Distinct subpopulations of neurons in the deep cerebellar nuclei regulate limb movements during locomotion

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**NANYANG
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SINGAPORE

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nuclei regulate limb movements during locomotion**

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**Interdisciplinary Graduate School
Institute for Health Technologies
2019**

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

2019

Statement of Originality

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

18 Aug 2018

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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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Asst Prof Albert I. Chen

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal(s) where I was the first and/or corresponding author.

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The contributions of the co-authors are as follows:

- Asst Prof Albert I. Chen introduced the projection direction, contributed experimental ideas and edited the manuscript drafts.
- Prof George Augustine supervised the electrophysiological experiments.
- Asst Prof Albert I. Chen, Aloysius Low, Ayesha Thanawalla and myself prepared and edited the manuscript drafts.
- I co-designed the locomotion experiments with Kelly Wong and Dr Martesa Tantra.
- I performed the optogenetic implantation in mice that are used for locomotion experiments.
- I conducted the locomotion experiments with Kelly Wong and the data analysis for locomotion is analyzed together with Kelly Wong and Ayesha Thanawalla.
- Aloysius Low introduced the transgenic mouse line that is pivotal for the study.
- Aloysius Low and Ayesha Thanawalla performed tracing experiments, histological assays and data analysis.
- Ayesha Thanawalla and Dr Martesa Tantra designed the skilled reaching tests.
- Ayesha Thanawalla performed the ablation experiments, the skilled reaching tests and the data analysis.
- Dr Kim Jinsook performed all the electrophysiological recordings and the data analysis

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Abbreviations

AAV	Adeno-associated virus
AGRP	Agouti-related protein
AP	Action potential
Arch	Archaeorhodopsin-3
B	Basket cell
CF	Climbing fiber
cFL	Contralateral forelimb
cHL	Contralateral hindlimb
CM	Centromedial
CR	Calretinin
CRF	Corticotropin-releasing factor
DCN	Deep cerebellar nuclei
DIG	Digoxigenin
DTR	Diphtheria toxin receptor
eYFP	Enhanced yellow fluorescent protein
fMRI	Functional magnetic resonance imaging
Gad67	Glutamic acid decarboxylase 67kDa
GFP	Green fluorescent protein
Go	Golgi cell
Gr	Granule cell
hSyn	Human synapsin

iFL	Ipsilateral forelimb
iHL	Ipsilateral hindlimb
Int	Interposed deep cerebellar nucleus
IntA	Interposed anterior deep cerebellar nucleus
IO	Inferior olive
Lat	Lateral deep cerebellar nucleus
Med	Medial deep cerebellar nucleus
ML	Molecular layer
NC	Nucleocortical
NC-MF	Nucleocortical-mossy fiber
nNOS	Neuronal nitric oxide synthase
PBS	Phosphate buffered saline
PC	Purkinje cell
Pcd	Purkinje cell degeneration
Pcp2/L7	Purkinje cell protein 2
PF	Parallel fiber
PF	Paraflocculus
PFA	Paraformaldehyde
PL	Purkinje layer
PN	Pontine nucleus
RMC	Magnocellular region of red nucleus
RN	Red nucleus
S	Stellate cell

SC	Spinal cord
Sim	Simple lobule
SMI32	Neurofilament heavy polypeptide
SSC	Saline-sodium citrate
Tbr1	T-box brain 1
Tfap2A	Transcription factor AP-2 α
Thal	Thalamus
Ucn3	Urocortin 3
vGluT2	Vesicular glutamate transporter 2
VL	Ventrolateral
VM	Ventromedial
wm	White matter
ZI	Zona incerta

Abstract

The cerebellum controls movement and motor coordination by transmitting integrated sensorimotor signals through the deep cerebellar nuclei (DCN). The Purkinje cells in the cerebellar cortex send modulated signals to a diverse population of cell types within the DCN, but very little is known about how each individual cell type contributes to regulation of motor function. In this study, we identified a genetically defined subpopulation of neurons in the interposed anterior nucleus (IntA). We characterized these neurons and found that based on their electrophysiological and molecular properties, they belong to a subset of glutamatergic neurons within the IntA nucleus. To determine the function of these neurons, we selectively expressed Channelrhodopsin 2 (ChR2) in these neurons and optogenetically activated them while the mice performed locomotor task.

