customized paw-tracking program. At each video frame, the positions of the nose, reaching forelimb and pellet was collected and analyzed for position, trajectories, angles and errors. Videos were taken initially at 30 fps (Figure 5), subsequently at 250 fps (Figure 6, 8).

3.9.2 Kinematic analysis during locomotion

The x and y coordinates of the forelimb were labelled for each video frames using MaxTRAQ 2D (Innovision Systems). The maximum and mean vertical position across a stride were calculated. Videos were taken at 300 fps.

3.10 Immunohistochemistry and Imaging

Immunohistochemistry was performed on 40-60 μm free-floating sections, cut using the microtome or cryostat. Tissue sections were washed three times with tris buffered saline (TBS) (0.2% TX100), blocked with TBS (0.2% TX100, 3% horse serum) for one hour at room temperature and then incubated in primary antibody for two nights at 4°C. Tissue sections were washed 3 times with TBS and then incubated with secondary antibody (fluorophore conjugated) for 4-6 hours at RT. Tissue sections were then stained with DAPI, washed three times with TBS and mounted on glass slides. Primary antibodies used were: guinea pig anti-vGluT2 (1:2000; Merck; AB2251); rat anti-GFP (1:1000; Nacalai Tesque; GF090R); rabbit anti-TBR1 (1:500; abcam; ab31940; mouse anti-GAD67 (1:1000; Invitrogen; PA5-21397); mouse anti-SMI 32 (1:1000; Biolegend; #801701). Sections were imaged using a confocal microscope.

3.11 Statistical Analysis

For ablation experiments, comparable number of strides/reaches per animal of each in control and ablated animals were analyzed to avoid disproportionate effect on results.

Similarly, for photo-stimulation experiments, comparable number of strides/reach from each animal during ON and OFF conditions were used for statistical comparison. All values are represented as mean $\pm$ s.e.m. unless otherwise stated. Comparisons were made by unpaired Mann-Whitney U test. Non-parametric statistical tests were used. P values across more than two groups were calculated using ANOVA. Statistics were analyzed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla, California USA).

4. Results

4.1 Characterization of a distinct subpopulation of neurons within the IntA nucleus

4.1.1 Characterization of the recombination capability of *Ucn3::Cre* mice in the deep cerebellar nuclei

In order to define the organization and function of neuronal subpopulations and circuits arising from the deep cerebellar nuclei (DCN), we first searched for a mouse line that allows genetic targeting of a subset of neurons in the DCN. We identified a BAC transgenic mouse line *Ucn3::Cre* (Madisen et al., 2012), which permits recombination within the DCN, without targeting neurons in the cerebellar cortex (GENSAT; Mutant Mouse Regional Center). To characterise the localization and identity of the recombined neurons, we crossed *Ucn3::Cre* mice with reporter mouse lines *Rosa26::lsl-eGFP* (*Rosa::eGFP*) and *Rosa26::lsl-Channelrhodopsin (ChR2)-EYFP* (*Rosa::ChR2*) (Figure 6A). We assessed the distribution of recombined neurons in the DCN by first dividing the DCN into 9 different subnuclei based on histological distinctions (Paxinos and Franklin, 2007) (Figure 2A). We counted the number of YFP-positive neurons in each sub-nucleus and expressed it as a percentage of the total number of recombined neurons in the DCN. Interestingly, we found that within the nine subnuclei more than 70% of YFP⁺ neurons were found in the interposed anterior nucleus (IntA), 15% in the medial nucleus and 15% distributed within the remaining subnuclei (Figure 6B). Thus, in the cerebellum, the *Ucn3::Cre* mouse drives recombination primarily in the interposed anterior nucleus of the DCN.

4.1.2 Defining the electrophysiology and molecular properties of *IntA^{Ucn3}* neurons

To identify the neurotransmitter profile and electrophysiological properties of recombined neurons, we next performed whole cell patch clamp recordings on YFP-positive neurons (YFP^{ON}) and YFP-negative neurons (YFP^{OFF}) in collaboration with Dr. Jinsook Kim

and Prof. George Augustine (NTU-LKC). We found that YFP^{ON} neurons had a high firing rate of ~40hz. These values are indicative of glutamatergic projection neurons in the DCN previously described (Uusisaari et al., 2007). Based on further analysis of cell morphology using Neurobiotin, we further classified neurons into three neuronal types large YFP-positive neurons (YFP^{ON}-L), large YFP-negative neurons (YFP^{OFF}-L) and small YFP-negative neurons (YFP^{OFF}-S) (Figure 6C-D). These three classes of neurons fall within the classification scheme of DCN neurons proposed by Uusisaari and Knopfel and show similarities to large GAD-negative (YFP^{ON}-L, YFP^{OFF}-L) and small GAD-positive neurons (YFP^{OFF}-S) (Uusisaari and Knopfel, 2011). These results revealed that Ucn3-recombined neurons in the IntA nucleus (IntA^{Ucn3} / YFP^{ON}-L) are a subset of large glutamatergic neurons within this cerebellar nucleus.

To define the molecular profile of IntA^{Ucn3} neurons, we performed immunohistochemistry on YFP^{ON} neurons using glutamatergic markers SMI32, also known as neurofilament H which has previously been shown to mark cell bodies and dendrites of large glutamatergic cells in the DCN and TBR1 (T-box receptor 1, a transcription factor which marks glutamatergic cells in the DCN (Figure 6C, E-H) (Chung et al., 2009; Fink et al., 2006). We also stained neurons with gabaergic markers of the cell body GAD67, and nucleus TFAP2A (transcription factor AP2A) (Figure 6I-L) (Zainolabidin et al., 2017). We found that IntA^{Ucn3} neurons are a subset of glutamatergic SMI32⁺ neurons (44±0% of the total SMI32) and TBR1+ neurons (30±0% of the total TBR1+) in the IntA. IntA^{Ucn3} showed no co-localization with GABAergic markers in the IntA (Figure 6E-H, I-L). Together, our molecular and electrophysiology results indicate that IntA^{Ucn3} neurons are a subpopulation of large glutamatergic neurons in the IntA. Thus the *Ucn3::Cre* mouse line allows genetic targeting of a specific set of neurons within the IntA nucleus.

Figure 6. The *Ucn3::Cre* mouse selectively recombines in glutamatergic neurons of the IntA nucleus. (A) Expression of GFP in *Ucn3::Cre; Rosa::eGFP* mice. (B) %distribution of $\text{IntA}^{\text{Ucn3}}$

neurons, in 9 subnuclei of the DCN. (C-D) GFP+/TBR+/ NB+ neurons show higher firing rates compared to GFP-/TBR-/NB+ neurons. (E-L) GFP+ neurons colocalize with neuronal marker in the DCN, SMI32 (G, K). GFP+ neurons do not colocalize with GABAergic markers, TFAP2A (F) and GAD67 (J). n=7-15 YFP^{ON}-L, 6-9 YFP^{OFF}-S, 6-9 YFP^{OFF}-L neurons. **: $P \leq 0.01$, ***: $P \leq 0.001$

4.2 Chemogenetic ablation of IntA^{Ucn3} neurons disrupts accuracy of skilled movements

4.2.1 Diphtheria toxin receptor-mediated ablation of IntA^{Ucn3} neurons

To examine the functional relevance of neurons in the IntA nucleus, we selectively targeted the IntA^{Ucn3} neurons for chemogenetic ablation using AAV virus to conditionally express diphtheria toxin receptor in IntA^{Ucn3} neurons. We first injected AAV2.*EFla*.FLEX.DTR.GFP into the DCN of *Ucn3::Cre* and assessed the conditional expression of the DTR construct (Figure 7A). GFP expression was found within the IntA nucleus of Cre-positive mice while injection into wildtype mice showed no GFP expression within the IntA (Figure 7A-C). Additionally, GFP-positive neurons in *Ucn3::Cre* mice colocalise with TBR1 (Figure 7D-F). After administration of diphtheria toxin (150 ng/g body weight), we found a significant reduction in TBR1⁺ and SMI32⁺ in IntA^{Ucn3}-ablated mice (Figure 7G-H, J-K). Ablated mice showed ~32% reduction in TBR+ neurons and ~43% reduction in SMI32+ cells (Figure 7I, L). Thus, conditional diphtheria toxin-mediated chemogenetic ablation provides a reliable means to eliminate IntA neurons.