To analyze consequences of the manipulation of neurons in the IntA nucleus on locomotion, we designed a walkway that permits detailed analysis of walking kinematics and concurrent optogenetic manipulation in the mice. We show that photostimulation of these neurons did not disrupt the cadence, stance duration, swing duration, stride length and trajectory length, but significantly increased the y-displacement and y max of ipsilateral limb positioning of the mice. Furthermore, we have selectively expressed ChR2 within glutamatergic neurons of the medial (Med) nucleus and compared the limb kinematics of these mice with those with IntA manipulation. Collectively, photostimulation of glutamatergic neurons in the Med nucleus disrupted almost all the limb kinematics of ipsilateral forelimbs and hindlimbs, different than what was observed for photostimulation of neurons in the IntA nucleus. Our studies revealed that there are molecularly distinct subsets of neurons in the DCN, and that subpopulations of

glutamatergic neurons from each subnuclei of the DCN regulates different aspect of limb movement.

Chapter 1: Functions and Organization of the Cerebellum

Introduction

This thesis aims to explore the functional contribution of glutamatergic neurons within the deep cerebellar nuclei towards regulated motor function. Despite efforts in studying the cerebellum primarily through investigation of the function of an important class of cerebellar neurons, the Purkinje cells (PCs), there is limited understanding of how specific populations of glutamatergic projection neurons in distinct cerebellar subnuclei modulates limb movements during locomotion. This introduction will give a general overview of our current understanding of local and long-range cerebellar circuitry, and associated dysfunction. The research aim that will be addressed in this thesis will be introduced, and recent findings in elucidating the motor functions associated with neurons in the deep cerebellar nuclei (DCN) through either direct or indirect methods will be described in detail.

Overview of the function of the cerebellum and associated movement disorders

The cerebellum is an important brain region which controls movement and motor coordination (Eccles, 1967; Takahashi and Linden, 2000). Several types of motor movements are closely associated to cerebellar functions (Manto *et al.*, 2012). Lesions of cerebellar regions have been shown to disrupt eye movements in both humans and primates (Baier *et al.*, 2009; Hiramatsu *et al.*, 2008; Ohki *et al.*, 2009). Patients with deficit in smooth pursuit eye movement showed similar damage within the uvula and vermal region of the cerebellum (Baier *et al.*, 2009). Cerebellar damage has also been shown to disrupt the regulation of oculomotor movements. Patients with cerebellar damage/abnormalities displayed impaired speed control (Holmes, 1917; Lechtenberg and Gilman, 1978). Speech function was affected when the superior region of the left cerebellar hemisphere is damaged (Lechtenberg and Gilman, 1978). Apart from

oculomotor and speech control disruption, cerebellar disorders have also been associated to loss of proper limb control.

Patients with cerebellar atrophy showed increased static grip forces when holding an object. (Nowak *et al.*, 2002). Most commonly, cerebellar dysfunction has been associated to aberrant voluntary limb movements (Flament and Hore, 1986; Gilman, 1969; Goodkin *et al.*, 1993). Necrosis of anterior regions of the cerebellar cortex due to infarction has been shown to impair reaching movements (Goodkin *et al.*, 1993). Reversible lesions of the DCN has been demonstrated to disrupt agonist and antagonist muscle activity, which are essential for controlling arm movements (Flament and Hore, 1986). Patients with degenerative cerebellar disease have been reported to have deficit in intra-limb coordination (Ilg *et al.*, 2007). Taken together, the cerebellum governs a myriad of motor functions and cerebellar damage can deprive individuals of the ability to perform important tasks.

Distinct neuronal subtypes and connectivity within the cerebellar cortex

There are multiple experimental studies that have further explored cerebellar contribution towards motor control. The subsequent cerebellar studies that will be reviewed can be aided by having a better understanding of the cerebellar cytoarchitecture. The refinement of motor commands are governed by both the cerebellar cortex and the deep cerebellar nuclei, which are the main structures of the cerebellum (Figure 1A) (Sillitoe and Joyner, 2007). The cerebellar cortex can be further subdivided into three distinct layers with the outermost layer, the molecular layer, containing inhibitory stellate and basket cells (Figure 1) (Sillitoe and Joyner, 2007). These interneurons send inhibitory inputs to PCs within the Purkinje layer (PL), which is adjacent to

the molecular layer (ML) (Figure 1) (Sillitoe and Joyner, 2007). The innermost layer of the cerebellar cortex is the internal granular layer (IGL).

Within the IGL, granule cells receive inhibitory input from Golgi cells and send excitatory input through parallel fibers to stellate and basket cells, Golgi and PCs (Figure 1) (Sillitoe and Joyner, 2007). The PCs also receive excitatory input from climbing fibers that originate from the inferior olive (IO, Figure 1) (Sillitoe and Joyner, 2007; Hioki *et al.*, 2003). The cerebellar cortex then send the integrated signals through PCs to the deep cerebellar nuclei (DCN, Figure 1) (Sillitoe and Joyner, 2007). Previous studies of patients with cerebellar lesions have not been able to address which cell types within a specific cerebellar region are disrupted and were not able to distinguish whether the motor abnormalities observed is due to the loss of multiple cell types or the ablation of a single cell type that lead to perturbation of the cerebellar regulatory system.

Figure 1. The organization and connectivity of the cerebellum

3D model of a mouse brain obtained from Brain Explorer, showing a section at the posterior part of the brain (top-left panel) being selected to depict the cerebellum (bottom-left panel, coronal section). The coronal cerebellar section depicts the cytoarchitecture of the cerebellar cortex (small dotted box) and the subnuclei of the DCN (large dotted box). Cytoarchitecture of the cerebellum (right panel) consist of stellate and basket cells in the molecular layer and they inhibit Purkinje cells in the PL. Golgi cells in the IGL receive inputs from DCN neurons, excitatory inputs from granule cells and in turn provide inhibitory feedback to granule cells. Granule cells receive excitatory inputs from mossy fibers that originate from pre-cerebellar regions (e.g. VN, PN, SC, etc.) and nucleo-cortical inputs from DCN neurons. Granule cells branch into parallel fibers within the ML to excite Purkinje cells, which also receive excitatory inputs from climbing fibers originate from the IO. Purkinje cells inhibit DCN neurons and the DCN neurons project to several extracerebellar regions like the VN and IO (Franklin and Paxinos, 2013; Sillitoe and Joyner, 2007). B, basket cell; DCN, deep cerebellar nuclei; Go, Golgi cell; Gr, granule cell; IGL, internal granular layer; IntA, anterior part of interposed DCN; IO, inferior olive; Lat, lateral DCN; Med, medial DCN; mf, mossy fiber; P, Purkinje cell; PF, paraflocculus; PL, Purkinje layer; PN, pontine nuclei; SC, spinal cord; SIM, simple lobule; wm, white matter

The DCN can be subdivided into lateral, interposed and medial nucleus (Lat, Int and Med) in the rodents by histological and molecular distinctions (Figure 1) (Chung *et al.*, 2009; Franklin and Paxinos, 2013). Besides receiving inhibitory inputs from the PCs, the DCN receive excitatory inputs from collaterals of mossy fibers (MF) and climbing fibers (Dezeew and Berrebi, 1995; Uusisaari and Knopfel, 2011; Hioki *et al.*, 2003). Based on morphological molecular and electrophysiological profiles, a heterogeneous population of neurons can be found in the DCN (Uusisaari and Knopfel, 2012). Six major neuronal subtypes have been identified: glutamatergic, γ -aminobutyric acid-ergic (GABAergic) and glycinergic interneurons and projection neurons (Figure 2) (Uusisaari and Knopfel, 2008; Bagnall *et al.*, 2009; Chan-Palay, 1977; Chen and Hillman, 1993). The glutamatergic projection neurons send projections to motor relay centres, mainly the thalamus and red nucleus (Figure 2) (Houck and Person, 2015). The cerebellum sends inhibitory feedback input to the IO through GABAergic projection neurons

(Figure 2) (DeZeeuw *et al.*, 1997). Despite the general characterization of the neurons, whether there are further subpopulations within each major neuronal subtype has not been determined.

In addition to output connectivity mediated by the DCN, the cerebellar cortex has also been shown to receive both glutamatergic and GABAergic inputs from nucleocortical projection neurons in the DCN (Ankri *et al.*, 2015; Houck and Person, 2015). Glycinergic neurons in the Med make functional projections to vestibular and reticular formation while glycinergic neurons in the Lat have been found to send projections to the IGL (Bagnall *et al.*, 2009; Houck and Person, 2015). There are also GABAergic interneurons that could elicit glycinergic properties and receive weak inhibitory inputs from the PCs (Figure 2) (Uusisaari and Knopfel, 2008). The multiple inputs received by the DCN and the diverse population of neurons suggests that the DCN must perform a certain degree of signal integration before sending modulated instructions for accurate motor outputs. With the presence of a myriad of neuronal subtypes in the DCN, there is an urgency to employ neuronal subtype-specific mouse genetics together with the use of optogenetics to investigate specific motor functional significance of these individual neuronal subpopulations.