Figure 7. Diphtheria toxin mediated ablation of IntA^{Ucn3} neurons. (A-C)

AAV2.EF1A.Flex.DTR.GFP virus was injected into *Ucn3::Cre*; reporter and wildtype mice. GFP expression was observed in the IntA in *Ucn3::Cre* positive mice (B) while injection into WT mice showed no GFP expression in the IntA (C). (D-F) Co-localization of *Ucn3::Cre*-recombined neurons with TBR1. (G-I) Administration of diphtheria toxin results in loss of TBR1+ cells in *Ucn3::Cre*; reporter mice injected with DTR virus (Ablated), but not WT injected mice. (J-L) Administration of diphtheria toxin results in loss of SMI32+ cells in *Ucn3::Cre*; reporter mice injected with DTR virus (Ablated), but not WT injected mice. N=7 ablated, 8 control. ***: $P \leq 0.001$, ****: $P \leq 0.0001$

4.2.2 *IntA^{Ucn3} neuronal ablation does not affect general motor activity*

Because perturbation of the cerebellum is associated with motor deficits, we first tested the health and general motor ability of ablated versus control mice. We used the SHIRPA protocol for assessment of general health to test whether mice showed any physiological deficits or deficits in their reflexes (Rogers et al., 2001). Ablated mice showed normal SHIRPA responses comparable to controls, with no apparent deficits in neurological reflexes (Table 2). To test whether ablation of IntA^{Ucn3} influenced activity and free movement we used the open field paradigm (Gellért and Varga, 2016). Activity level and general locomotor ability was assessed in terms distance travelled and speed during the task. Control and ablated mice travelled similar distances at similar speeds for the duration of the task without exhibiting any observable or quantifiable deficits in the open field paradigm (Figure 8B-C). Next to test the ability to adjust movement to changing speeds, mice were tested on the accelerating rotarod paradigm. latency to fall in the accelerating rotarod paradigm was not affected by IntA^{Ucn3} neuronal ablation (Figure 8D). To test the ability of control and ablated mice to identify food rewards, and to test for the ability to dig, we tested mice in a buried food task where mice locate a hidden food reward. Control and ablated mice showed no significant difference in the latency and ability to retrieve the reward (Figure 8E). Furthermore, no difference in weight was observed in control and ablated mice indicating no significant difference in basic physiology due to ablation (Figure 8F). Finally, as an internal control, for the staircase task we tested success on step 2 of the staircase, where mice do not require forelimb skill but are required to enter the chamber and identify and retrieve the food reward. All mice were able to retrieve food rewards at step 2 on the staircase case (Figure 8G). Thus, the ablation of IntA^{Ucn3} neurons does not appear to affect general motor activity, and are likely required for more specific motor processes.

	Ablated	Control	Scoring Guide
Palpebral closure	2	2	2 = Eyes wide open
Respiration rate	2	2	2 = Normal
Heart rate	1	1	1 = Normal
Pinnae reflex	1	1	1 = Active retraction
Corneal reflex	1	1	1 = Active single
Visual placing	3	3	3 = Before vibrissae extension
Limb grasping	1	1	1 = Present
Grip strength	2. 2	2	2 = Moderate grip
Gait	3	3	3 = Normal
Pelvic elevation	2	2	2 = Normal
Tail elevation	1	1	1 = Horizontally extended
Trunk curl	1	1	1 = Present

Table 2. SHIRPA assessment of neurological deficits. Ablated and control mice show comparable neurological reflexes in by SHIRPA assessment. N=5 ablated, 7 control.

Figure 8. Ablation of $IntA^{Ucn3}$ neurons does not affect general motor ability. (A) Timeline of diphtheria toxin administration combined with behavioural testing. (B-C) Ablated and control mice travel similar distances at similar speeds in the open field test. N=5 ablated, 7 control (D) Ablated and control mice show the same latency to fall on the accelerating rotarod. N=5 ablated, 7 control (E) Ablated and control mice do not show significant differences in their ability to obtain a buried food reward. N=5 ablated, 4 control (F) Ablated and control mice are not significantly different in body weight. N= 3 ablated, 3 control (G) Ablated and control mice are similar in their ability to obtain a food reward from step 2 of the staircase task of skilled reaching (please refer to Figure 10). N=5 ablated, 4 control.

4.2.3 $IntA^{Ucn3}$ neurons regulate the timing and positioning of the forelimb during locomotion

The cerebellum, in part through the activities of Purkinje cells, regulates the timing and extent of the limbs during walking (Hoogland et al., 2015; Machado et al., 2015). Since we did not observe any general motor deficits in $IntA^{Ucn3}$ -ablated mice using the open field paradigm, we set out to assess limb movements during the step cycle in more detail. To do this, we designed a modified locomotor apparatus optimized for observing the step cycle from a lateral or side view (Figure 9A) (Machado et al., 2015). The rodent step cycle consists of alternating limb movements, a dynamic swing phase and a stationary stance phase, where the beginning of one stance to the next forms a single stride (Figure 9B). Mice were trained to walk through a long corridor and forelimb trajectories were recorded in the x and y planes.

At first glance, we observed that ablated mice show aberrant stride trajectories with a premature increase in y displacement from the base (Figure 9C). To characterize this deficit in more detail, we plotted the movement of the forelimb in the x and y plane over time. We found that along the x -axis, ablated mice showed no difference in limb positioning over time (Figure 9D-E). However, slight changes were observed along the y -axis with 5% increase in y displacement in the initial part of the swing phase (Figure 9F-G). Interestingly, we found that although the average y displacement of the forelimb remained unchanged in ablated mice, the time taken to reach the maximum height during a swing was significantly reduced (Figure 9F, H). This indicates that a premature increase in y displacement early on in the stride may have resulted in abnormal trajectories in ablated mice. Next, to examine the timing of each phase, we quantified the duration of the stance and swing phase and found that ablated mice showed a 23% decrease in the stance phase which results in an overall decrease in cadence (Figure 9H-K). Thus, ablated mice show deficits in timing their movements, particularly in the initial phase of the step cycle.

Together, our results from analysis of locomotor movement in $\text{IntA}^{\text{Ucn3}}$ -ablated mice indicate that $\text{IntA}^{\text{Ucn3}}$ neurons regulate the kinematics of forelimb movement by specifically controlling the positioning of the forelimb in the vertical plane and determining the initiation of forelimb locomotion.

Figure 9. Ablation of $IntA^{Ucn3}$ neurons disrupts forelimb trajectories during locomotion. (A) Schematic of the locomotor apparatus. (B) Representative images showing division of the stride into a stance phase and swing phase. (C) Forelimb trajectory traces from one control and one ablated mouse. (D-E) x displacement and x displacement over time is similar in control and ablated mice. (F-G) Ablated mice show subtle changes in the early stages of the swing phase. (H) Ablated mice show a significantly reduced latency to reach the maximum height of swing compared to controls. (I-K) Ablated mice show a significantly reduced stance duration, resulting in an overall reduction in cadence. $N=3$ ablated, 3 control. ***: $P \leq 0.001$, ****: $P \leq 0.0001$.