Figure 2. Neuronal subpopulations and circuitry of the DCN

Glutamatergic projection neurons in the DCN project several pre-motor regions and provide excitatory inputs as NC-MF to granule cells and Golgi cells in the cerebellar cortex. GABAergic/glycinergic neurons provide inhibitory input to Golgi cells in the cerebellar cortex. GABAergic projection neurons send inhibitory feedback to the IO. The GABAergic/glycinergic interneurons and putative glutamatergic interneurons inhibit or excite DCN neurons, respectively (Ankri *et al.*, 2015; Bagnall *et al.*, 2009; Houck and Person, 2015; Teune *et al.*, 2000; Chung *et al.*, 2009; Uusisaari and Knopfel, 2008; Uusisaari *et al.*, 2007). Go, Golgi cell; Gr, granule cell; IO, inferior olive; LRN, lateral reticular nucleus; RN, red nucleus; Thal, thalamus

Characterization of the role of cerebellar circuits and neuronal subtypes with optogenetics

A better understanding of the functional importance of each individual neuronal subtype of the cerebellum, requires strategies for selective labeling, monitoring and manipulation.

Several transgenic mice have been developed to provide manipulation of specific neuronal subpopulations within the cerebellar cortex through optogenetics (Barski *et al.*, 2000; Hull and Regehr, 2012). Photostimulation of molecular layer interneurons in the cerebellar cortex was achieved using the parvalbumin promoter (Hull and Regehr, 2012). Optogenetics is a spatially and temporally precise technology that uses naturally occurring opsins (Boyden *et al.*, 2005). These opsins can be genetically introduced into neurons and provide an artificial mean to manipulate specific neuronal subpopulations (Boyden *et al.*, 2005). For instance, a transgenic mouse that selectively express light-sensitive proteins in PCs permit indirect perturbation of DCN neurons and can provide further insight into how the DCN controls motor functions.

Channelrhodopsin (ChR2) is a light-sensitive cation channel that can be expressed in neurons and cause neuronal depolarization with millisecond temporal resolution upon presentation of a blue light (wavelength ~470 nm) (Figure 3) (Boyden *et al.*, 2005). On the other hand, archaerhodopsin-3 (Arch) is a proton pump sensitive to yellow light (wavelength ~570 nm) that silences neurons upon photostimulation (Figure 3) (Chow *et al.*, 2010b). Therefore, using the appropriate wavelength of light, photostimulated neurons that express ChR2 will result in neuronal activation while photostimulated neurons that express Arch neurons will result in neuronal inhibition. Thus, the combination of optogenetics with spatial and temporal precision of manipulation and neuronal subtype-specific transgenic mouse lines represents a powerful strategy to examine the functional properties of specific neuronal subtypes in the cerebellum that contributes to regulation and refinement of locomotor movement.

Figure 3. Activation and inactivation of neural activity using opsins

Channelrhodopsin, a non-selective cation light-sensitive channel, opens upon receiving photostimulation at a wavelength of about 470 nm, which permits influx of cations from extracellular space (top). Light that has a wavelength of about 570 nm is used to photostimulate Archaerhodopsin-3 (and intracellular protons will be pumped out through the channel (bottom)) (Boyden *et al.*, 2005; Chow *et al.*, 2010b). ChR2, channelrhodopsin; Arch, archaerhodopsin.

Elucidation of the role of DCN neurons in motor control through indirect optogenetic manipulation

Purkinje cells regulate eye and whisker movements

Several optogenetic studies have attempted to investigate the consequences of perturbing specific neuronal population of the cerebellar cortex on motor behaviours. Photostimulation of stellate and basket cells, which in turn results in inhibition of PCs and disinhibition of DCN neurons, induced eyeblink movements in mice (Figure 4) (Heiney *et al.*, 2014). In addition to eyeblink movements, photostimulation of PCs through the use of a transgenic mouse line that uses the *Purkinje cell protein 2* (*Pcp2/L7*) promoter to drive expression of ChR2 specifically in PCs resulted in perturbation whisker movements (Figure 4) (Proville *et al.*, 2014). However, the modulated activity of the cerebellum is also required for additional sophisticated and complex motor functions that are beyond eye and perturbation of whisker movements.

Figure 4. Indirect modulation of the activity of DCN neurons affects eyeblink and whisker movements

(A) Schematic illustrating the photostimulation of stellate and basket cells in Sim (top) and Purkinje cells in Crus 1 (bottom). (B) Schematic showing photostimulation of stellate and basket cells induces eyeblink response (top) and optogenetic activation of Purkinje cells induce whisker movements (Heiney *et al.*, 2014; Proville *et al.*, 2014). ChR2, channelrhodopsin 2; nNOS, neuronal nitric oxide synthase; Pcp2, Purkinje cell protein 2; Sim, simple lobule.