4.2.4 $IntA^{Ucn3}$ neurons control accuracy of skilled reach

Voluntary goal-directed limb movements require the ability to execute a precise sequence of movements to attain an objective (Rothwell, 2012). To assess whether $IntA^{Ucn3}$ neurons regulate aspects of voluntary discrete movements, we used the staircase task of

skilled reaching (Baird et al., 2001; Klein et al., 2012) (Figure 10C). DTR-mediated ablation was carried out and the ability for mice to retrieve a food reward from step 6 and 7 was assessed using the staircase apparatus (Figure 10A-C). After ablation, over five days of assessment, $\text{IntA}^{\text{Ucn3}}$ -ablated mice were significantly less successful at obtaining the food reward (Figure 10D). Furthermore, analysis of the forelimb trajectories revealed that ablated mice make angular arm movements towards the pellet that may have resulted in deficits in success (Figure 10E).

To further examine the underlying cause of the observed reduced success in reaching, we recorded errors as a percentage of total number of reaches made during the task and analyzed specifically the reach, grasp and retrieval errors. Through video recording of the reaching movement, we were able to define criteria for reach, grasp and retrieval errors. We defined reaching errors as deficits in correctly targeting the pellet where the mice either reached too far past the pellet (over-reach) or stopped short of the pellet (under-reach) (see Methods for details). Grasping errors were defined as an inability to grasp the pellet despite correct targeting, and retrieval errors were defined as dropping the pellet after a successful grasp (see Methods for details). We found that ablated mice exhibited deficits specifically in reaching while the grasping and retrieving movements were not disrupted (Figure 10F). Furthermore, despite similar nose positions, reaching endpoints in $\text{IntA}^{\text{Ucn3}}$ -ablated mice were consistently found past the position of the pellet (Figure 10I, G) (Supplementary video file 1 [ablated], 2 [control]). This resulted in an obtuse angle between the paw and pellet a bias towards overreaching movements in $\text{IntA}^{\text{Ucn3}}$ -ablated mice (Figure 10H). Together these results reveal that the elimination of $\text{IntA}^{\text{Ucn3}}$ neurons results in inaccurate targeting of the forelimb which indicates that these neurons are required to maintain the fidelity of skilled reaching.

Figure 10. $\text{IntA}^{\text{Ucn3}}$ ablated mice exhibit deficits in the accuracy of skilled reach. (A-B) Schematic showing viral injection and the time line of diphtheria toxin administration (C) Dimensions of the staircase task of skilled reach, diagram explaining the position of the pellet with respect to the paw. (D) $\text{IntA}^{\text{Ucn3}}$ ablated mice show an overall reduction in success rate compared to control mice. (E) Representative trajectories of reaching movements from 2 ablated and 2 control mice. N=2 control, 2 ablated (F) $\text{IntA}^{\text{Ucn3}}$ ablated mice show significantly higher number of reaching errors, while grasping and retrieval are intact. Inset showing a reach, grasp and retrieve. (G) Contour density plot showing the frequency of the reaching endpoints at various positions in ablated and control mice. (H) The

angle between the paw and pellet is significantly more obtuse in ablated mice. (I) nose position is consistent between ablated and control mice. N=10 ablated, 11 control. *: $P \leq 0.05$, **: $P \leq 0.01$.

Although ablation of $\text{IntA}^{\text{Ucn3}}$ neurons resulted in deficits in reach efficacy, it was unclear whether $\text{IntA}^{\text{Ucn3}}$ neurons are required for reach accuracy even after an initial phase of learning. In addition, both control and ablated mice were able to achieve an initial success rate of ~40%, indicating that $\text{IntA}^{\text{Ucn3}}$ neurons may not be crucial for acquiring stimulus to reward contingencies and rudimentary motor skills (Figure 10D). To determine whether $\text{IntA}^{\text{Ucn3}}$ -ablated mice exhibit any deficit in task execution when learning is intact, we trained control and ablated mice to perform on the staircase task until all mice achieved a success rate of ~40-50%, before diphtheria toxin administration (Figure 11A-B). After ablation, $\text{IntA}^{\text{Ucn3}}$ -ablated mice showed a significant reduction in success and similar deficits in trajectory and endpoint positioning as untrained ablated mice (Figure 11C-D). This implies that IntA neurons play an important role in task execution despite an extended training period. Together, our results indicate that $\text{IntA}^{\text{Ucn3}}$ neurons are required for precise execution of skilled forelimb movements and potentially participate in online refinement of limb movements.

Figure 11. $\text{IntA}^{\text{Ucn3}}$ ablation after learning results in similar deficits in the accuracy of skilled reach. (A) Schematic of the timeline of DT administration and the skilled reaching task. (B) Post DT administration, ablated mice show a significant reduction in success rate. (C) Representative trajectories of the reaching movement from one ablated and one control mouse. (D) Contour density plot showing the frequency of the reaching endpoints at various positions in ablated and control mice, pre and post DT. $N=3$ ablated, 3 control. *: $P \leq 0.05$

4.3 $\text{IntA}^{\text{Ucn3}}$ neurons modulate the extent of forelimb positioning during voluntary movement

Indirect stimulation of neurons in the interposed nucleus, through inhibition of Purkinje neurons has been shown to drive limb movements (Lee et al., 2015). Optogenetic silencing of Purkinje cells resulted in forearm movements time locked to the inhibition (Heiney et al., 2014; Lee et al., 2015). However, whether acute manipulation of interposed nucleus neurons drives changes in forelimb movement has not been addressed. Since ablation of $\text{IntA}^{\text{Ucn3}}$ neurons resulted in deficits in the accuracy of forelimb movement, we set out to assess whether transient stimulation of $\text{IntA}^{\text{Ucn3}}$ neurons would influence kinematic parameters in a similar manner.

4.3.1 Photostimulation of $IntA^{Ucn3}$ neurons results in changes in forelimb positioning during locomotion

To stimulate $IntA^{Ucn3}$ neurons in behaving mice, $Ucn3::Cre$; $Rosa::ChR2$ mice implanted with optic fibers were tested in skilled reach and the locomotor paradigm. We first performed a preliminary experiment using the locomotor paradigm to test whether stimulation of $IntA^{Ucn3}$ neurons can elicit changes in limb movements, complementary to the changes observed in ablated mice. Mice were made to walk through a corridor as previously described (Figure 9A). Mice were then randomly stimulated through the experiment, with light onset at the stance phase and two stages of the swing phase (Figure 12A-C). We divided the swing phase based on movement at the ankle joint, into a swing-flex phase (ankle flexion) and a swing-extend phase (ankle extension) (Figure 12C). Strides were then sorted into 4 conditions of stimulation: Light-OFF, Light-ON at stance, Light-ON swing-flex and Light-ON swing extend. We found that stimulation of $IntA^{Ucn3}$ neurons resulted in apparent changes in stride trajectory and an increase in average y displacement in the stance phase, while stimulation in control mice during showed no effect on stride trajectory (Figure 12 D-G, Table 3). In addition, stimulation at an earlier stage of the swing phase the swing-flex phase resulted an increase in average y displacement, while stimulation during extension in the swing-extend phase, resulted in no change in y displacement or stride trajectory (Figure 12 H-I, Table 3). Thus, stimulation of $IntA^{Ucn3}$ neurons resulted in changes in y positioning during the stride and timing of light onset had a significant effect on the outcome of stimulation. Together, our results show that transient stimulation during initiation of limb movements affects stride patterns and participates in online modulation of movement.

Δy $Ucn3::Cre$; $Rosa::ChR2$ (mean $\pm$ sem) (mm)	Δy WT (mean $\pm$ sem) (mm)
--	---

Light-OFF	2.45 ± 0.14	3.32 ± 0.17
Light-ON at stance	4.12 ± 0.19	2.53 ± 0.14
Light-ON swing-flex	4.16 ± 0.19	--
Light-ON swing-extend	2.78 ± 0.17	--

Table 3. Table of average y displacement across the stride

Figure 12. Photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons results in changes in stride trajectory. (A-B) Schematic showing optogenetic implants and the timeline of stimulation. (C) Division of the stride into three Light-ON phases, Light-ON at stance, Light-On swing-flex and Light-ON swing-extend. (D-G) Stimulation of $\text{Ucn3}::\text{Cre}; \text{Rosa}::\text{ChR2}$ mice changes stride trajectory while stimulation in WT control keeps trajectories intact. (H-I) Stimulation swing-flex phase produces greater changes in y displacement compared to stimulation during the swing-extend phase. Dotted line represents maximum height in the Light-OFF condition. Δy represents average displacement, P values are with respect to the Light-OFF condition. $N=1 \text{ Ucn3}::\text{Cre}; \text{Rosa}::\text{ChR2}, 1 \text{ WT}$. ***: $P \leq 0.001$

4.3.2 Photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons result in early termination of the reaching movement.

To further characterise how $\text{IntA}^{\text{Ucn3}}$ neurons perform online modulation of limb movements in a goal directed context, we stimulated $\text{Ucn3}::\text{Cre}; \text{Rosa}::\text{ChR2}$ mice within a skilled reaching behavioral task. We used a modified version of the single pellet reaching behavioral paradigm, where mice reach through a narrow opening to obtain a pellet. In addition, we used motion sensors across the length of the opening to trigger stimulation during the reach (Figure 13A). Because, preliminary results from optogenetic stimulation during locomotion showed that the timing of stimulation onset impacts the trajectory of the forelimb, we tested the effect of stimulation at two time points during the reach: (1) stimulation onset during initiation, before the forelimb crosses the opening (Light-ONI) and (2) onset after extension of forelimb, at the endpoint of the reach (Light-ONR) (Figure 13C). In contrast to our ablation results, we found that photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons result in early termination of the reaching movement, and although the grasping action was intact, mice were unable to extend the arm to accurately target the pellet (Supplementary video file, 3 [Light- OFF], 4 [Light-ONI], 5 [Light-ONR]). By plotting the trajectories and endpoints of the reach, we were able to qualitatively observe that when stimulated, mice show trajectories terminating before reaching the pellet, and the reaching endpoints clustered further away from the pellet (Figure 13D-I). This was evidenced by a significant increase in distance between the paw and the pellet at the reaching endpoint (Figure 13N). Moreover, we found that stimulation at the initial stage of reach (Light-ONI) resulted in more profound effects on endpoint positioning, compared to stimulation at the reach (Light-ONR).

To examine kinematics of the reach, we plotted the distance travelled in the x and y plane over time, we found that in the horizontal plane, when stimulated, mice remain further

away from the pellet in the first ~ 20ms of the reach, while y positioning over time over time is unaffected (Figure 13J-K). This effect results in an increase in duration and distance travelled from the initial position, in spite of consistent under-reaching (Figure 13L-M). Thus, $\text{IntA}^{\text{Ucn3}}$ neurons are important in the initial stages of the reach, and could also control the initial positioning of the forelimb during the reach.

Through our analysis of stimulating $\text{IntA}^{\text{Ucn3}}$ neurons during locomotor and skilled reach tasks, a common theme emerged. $\text{IntA}^{\text{Ucn3}}$ neurons exert influence over the extent of forelimb position during a projecting movement thus affecting the overall precision of the limb movement, and this modulation preferentially occurs early in the limb movement.

Figure 13. Photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons results in early termination of the reach. (A) Schematic of the modified single pellet reaching behavioural task. (B) Timeline of optogenetic stimulation in the skilled reach paradigm. (C) Representative images showing events marked during

the reach, division of the initial phase and reach phase. (D-F) Representative trajectories in Light-OFF, Light-ONI and Light-ONR conditions from one mouse. (G-I) Reaching endpoints in Light-OFF, Light-ONI and Light-ONR conditions. (J-K) Movement in the x plane over time is slower in the initial stages on the reach in Light-ON conditions, while y positioning is unaffected. (L-M) Length and duration of the reach is increased in the Light-ONI condition (N) Distance from the pellet at the endpoint of the reach is increased in the Light-ONI condition. $N=2$. *: $P \leq 0.05$, ***: $P \leq 0.001$

4.3.3 *IntA^{Ucn3} neurons potentially influences muscle activities during skilled reach.*

To identify downstream mechanisms by which the IntA^{Ucn3} neurons influence the early termination of reach movements, we performed electromyogram (EMG) recording on muscles of the forelimb with simultaneous optogenetic stimulation during skilled reaching. Because optogenetic stimulation and ablation of IntA^{Ucn3} neurons primarily affected the reach phase of movement during skilled reaching, we hypothesized that extensor muscles of the forelimb may be controlled by IntA^{Ucn3} neurons. We utilised the single pellet reaching behavioural apparatus as described earlier and used the motion sensor to annotate reach events to the EMG (Figure 14A). EMG electrodes were implanted into the elbow extensor muscle (triceps brachii), the wrist extensor muscle, (extensor carpi ulnaris) and as a control to the shoulder abductor (deltoideus pars acromialis)(Figure 14B).

In these experiments, which represent preliminary data, we compared muscle activity during the Light-OFF, Light-ONI, and Light-ONR condition (Figure 14D-F, H-J). During skilled reaching, in Light-OFF condition, the tricep muscle is active from initialise till reach events, while the ECU is active between initialise till the grasp event (Figure 14D, H). We found that during the Light-ONI condition, tricep muscle activity was significantly attenuated between initialise and reach events (Figure 14E). ECU activity was also found to be attenuated between initialise and reach (Figure 14I). This attenuation was supported by a ~50% reduction in change in activity over the baseline for both muscles (Figure 14G, K). Thus, stimulation of IntA^{Ucn3} neurons resulted in reduced activation of extensor muscles.

Interestingly, similar to our kinematic results, muscle activations remained intact in the light-ONR condition (Figure 14F, J). Thus, optogenetic activation of IntA^{Ucn3} neurons appear to suppress muscle activities required to execute a reaching movement.

Together, our results from stimulating IntA^{Ucn3} neurons show that the cerebellum may exert its control over movement by influencing the trajectory of the limb through suppression of muscle activity. The effect of timing of stimulation potentially indicates that signals from the IntA may be required to determine proper trajectories early on in the movement, after which other motor areas may play a more influential role. In addition, our results set the stage to identify the anatomical substrates through which the IntA controls movement.

4.4 IntA^{Ucn3} neurons are linked to both corticospinal and rubrospinal pathways

4.4.1 Preferential targeting of IntA^{Ucn3} neurons to the VA-VL thalamus and red nucleus

In order to delineate the circuits through which IntA^{Ucn3} neurons regulate voluntary movement, we performed viral mediated anterograde tracing using adeno associated virus. To ensure cell body and axonal terminal labeling of neurons in the interposed anterior nucleus, we co-injected virus expressing a reporter with virus expressing a reporter linked to channelrhodopsin, AAV2-*hSyn*::mCherry and AAV2-*hSyn*::ChR2-mCherry, into the IntA nucleus (Figure 15A). All tracing experiments were performed on *Rosa*::ChR2-eYFP reporter mice, using this double labelling strategy, we were able to ensure projections in target regions originated specifically from IntA neurons. We obtained reliable labeling of IntA^{Ucn3} neurons using this method (90±1% of all YFP+ cells were labeled with mCherry) (Figure 15A). We labeled non-IntA^{Ucn3} projection neurons (red, mCherry⁺) from the IntA and IntA^{Ucn3} neurons expressing both reporters (yellow, mCherry⁺ eYFP⁺), thus we were able to distinguish between projections from IntA^{Ucn3} neurons and non-IntA^{Ucn3} neurons and control for projections from extracerebellar regions.