Coordinated movement is dependent on modulated Purkinje cell activity

The firing rates of PCs and neurons in the DCN are different between mice that are resting and mice that are running on a treadmill (Sarnaik and Raman, 2018). Both PCs and DCN neurons showed higher firing rate when the mice started running (Sarnaik and Raman, 2018). Therefore, the perturbation of the regulated communication between PCs and DCN neurons are presumed to disrupt locomotion. Numerous studies have examined the relevance of PCs in regulating locomotion. Complete elimination of PCs achieved by using the *Purkinje cell degeneration (Pcd)* mice, resulted in observable ataxic and uncoordinated movements (Machado *et al.*, 2015). However, the complete ablation only demonstrated that PCs in the cerebellum are required for locomotion but cannot discern whether perturbation PCs in a specific lobule is sufficient to disrupt limb movements.

Limb movements evoked by manipulation of Purkinje cells

Other studies have examined the perturbation of PCs in modulating limb movement and locomotion by using *Pcp2::Cre* transgenic mice (Hoogland *et al.*, 2015; Lee *et al.*, 2015; Sarnaik and Raman, 2018; Witter *et al.*, 2013). Upon crossing *Pcp2::Cre* mice with a *Rosa26::lsf-ChR2*-enhanced yellow fluorescent protein (eYFP) (ChR2-eYFP) or *Rosa26::lsf-Arch*-green fluorescent protein (GFP) (Arch-GFP) mice, ChR2-eYFP or Arch-GFP was expressed selectively in PCs (Lee *et al.*, 2015). Optogenetic activation of the PCs specifically in the simple (Sim) lobule of the cerebellum of *Pcp2::Cre*; ChR2-eYFP resulted in inhibition of DCN neurons and induce ipsilateral forelimb movement upon termination of photostimulation, which is time-locked to the rebound firing of DCN neurons (Figure 5) (Lee *et al.*, 2015). Another important finding about the contribution of PCs in eliciting forelimb movement was made when optogenetic inhibition of PCs within the same lobule of *Pcp2::Cre*; Arch-GFP mice resulted in increased DCN neuronal activity and induce ipsilateral forelimb movements during the onset of photostimulation (Figure 5) (Lee *et al.*, 2015). Thus, there is ample evidence that the perturbation of DCN neuronal activity through optogenetic manipulation of PCs disrupts ipsilateral forelimb movement, but whether the hindlimb is disrupted was not examined.

Figure 5. Indirect activation or inactivation of DCN neurons induces limb movement

(A) Schematic showing photostimulation of Purkinje cells that either express ChR2-eYFP or Arch-GFP. (B) Schematic ipsilateral forelimb movement upon offset of Purkinje cell activation or during onset of Purkinje cell

inactivation (Lee *et al.*, 2015). Arch, archaerhodopsin-3; ChR2, channelrhodopsin 2; Sim, simple lobule; Pcp2, Purkinje cell protein 2.

A recent study revealed that photostimulation of PCs in the Sim lobule using the *Pcp2::Cre; ChR2-tdTomato* mice can also influence ipsilateral hindlimb movements and reduce DCN neuronal firing rates (Figure 6A & B) (Sarnaik and Raman, 2018). PCs from different cerebellar lobules can converge to inhibit a single subnuclei of the DCN (Sugihara *et al.*, 2009). Together, these studies raise the possibility that DCN neurons are not exclusively inhibited by PCs input from the Sim lobule of the cerebellar cortex, but that the coordinated inhibition of distinct neuronal subpopulations within the DCN by PCs from various lobules contribute to the modulation of locomotion.

Figure 6. Disruption of the activity of DCN neurons through Purkinje manipulation perturbs locomotion

(A) Schematic showing photostimulation of Purkinje cells at the base of Sim (top) and illustration showing the side of a head-fixed mouse in a treadmill setup used for recording the effects of photostimulation during locomotion (bottom). (B) Ipsilateral hindlimb movement is disrupted during Purkinje cell activation by photostimulation (Sarnaik and Raman, 2018). (C) Schematic showing photostimulation of Purkinje cells in Lobule 5 and 6. (D) Illustration showing a head-fixed mouse on a free-rotating transparent disc and photostimulation of Purkinje cells elicited motor behavioural response (black trace) (Witter *et al.*, 2013). ChR2, channelrhodopsin 2; Pcp2, Purkinje cell protein 2; Sim, simple lobule.