To explore potential distinctions in extracerebellar targeting between IntA^{Ucn3} and non- IntA^{Ucn3} neurons, we looked at labeled puncta in the brainstem, midbrain and forebrain. We compared the projections from IntA^{Ucn3} neurons (yellow) and non-IntA^{Ucn3} neurons (red) by determining the number of labeled puncta from each type of neuron. This was expressed as a percentage of total IntA labeling (red and yellow) and depicted as a heat map showing minimum and maximum percentage values across the target regions traced (Figure 15B). In the descending pathway, we found mCherry labeling in the inferior olive dorsolateral part (IOD) and lateral reticular nucleus (LRt) (Figure 15B, C-E, L-N). However, these puncta make up less than 15 % of the total mCherry puncta, and only 25 % of these signals were co-

labeled with IntA^{Ucn3} terminals. Thus, we found that brainstem nuclei were not the primary targets of the IntA^{Ucn3} neurons.

In the ascending pathway, we found mCherry terminals in the contralateral midbrain-thalamic regions. mCherry puncta were found in the magnocellular red nucleus (RMC), intermediate gray region of the superior colliculus (SC), the zona incerta (ZIV) and the ventral anterior-ventrolateral nucleus of the thalamus (VA-VL) (Figure 15B, F-K, O-T). The majority of projections arising from neurons in the IntA nucleus were found in the magnocellular red nucleus (Figure 15B). However, when taken as a percentage of total mCherry puncta, IntA^{Ucn3} projections showed preferential targeting towards the VA-VL nucleus (Figure 15B). Because neurons in the VA-VL nucleus of the thalamus send extensive projections to the cerebral cortex (Kuramoto et al., 2009, 2011), we hypothesize that IntA^{Ucn3} neurons are a part of the cerebello-thalamo-cortical pathway via the VA-VL.

	IOD	ZIV	SC	LRt	VA-VL	RMC
IntA	8±2%	12±1%	13±5%	14±6%	24±7%	30±10%
non-IntA^{Ucn3}	8±2%	12±1%	13±5%	14±6%	24±8%	31±10%
IntA^{Ucn3}	2±2%	10±2%	9±1%	12±7%	35±11%	32±13%

Table 4. Table showing the percentage distribution of puncta from IntA^{Ucn3} non-IntA^{Ucn3} and all IntA neurons. Values are calculated as a percentage of all puncta originating from the three populations in the IntA.

Figure 15. Extracerebellar targets of IntA^{Ucn3} neurons. (A) Strategy for labeling IntA^{Ucn3} neurons and non-IntA^{Ucn3} neurons. (B) Heat map showing the distribution of IntA terminal labeling in target

regions. (C-T) Co-localization of YFP and mCherry puncta in target regions. (C-E) IOD, (F-H) ZI, (I-K) SC, (L-N) LRN, (O-Q) RMC and (R-T) VA-VL. N=3.

4.4.2 Pathways to the spinal cord from *IntA^{Ucn3}* neurons

Motor commands are communicated to the motor neurons via the corticospinal, rubrospinal and the bulboreticulospinal circuits (Alstermark and Isa, 2012). Our viral tracing results revealed that the *IntA^{Ucn3}* neurons could influence voluntary movement through the cerebello-thalamo-cortical and the rubrospinal pathways. Additionally, EMG recordings of extensor muscles suggest that stimulation of *IntA^{Ucn3}* neurons suppresses muscle activities within the skilled reach paradigm. To identify potential sites of this suppression within the rubrospinal and corticospinal pathway, we used a combination of anterograde tracing using AAV and retrograde tracing using a fluorescent dye, fluorogold. Similar to the tracing strategy described earlier, AAV2-*hSyn::mCherry* and AAV2-*hSyn::ChR2-mCherry* were co-injected to the IntA nucleus of the cerebellum of *Ucn3::Cre; Rosa::ChR2* mice to label *IntA^{Ucn3}* terminals in extra-cerebellar target regions (Figure 16A-B). Fluorogold was then injected in the cervical spinal cord (C5-C8) to retrogradely label neurons of the corticospinal and rubrospinal pathways (Figure 16C). Retrogradely labeled cell bodies were found in both the red nucleus and motor cortex (M1).

To determine the neurotransmitter identity of retrogradely labeled cells, we performed immunohistochemical analysis using an antibody against GAD67. In the magnocellular red nucleus, we found that *IntA^{Ucn3}* neurons form terminals on or near fluorogold-labeled cell bodies (Figure 16D-K). Moreover, the fluorogold labeled cells colocalize with GAD67 indicating these neurons may be GABAergic neurons (Fig 16H-K). Thus, *IntA^{Ucn3}* neurons are linked to GABAergic rubrospinal neurons and could be a

plausible mechanism for neurons in the IntA nucleus to exert an inhibitory influence on neurons of the spinal cord.

Figure 16. Neurons in the IntA nucleus are a part of the rubrospinal pathway. (A) Strategy for double tracing from the spinal cord and the IntA. (B) Injection site within the IntA. (C) Injection site within the spinal cord. (D-G) Tracing from the IntA to the RMC. (H-K) Co-localization of fluorogold and GAD67 with terminals originating from IntA^{Ucn3} neurons. N=2.

To test whether IntA^{Ucn3} neurons contribute to corticospinal circuits, we injected a retrograde tracer, cholera toxin B subunit (CTB), to the M1 cortical region in *Ucn3::Cre*; *Rosa::ChR2-eYFP* mice (Figure 17A-B). Retrogradely labeled cell bodies were found in the VA-VL thalamus in close proximity to IntA^{Ucn3} (YFP⁺) terminals, suggesting anatomical connectivity between neurons in the IntA and cortical projecting neurons in the VA-VL

thalamus (Figure 17C-D). In addition, neurons of the VA-VL have been shown to project to layers II-V of motor associated areas of the cortex (Kuramoto et al., 2009). In the motor cortex, corticospinal neurons are known to be innervated by GABAergic interneurons (Guo et al., 2015). Additionally, skilled forelimb movements can be reliably triggered by inhibition of cortical interneurons (Guo et al., 2015). In line with this possibility, our retrograde tracing experiment revealed fluorogold labeled cell bodies in layer V of the motor cortex (Figure 17G). These neurons appear to be surrounded by GAD67 puncta (Figure 17H). Together, we hypothesize that $\text{IntA}^{\text{Ucn3}}$ targeted VA-VL neurons may influence cortical interneurons, to suppress activity in the corticospinal pathway. Thus, indirect excitation of inhibitory cortical interneurons could be an alternative means by which $\text{IntA}^{\text{Ucn3}}$ neurons exert an inhibitory effect on spinal neurons.

Together, these results indicate that $\text{IntA}^{\text{Ucn3}}$ neurons may be an important element of the cerebello-thalamo-cortical pathway, sending input from the cerebellum to alter cortical motor commands. Furthermore, the direct connections with rubrospinal circuits may allow the IntA nucleus to contribute to fast online refinement during movement.

Figure 17. Neurons from the IntA nucleus are also a part of the cerebello-thalamo-cortical pathway. (A-B) Injection of CTB555 into the M1 cortex. (C-D) Retrograde labeling of neurons in the VA-VL. (E-F) Injection of fluorogold into the cervical spinal cord. (G) Retrograde labeling of neurons in the motor cortex. (H) Juxtaposition of fluorogold labeled neurons with GAD67 puncta. N=2.

5. Discussion

The DCN is an important output structure for the cerebellum, and contains neurons that play critical roles for the coordination of motor activities (Ito, 2006; Thach et al., 1992; Uusisaari and Knopfel, 2012). However, the function of neuronal cell types and their effects on motor circuits have not been determined. The results in this thesis identify the properties of genetically defined neurons in the IntA nucleus of the DCN. Through selective targeting of a subpopulation of neurons in the DCN, we found that neurons in the IntA nucleus are required to regulate precision of limb movements during rhythmic and goal-directed behaviors. In addition, we uncovered how the IntA nucleus influences limb movements through regulation of muscle activities and defined potential pathways through which IntA nucleus exerts its influence. We feel that these results provide an important first step to

address the role of the IntA within the diverse network of motor regions involved in precise movement.

5.1 The *Ucn3::Cre* mouse as a genetic tool to investigate the connectivity and function of projection neurons of the IntA nucleus

The identification of mouse lines with specific recombination patterns has been invaluable to the functional characterization of neurons and neuronal pathways. The ability to target specific classes of neurons of the spinal cord, motor cortex and Purkinje cells of cerebellum has revealed specific roles of each cell type in motor control (Azim et al., 2014a; Guo et al., 2015; Hoogland et al., 2015; Kiehn, 2016). The identification of cell type specific tools to target projections neurons in the cerebellum is an important addition to the list of molecular tools to study motor circuits in the mouse brain. Based on molecular characterization of neurons using developmental markers, TBR1/vGlut2, DCN neurons can be identified as glutamatergic projection neurons (Fink et al., 2006). Immunohistochemistry labeling of IntA^{Ucn3} neurons showed co-localization with TBR1 known to mark glutamatergic projection neurons arising from the rhombic lip during cerebellar development. In addition, the recombination capability of the *Ucn3::Cre* mouse in the cerebellum appears to be restricted to the I4 zone of the interposed described by Chung et al and proposed to be innervated by Zebrin negative Purkinje cells from motor domains of the cerebellar cortex (Chung et al., 2009; Voogd, 2014). The electrophysiological profiling of IntA^{Ucn3} confirms these neurons as glutamatergic, large neurons consistent with the known properties of glutamatergic projection neurons in the DCN (Uusisaari and Knopfel, 2011).

Thus the identification of the *Ucn3::Cre* mouse provides a genetic entry point to investigate the function of glutamatergic projection neurons within the DCN. Moreover, the

confinement of recombination specifically to the IntA nucleus of the cerebellum makes this mouse an ideal tool to exclusively study the involvement of the cerebellum in motor behaviors.

The *Ucn3::Cre* mouse line recombines neurons in the amygdala, activation of these neurons revealed an increase in prosocial behavior towards novel conspecifics with no effects on non-social exploratory behavior (Shemesh et al., 2016). This was shown to be mediated by the *Ucn3*-CRF-R2 pathway in the medial amygdala (Shemesh et al., 2016). In the cerebellum, the signaling within the olivo-cerebellar pathway has also been shown to be influenced by CRF (Corticotrophin releasing factor), and CRF-R1/2 (Corticotrophin releasing factor receptor 1/2) are expressed in the interposed nucleus with implications in motor ataxia, raising the possibility that components of the mammalian CRF peptide family, and potentially stress plays a role in motor behaviors. From our characterization of the *Ucn3::Cre* mouse, it remains unclear whether peptide signaling itself may play a role in the motor behavioral phenotypes observed, and is an interesting question to address in future experiments.

5.2 Role of the IntA nucleus in voluntary movements

5.2.1 The IntA nucleus modulates motor behavior

The cerebellar motor syndrome in patients is identified by deficits in gait, control of extremities, eye movements, speech articulation and tremors (Schmahmann, 2004). These symptoms are characterized by changes in rhythm, timing of movement and the force of muscle contraction (Schmahmann, 2004). More recently, using MRI based lesion to symptom mapping, motor deficits have been mapped to restricted cerebellar regions. Lesions restricted to the posterior lobes results in cerebellar cognitive affective disorder, with an absence of

motor symptoms while lesions of the anterior cerebellar lobules including the interposed nucleus, are associated with deficits in skilled motor tasks such as peg placement and finger tapping (Stoodley et al., 2016). While gross dysfunction of the cerebellum produces a number of motor symptoms, such as ataxia and tremors, more restricted lesions produce finer effects and provides insight to the function of specific cerebellar areas (Stoodley et al., 2016; Stoodley et al., 2012).

Consistent with these observations in human studies, our restricted manipulation of the Interposed anterior nucleus did not produce changes in gross motor behavior as observed in the open field and rotarod behavioral paradigms. Instead, a deeper investigation in locomotor and skilled reach paradigm showed deficits in precision of limb movements. Manipulation of the IntA^{Ucn3} neurons resulted in deficits in rhythmic activity, locomotion as well as discrete movements, skilled reach. However, in both instances, mice were able to retain response contingencies but were impaired in the execution of movements, with significant overshoot, or undershoot of forelimb positioning being a characteristic feature. These observations are consistent with patients suffering from cerebellar damage, who show deficits in timing and consistency of limb movement, as well as initiation and termination of movement (Goodkin et al., 1993). In animal studies, muscimol inactivation of the interposed nucleus also results in deficits in paw placement and hypermetria during skilled reach (Bracha et al., 1999). Thus, the accuracy of movements is affected, while gross movement remains intact.

Together, our results support the idea that the interposed nucleus is involved in modulation of movement to attain precision and further denotes this role to specific neurons within the IntA. In addition, studying neurons of the IntA controlling specific features of

movement has the potential to provide the mechanistic details for observed deficits in cerebellar patients.

5.2.2 *IntA^{Ucn3} neurons as an element of cerebellar internal models*

As the final output of the cerebellum, DCN neurons are an important locus of cerebellar function. However, due to the lack of genetic tools to address DCN function, their precise role in various motor behavioral contexts is unclear, and the contribution of each nucleus and the cell types within them have not been defined. In recent years, the study of motor behavior has advanced to include genetic manipulation of cell types that control various aspects of limb movement in diverse regions of the brain. Additionally, the use of standardized behavioral assays for the study of locomotion and skilled reach, combined with high frame rate video recording, enables the functional characterization of neurons in various regions of the brain within the same behavioral context (Azim et al., 2014b; Esposito et al., 2014; Machado et al., 2015). Thus, the advent of the modern genetic techniques combined with kinematic analysis of movements permits a systems level approach to the study of neuronal circuits directing motor behavior. With the addition of the *Ucn3::Cre* mouse, the contribution of the IntA can now be addressed in several motor behaviors.

In the context of voluntary movement, the cerebellum is considered to be a source of control signals, which makes adjustments to the kinematics of the limb (the controlled object) in a variable environment (Ito, 2008; Wolpert et al., 1998). To do this, the cerebellum is thought to acquire an internal model through learning, which may simulate a response to motor commands in order to provide predictive feedback during movement, the forward model (Miall and Wolpert, 1996; Shidara et al., 1993). In recent years, evidence favoring the forward model has shown that the cerebellum in fact predicts the sensory consequences of

motor commands (Bastian, 2006; Requarth and Sawtell, 2014; Therrien and Bastian, 2015). This prediction may then be used to compensate for delays in feedback processing of sensory information and ensure precise movement (Azim et al., 2014a; Wolpert et al., 1998).

Failure of the internal model could result in limb kinematics that are delayed due to feedback signaling, resulting in aberrant trajectories and ill-timed movements. IntA, as an important motor output of the cerebellum, could act as the anatomical substrate that communicates the predictions of internal models to the rest of the brain. Our results from manipulation of IntA^{Ucn3} projection neurons reveal that loss of cerebellar motor signals does result in changes in the trajectories of limb movements, where loss of IntA signals results in overshoot of the limb past a target, and stimulation results in early termination of the limb movement. In principal, these observations could be a result of loss of predictive signaling causing delayed responses (overreach) or artificially simulated signaling resulting in premature responses (under reach). Thus, manipulation of IntA neurons potentially offers an opportunity to identify the role of predictive internal models in a given behavioral context and highlights the need for control signaling to ensure precise movements.