Evidence came from a study which demonstrated that evoking an inhibitory response in DCN neurons through photostimulation of PCs that express ChR2 in Lobule 5 and 6 can either resulted in twitches of tail and hind limbs (Figure 6C & D) (Witter *et al.*, 2013). The behavioural response was observed only after offset of the photostimulation and during the rebound firing of the DCN neurons (Figure 6C & D) (Witter *et al.*, 2013). The effects on bodily and limb movements during rest and locomotion were further examined through the photostimulation of PCs of *Pcp2::Cre; ChR2-eYFP* mice (Hoogland *et al.*, 2015). Optogenetic stimulation during rest can either evoke whole-body or tail twitches or initiate stepping behavior while photostimulation during the step cycle reduce the speed or halt locomotion (Figure 7B) (Hoogland *et al.*, 2015). Photostimulation of PCs, which indirectly perturb the ability of the DCN to regulate motor functions, has been shown to elicit diverse phenotypic motor response, which further corroborates the importance of cerebellum in modulating motor functions. However, whether direct manipulation of specific identifiable neuronal subpopulations within the DCN can evoke similar phenotypic output has not been examined and requires a more systematic characterization.

Figure 7. Differential effects of indirect inhibition of DCN neurons during rest and locomotion

(A) Schematic showing the photostimulation of PCs in Lobule 5. (B) Behaviours elicited upon photostimulation of PCs are state dependent. (C) Average response that demonstrate the stopping (top graph) or initiation (bottom graph) upon photostimulation of PCs (Hoogland *et al.*, 2015). ChR2, channelrhodopsin 2; Pcp2, Purkinje cell protein 2.

The heterogeneity of neuronal subpopulations in the DCN precludes the ability to examine individual neuronal subtypes on motor control during locomotion when non-specific cell-manipulation approaches like muscimol neuronal inactivation and microstimulation are used (Uusisaari and Knopfel, 2008; Uusisaari and Knopfel, 2010). In this thesis study, we aim to identify and characterize a subpopulation of neurons within the DCN. We hypothesize that this subpopulation of neurons could be implicated in limb control during locomotion and perturbation of these neurons could disrupt motor movement. Expression of ChR2 will be driven in specific cell types by using transgenic Cre-expressing mouse line. The cerebellar cortical control of limb movements could be mediated through the DCN neurons (Sarnaik and Raman, 2018; Witter *et al.*, 2013). *In vivo* optogenetic perturbation of each DCN during locomotion can discern specific DCN neuronal subpopulation of each DCN towards gait control.

Materials and Methods

Mouse strains

C57BL/6JInv (InVivos), Urocortin 3::Cre (Ucn3::Cre; Mutant Mouse Regional Resource Center; Tg[Ucn3-cre]KF31Gsat/Mmucd) (Harris et al., 2014), vesicular glutamate transporter 2::Cre (vGluT2::Cre; European Mutant Mouse Archive; Tg(Slc17a6-icre)100Ki) (REF) and Rosa::eGFP (B6;129-Gt[ROSA]26Sortm2Sho/J), 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) (The Jackson Laboratory) were obtained. Male and female wild-type and genetically modified mice were used. Mice between the age of post-natal day (P) 27 to P 33 days were used for *in vitro* electrophysiological experiments. Adult mice P53 to P120 were used for all other experiments. Only heterozygotes were used for each transgene allele. All procedures performed were approved by the Nanyang Technological University (NTU) and A*STAR Biological Resource Center Institutional Animal Care and Use Committee (IACUC).

In situ hybridization

A DNA template was used to generate RNA probes that will bind to vGluT2 RNA (product size: 953 bp):

5'–AAGAAGCAGGACAACCGAGA–3'

5'–ATGCCAACCTTGCTGATTTC–3'

Total cerebellar RNA of mouse origin (Clontech #636660) was reversed transcribed (Thermo Scientific #K1652) to generate template cDNA. Polymerase chain reaction (i-DNA Technology #iTaq-2) was performed with the DNA template to amplify the sequence of interest. The amplified sequences were transcribed to RNA probes using 11-Digoxigenin (DIG)-UTP