5.2.3 Role of the IntA in regulating kinematic parameters of voluntary movement.

The cerebellum has been widely implicated as the source of modulatory signals for voluntary movement, however due to the lack of tools to manipulate DCN outputs, the exact nature of these signals has been difficult to characterize (Azim and Alstermark, 2015). The cerebellum has previously been shown to have influence over limb kinematics through Purkinje cell manipulation (Hoogland et al., 2015; Machado et al., 2015). Locomotion in the mutant mouse *tottering*, *tg/tg*, is associated with increased swing durations, and Purkinje cell complex spikes are reduced and delayed from movement onset (Hoogland et al., 2015).

Furthermore, activation of Purkinje cells results in varying responses in limb movements depending on behavioral state (Hoogland et al., 2015).

Complementary to our results from IntA stimulation, during locomotion, where activation of IntA^{Ucn3} neurons resulted in increased y displacement from the base, stimulation of Purkinje cells resulted in inhibition of movement. In *pcd* (Purkinje cell degeneration) mice, interlimb co-ordination during locomotion is also impaired, and similar to IntA^{Ucn3} manipulation, y displacement was specifically deficit while x displacement remained intact (Machado et al., 2015). Thus, within locomotor paradigms, our results from IntA manipulation generally agree with data from Purkinje cell manipulation. Both approaches produce changes in y positioning of the limb during movement. However, by performing specific manipulations within the IntA, we were able to characterize the precise effect of IntA neurons on limb movement and localize the kinematic function of the DCN to IntA neurons.

The interposed nucleus is implicated in skilled reach through reversible inactivation studies. Inactivation of the anterior and posterior interposed both resulted in deficits in reaching behavior while grasping and retrieval were left intact (Bracha et al., 1999; Martin et al., 2000). While these studies provide important information about the role of the interposed nucleus in reaching behavior, muscimol inactivation is unable to identify the role of cell types within the interposed. Ablation of IntA^{Ucn3} neurons revealed specific deficits in reaching behavior with a tendency towards hypermetria, indicating that the IntA controls the termination of reaching movements and is important for endpoint accuracy. Photostimulation of IntA^{Ucn3} neurons also revealed changes in the distance travelled and duration of reaching during the skilled reaching behavior.

Although stimulation resulted in early termination of the reach behavior, the time taken from initialize to reach events was significantly increased in photostimulated mice. This indicates that signals from the IntA may also cause a slowing down of limb movements. Studies of forelimb movements in cats have shown that microstimulation of the interposed nucleus evoked forelimb movements which serve to move the forelimb away from a stimulus (Ekerot et al., 1991). In the context of reaching movements, the authors proposed that these movements may result in a braking effect during the extension phase of the limb movement, which acts to avoid accidental skin contact during movement (Ekerot et al., 1997). Consistent with this hypothesis, photostimulation of IntA^{Ucn3} neurons resulted in a significant slowing down of the forelimb during reaching and could be a result of false anticipation of obstructions along the trajectory. Thus, the IntA^{Ucn3} neurons may be important aspect of the motor system that makes feedforward adaptations during goal-directed limb movement.

5.2.4 IntA^{Ucn3} neurons may be involved in predictive signaling of limb positions

The direction of reaching errors in IntA^{Ucn3} manipulated mice reveal clues as to their potential role in predictive signaling of limb positions. Ablation and optogenetic stimulation of IntA^{Ucn3} neurons both reveal that the IntA specifically affects the reach phase during skilled reaching and in general affects forward projecting limb trajectories. Through the IntA the cerebellum may signal the predicted position of the limb, in order to derive motor commands for the next phase of the trajectory (Tseng et al., 2007). A lack of this signal, and delayed sensory feedback in ablated mice may have resulted in motor signals extending the arm further than necessary resulting in hypermetric movements. On the other hand, an optogenetically-simulated signal may have resulted in early predicted feedback and motor commands which end arm extension resulting in failure to reach the pellet. Thus, the

observed changes in reaching kinematics during voluntary movement, could be a result of incorrect predictions in the position of the arm during the reach.

5.2.5 IntA^{Ucn3} neurons specifically affects projecting movement trajectories

The motor cortex generates commands for skilled movement in fixed action sequences (Guo et al., 2015). Inhibition of cortical neurons has been shown to disrupt all aspects of skilled reaching. Post stimulation of cortical interneurons, deficits are observed in reach, grasp and retrieval indicating that the cortex codes for action patterns for voluntary movements (Guo et al., 2015). While the cerebellum is thought to signal changes in motor commands, it was unclear whether the cerebellum is able to generate motor commands for movement (Shidara et al., 1993). Although manipulation of IntA^{Ucn3} neurons caused changes in kinematics during reaching and reduced success rate; in contrast to cortical inhibition, the ability of the forelimb to go through the stages of reach, grasp and retrieve was not hindered.

The timing of stimulation of IntA^{Ucn3} neurons, relative to the movement produced differing effects on limb positioning in locomotor and skilled reaching. Photostimulation during the initial stages of the reach or swing phase during locomotion resulted in altered limb trajectories, while stimulation at the endpoint of the reach produced no effect on subsequent grasp or retrieving movements. The most significant effects were observed when light onset occurred in the window between initiation of limb movement and halfway through the extension of the limb. Moreover, despite stimulation continuing beyond this phase, no changes were observed in the later phases of movement. Thus, photostimulation of IntA^{Ucn3} neurons at various stages of limb movement revealed that the IntA nucleus works within a specific time window to affect movement. The existence of this time window implies that the IntA plays a role in setting up projecting limb trajectories, after which other areas of the

cerebellum may be more important. Moreover, manipulation of IntA^{Ucn3} neurons was unable to change the complete action sequence of skilled reaching, but was specific to the forward projecting part of the movement. This implies that the IntA nucleus communicates information specifically related to the dynamic properties of limbs and does not initiate an action sequence (Ebner et al., 2011). Thus, the IntA is unlikely to influence a sequence of movements and instead affects movement in a modular fashion.

5.2.6 IntA^{Ucn3} neurons influence extensor muscle activities

In multi-joint movement control, the cerebellum has been shown to have an effect on timing and magnitude of muscle activity in reversible inactivation studies of the lateral nucleus and in patients with cerebellar patients (Flament and Hore, 1986; Hallett et al., 1975). However, the link between neurons of the interposed nucleus and their potential effect on muscle activities has not been addressed. Reaching movements in the skilled reaching paradigm can be broken down into an initial shoulder flexion to lift the forelimb, extension at the elbow and wrist during reach and flexion of the wrist in order to grasp (Lacquaniti and Soechting, 1982). During the reach, elbow and forearm extensors muscles are active till the grasp movement. Clinical studies in patients with cerebellar dysmetria have shown that the patients exhibit prolonged flexion movements and delayed extensor activities, but whether the IntA is able to affect the activity of specific muscle groups during forelimb reaching has not been addressed (Hore et al., 1991; Shakkottai, 2014).

We found that optogenetic stimulation of IntA^{Ucn3} neurons caused a significant attenuation of extensor muscle activity, consistent with the reduced extension of the forearm observed in skilled reaching. Thus, the IntA may affect flexion of the limb through attenuation of extensor muscle groups. Interestingly, IntA^{Ucn3} stimulation was able to reduce

muscle activity of both the tricep muscle as well the ECU indicating that the IntA does not selectively control individual muscles, but may affect higher order neural commands affecting co-activation of muscles. This, in turn, could be due to convergence of Purkinje cell inputs representing individual motor domains (Hoogland et al., 2015; Uusisaari and De Schutter, 2011). Thus, our results point to the existence of a functional zone in the DCN, that influences extensor muscle synergies during forelimb extension movements (Thach et al., 1993).