(Roche #11175025910). Tissue sections were fixed for 10 minutes in freshly prepared ice-cold 4% PFA (Sigma; #P6148). The slides were then washed thrice for five minutes with phosphate buffered saline (PBS) (1st Base; #BUF-2040-10X4L), followed by 10 minutes of Proteinase K (10µg/mL) (Promega; #V3021) treatment. The tissues were re-fixed in 4% PFA for 5 minutes and washed thrice for five minutes in PBS. The sections were then treated for 10 minutes in a solution containing triethanolamine (Sigma; #108-24-7) and acetic anhydride (Sigma; #411000) dissolved in MilliQ water. The tissues were then treated with hybridization solution, containing 50% formamide (Promega; #H5052), 5X SSC (1st Base; #BUF-3-50), 5X Denhardt's solution (Ficoll type 400 Sigma; #F9378), polyvinylpyrrolidone (Sigma; #PVP40) and bovine serum albumin (Sigma; #A7906), 200 µg/mL yeast RNA (Sigma; #R6625-25G) and 500 µg/mL herring sperm DNA (Promega; #D1816), for two hours at 63 °C. RNA probes were then diluted in hybridization solution at a concentration of 400 ng/mL, which was heated at 80 °C for 10 minutes. After the tissues were treated with hybridization solution, the slides were drained and the probes (diluted in hybridization solution) added to the samples followed by the placement of a coverslip. The slides were then incubated overnight in humidified conditions at 63 °C. After the overnight incubation, 5X and 0.2X SSC buffers (1st base; BUF-3-50) were preheated in a water bath at 63 °C. The slides were submerged in 5X SSC and coverslips were removed. Once the coverslips were removed, the sections were washed in the pre-heated 0.2X SSC for 30 minutes, followed by a second wash in a fresh container of 0.2X SSC at room temperature for 5 minutes before being transferred to a solution containing TN buffer (0.1 M Tris-HCl, pH 7.5 (1st Base; BUF-1416-pH7.5) mixed 0.15 M NaCl (1st Base; BUF-1112). The samples were then incubated with blocking buffer containing 10% sheep serum (Sigma; #SS263) and TN buffer for 2 hours at room temperature. After the incubation, the slides were drained and incubated overnight in anti-DIG alkaline phosphatase antibody (1:2000) diluted in blocking buffer. After

overnight incubation, samples were washed with TN buffer followed by incubation in equilibration buffer containing 100 mM Tris-HCl at pH 9.0, 150 mM NaCl and 1 mM MgCl for 5 minutes before being incubated in BCIP/NBT substrate (Promega; #SS3771) until there was sufficient colour development. The enzymatic reaction was then stopped by washing the samples in ice-cold methanol and slides were mounted using Dako mounting medium (Dako; CS703).

Immunohistochemistry and imaging

Immunohistochemistry was performed on free-floating, 40 µm cryosections using fluorophore-conjugated secondary antibodies (1:1000; Molecular Probes). Mice between P56-63 were used for characterization of the DCN. Tissue sections were washed thrice with solution containing PBS, and 0.2% Triton X-100, blocked with solution containing PBS, and 0.2% Triton X-100 and 3% horse serum for one hour at room temperature and then incubated in primary antibody with PBS, 0.2% Triton X-100, 3% horse serum over night at 4°C. Tissue sections were washed thrice with PBS, and 0.2% Triton X-100 (five minutes each) and then incubated with secondary antibody diluted in a solution containing PBS, 0.2% Triton X-100, 3% horse serum for overnight at 4°C. Tissue sections were again washed thrice with PBS (5 minutes each). Primary antibodies used in this study: rabbit anti-Calretinin (1:2000; abcam; ab702); rabbit anti-Gad67 (1:1000; Invitrogen; PA5-21397); rat anti-GFP (1:1000; Nacalai Tesque; GF090R); rat anti-RFP (1:1000; chromotek; 5f8-100); mouse anti-SMI 32 (1:1000; Biolegend; #801701); rabbit anti-Tbr1 (1:500; abcam; ab31940); guinea pig anti-vGluT2 (1:2000; Merck; AB2251). The DCN were divided into different subnuclei for analysis after brain slices were stained with glutamatergic neuronal markers Tbr1, SMI32 and vGluT2, and nuclear marker, DAPI. DAPI staining was used for visualizing large neuronal nuclei that were found only in the DCN and not the surrounding white matter (Heckroth 1994). We used these methods to delineate the distinct

shapes of each subnuclei and cross referenced our delineation with the mouse brain atlas (Franklin and Paxinos, 2013).

***In vitro* electrophysiology**

Mice between 27 and 33 days of age were sacrificed under deep anaesthesia using isoflurane and their brains were extracted. Coronal cerebellar sections (200 μ m) were cut at room temperature and incubated at 34°C for one hour in oxygenated standard extracellular solution containing 126 mM NaCl, 24 mM NaHCO₃, 1 mM NaH₂PO₄, 2.5 mM KCl, 2.5 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, and 0.4 mM ascorbic acid (pH 7.4 when equilibrated with 95% O₂ /5% CO₂). Whole-cell patch clamp recordings were conducted at room temperature as previously described (Lee *et al.*, 2010). For whole-cell recordings of DCN neurons, pipettes (4-7 M Ω) were filled with an internal solution containing 130 mM K-gluconate, 10 mM KOH, 2.5 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, 4 mM Na₂ATP, 0.4 mM Na₃GTP, and 5 mM disodium phosphocreatine (pH 7.3, ~295 mOsm). Electrical responses were acquired using a Multiclamp 700B amplifier with Digidata 1440A interface (Molecular Devices). Input resistance was measured from the slope of the current-voltage plot. Membrane capacitance was calculated using the capacitance = membrane time constant (τ)/resistance formula. The Clampfit program was used to generate the exponential curve fit of responses to hyperpolarizing current steps to calculate the membrane time constant. Action potential half width was measured from the duration at half-amplitude between threshold and action potential peak. Cells were filled with Neurobiotin (0.5%, diluted in internal solution; Vectorlabs, Burlingame, CA) during recording by ionophoretic injection (Curti *et al.*, 2012). Brain slices were treated (as described above) and an additional hour incubation with AlexaFluor633-conjugated Streptavidin (2 mg/ml; 1:250 dilution; Molecular Probes; S21375) was used to label the Neurobiotin filled cells. Cells were