Although we show that IntA^{Ucn3} neurons are able to reduce extensor activity, we were unable to resolve whether the IntA may also simultaneously increase flexion movements, though flexor activation. While this remains a question, our results from IntA manipulation within the skilled reach behavioral paradigm reveal that, neurons of the IntA inhibit extension of the forelimb and may activate neuronal pathways that inhibit extensor muscles synergies within the forelimb.

5.3 The contribution of IntA^{Ucn3} neurons to motor pathways

5.3.1 Differential projections from IntA neurons may distinctly affect movement

The interposed nucleus of the cerebellum in the past was thought to primarily project to the contralateral magnocellular nucleus and was proposed to influence movement through the rubrospinal tract (Keifer and Houk, 1994). However, recent studies as well as our present study show that the IntA send significant projections to the cerebello-thalamo-cortical pathway (Gao et al., 2016). Moreover, we found that potential distinctions exist within glutamatergic projection neurons of the DCN and IntA^{Ucn3} neurons may be distinguished by their preferential influence within the cerebello-thalamo-cortical pathway. Cell type distinctions based on efferent projection pathways have recently been found to exist in

neurons of the vestibular nuclei. LVN_E (Lateral vestibular nucleus, Extensor) neurons projecting to the spinal cord influence extensor muscle activity, while LVN_C (Lateral vestibular nucleus, Co-activation) neurons projecting to the pontine reticular nucleus influence co-activation of flexors and extensors (Murray et al., 2018). Thus, distinctions in projections influence function and can distinguish cell types within the same nucleus. Although, IntA^{Ucn3} neurons show similar extra-cerebellar targets as non-IntA^{Ucn3} neurons projection neurons, the preferential targeting within cerebello-thalamo-cortical pathways, point to a role in influencing signals to the motor cortex. Thus, neurons of the IntA may differentially affect movement through the cerebello-thalamo-cortical pathway and the rubrospinal pathway.

5.3.2 Significance of cerebellar projections to the red nucleus

The IntA nucleus sends significant projections to the RMC, however the functional significance of these signals has not been fully addressed. Through retrograde tracing studies and electrophysiological circuit mapping, it has been proposed that the magnocellular red nucleus provides a source of corollary discharge motor signals to the IntA to facilitate kinematic predictions within the IntA (Beitzel et al., 2017; Houck and Person, 2015). In addition, axons from the RMC form mossy fiber input within the IntA and have the potential to influence motor outputs from the cerebellum as well as nucleocortical feedback signals, furthermore, these signals were found to be reciprocal in nature, with the IntA projecting back to the RMC (Beitzel et al., 2017). Our tracing experiments from the IntA indicate that IntA^{Ucn3} neurons could be a subset of the RMC reciprocal neurons. Additionally, we found that IntA signals to the red nucleus may directly target rubrospinal pathways that influence spinal neurons. Retrograde labeling from the cervical spinal cord revealed cell bodies showing potential synapses with IntA^{Ucn3} axon terminals (Figure 16H-K). Surprisingly, spinal

projecting neurons in the red nucleus were found to be putative gabaergic cells (Figure 16H-K). Thus IntA activation could in principle, inhibit rubrospinal pathways to reduce excitatory signals to spinal cord and reduce muscle activation in the forelimb (Llinas, 1964). Although speculative, our results highlight a potentially novel pathway through which the IntA induces an inhibitory effect on spinal neurons.

5.3.3 Functional significance of cerebello-thalamic projections

The IntA^{Ucn3} neurons influence the motor cortex through indirect projections to the VA-VL nucleus of the thalamus. Neurons from the VA-VL are thought to innervate neurons of the deep layers of the motor cortex (Kuramoto et al., 2009). Stimulation of the DCN and the VA-VL result in early post synaptic potentials within these layers of the motor cortex (Yamamoto et al., 1979). Thus, IntA targets within the VA-VL may influence fast conducting fibers to the cortex. Neurons of the motor cortex show context dependent firing patterns and motor command pathways for skilled movement show preferential recruitment of short latency corticospinal effector pathways (Miri et al., 2017). IntA^{Ucn3} neurons projecting to the VA-VL could play a role to influence cortical population activity that selectively triggers short latency pathways for skilled movement. Together, recruitment of fast conducting pathways in the motor cortex and VA-VL by IntA neurons could facilitate rapid changes in motor commands required for online modulation of movement.

5.3.1. The IntA nucleus may influence fast feedback pathways to the spinal cord.

In the execution of skilled movements, the cerebellum is thought to receive internal copy commands and perform short timescale adjustments to movement to compensate for delayed sensory feedback (Azim and Alstermark, 2015). Internal copy commands have been shown to originate from propriospinal neurons, Chx10+ neurons in C3 and C4 segments of

the spinal cord (Azim et al., 2014b). Signals from Chx10⁺ neurons are relayed to the lateral reticular nucleus which provides major mossy fibre input to the cerebellum (Alstermark and Ekerot, 2015). Disruption of this pathway has been shown to result in deficits in smooth movement during skilled reaching and is proposed to be due to a disruption of fast feedforward signals from the cerebellum to the spinal neurons, however the circuits through which the cerebellum may influence spinal circuits remain unknown (Azim et al., 2014b). Our results show that the IntA nucleus influences skilled movement and could through circuits to the spinal cord influence fast feedforward signaling during reaching.

6. Conclusion

In conclusion, through the functional characterization $\text{IntA}^{\text{Ucn3}}$ neurons presented in this thesis, we have identified tools and developed strategies to study the role of the deep cerebellar nuclei in voluntary movements. We found that the neurons in the IntA nucleus are able to regulate forelimb movements in locomotor and skilled reach paradigms indicating that the IntA nucleus controls diverse behaviors. Moreover, we found that the IntA controls specific phases of movement in a context dependent manner, i.e the reach phase during skilled reaching and the swing phase during locomotion. In addition, we think that the IntA may influence muscle synergies to mediate limb movement, potentially discovering an important functional principle of DCN neurons. Thus, with this work, we have begun to uncover the role of circuits originating in the IntA and identified the IntA as the cerebellar nucleus important for the execution of skilled movement.

7. Future Directions

While these results provide details about the functional relevance of the IntA nucleus, many important questions still remain. For instance, whether potential functional distinctions in $\text{IntA}^{\text{Ucn3}}$ neurons versus non- $\text{IntA}^{\text{Ucn3}}$ neurons exist is still not known. Additionally, it is not clear whether the IntA nucleus controls different aspects of movement through the cerebello-thalamo-cortical pathway versus the rubrospinal pathway. With the $\text{IntA}^{\text{Ucn3}}$, there may be a slight preference towards the VA-VL thalamus, which potentially indicates subtle distinctions in targeting between projection neurons of the IntA. Thus, additional mapping and behavioral analysis are needed to define the direct and indirect connectivity between IntA nucleus and the spinal cord and mechanisms underlying how neurons in the IntA nucleus regulate muscle activities under different behavioral states. Furthermore, while it appears that the IntA neurons have diverging cells targeting several sub-cortical regions, at this stage it is

not clear what the role of each of these projections are, or if there is any preference for recruitment of each pathway based on task demands. Our work here, we hope, is an important first step to uncover the role of diverging circuits from the cerebellum.

Our future work, attempts to address these questions, by 1> using Cre-On and Cre-Off viruses to distinguish the role of IntA^{Ucn3} neurons vs non-IntA^{Ucn3}, 2> Using retrograde viruses to specifically label and manipulate projections from the IntA to the VA-VL versus projections to the Red nucleus. 3> Using retrograde tracers in the spinal cord, we would also identify neurons in the interposed which may have direct projections to the spinal cord. 4> Moreover, we would like to elucidate the potentially distinct roles of ascending and descending circuits from the Interposed nucleus by manipulating traced neurons in various motor tasks and addressing specific behavioral questions such as online correction during movement, motor adaptation and motor learning.

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