fixed and then imaged under confocal laser scanning microscope (LSM710; Zeiss) and images were processed using ImageJ software. Optical z-section images were collected 0.8 μm apart to reconstruct the entire cell body.

Photostimulation of neurons in the cerebellum

Photostimulation *in vitro* was conducted as previously described (Wang et al., 2007). In brief, slices were exposed to blue light (465-495 nm) from a mercury arc lamp. Simultaneously, cellular responses were measured in whole-cell patch clamp recordings. Data expressed as Mean $\pm$ SEM. was analyzed with custom software to produce the synaptic input maps and averaged probability maps. For *in vivo* photostimulation, mice were prepared for stereotaxic surgery and a custom-made mono fiber-optic cannula comprised of a multimode ceramic zirconia ferrule (230 μm ; Precision Fiber Products; MM-FER2007CF-2300) housing an multimode 0.22 NA optical fiber (200 μm core diameter; ThorLabs; FG200LEA) with a fiber protrusion length of 2.5 mm was inserted into the cerebellum [IntA, from bregma: 5.88 mm posterior, 1.35 mm lateral (left), 2.40 mm ventral; Med, from bregma: 5.25 mm posterior, 0.90 mm lateral (left), 2.50 mm ventral] using a stereotaxic cannula holder and adaptor (Doric Lenses). The ferrule was secured to the back of the skull using dental cement (Heraeus Kulzer, Germany) with two anchors (stainless steel, 1.5 by 1.5 mm, #000-120 thread screws). After one week recovery, mice were subjected to behavioral testing. Light was delivered using a mono fiber-optic patch cord temporarily connected to the ferrule via a ceramic split sleeve (1.25 mm internal diameter, 6.0 mm length; Precision Fiber Products; SM-CS125S). A 473 nm diode laser (Shanghai Laser) and an arbitrary waveform generator (NI myDAQ; National Instruments) were used to deliver pulses of light (50-500 ms pulse width, 1-30 Hz, ~1-12 mW, fibre tip 0.1 – 0.2 mm dorsal to region of interest).

Laser strength was calibrated at the end of the ferrule tip using a power meter (ThorLabs) prior to implantation.

Stereotaxic injection

Craniotomies (1 - 3 mm diameter) were made above the site of injection. Stereotaxic coordinates used for this study were obtained from a stereotaxic mouse brain atlas (Franklin and Paxinos, 2013). The NanoFil glass syringe (10 μ l) was used with a 33 g beveled needle for the microinjection of 70 nl AAV1.CAGGS.Flex.ChR2-tdTomato virus (Penn Vector Core) at a rate of 40 nl/min. Leakage or backflow was reduced by positioning the needle within the injected site for 5 minutes prior to removal (Ruigrok and Apps, 2007). The mouse was allowed to recover for a week before habituation. The surgical procedure was performed only when the mouse is anesthetized with an induction concentration of 3% isoflurane and an oxygen rate of 2 L/minute followed by a maintenance concentration of 1-2% isoflurane and an oxygen rate of 1 L/minute.

Motor behavioural analysis

Mice were habituated in the set up for three days (30 min/day) to the restricted walkway (3.2 cm x 30 cm x 70 cm) with a mirror placed at the bottom of the walking platform. For photostimulation experiments, photostimulated trials utilized a 473 nm laser at 1 - 10 mW at 1 - 30 Hz for 100 – 2000 milliseconds with a pulse duration ranging from 15 ms to 100 ms (Azim *et al.*, 2014; Guo *et al.*, 2015; Gao *et al.*, 2016). Lateral motion paths and ventral motion paths, which are deflected from the mirror, of walking through a restricted walkway were captured within 50 cm window by the camera at 300 and 500 fps, 1648 x 360 pixels. Gait parameters of freely walking mice are previously described (Mendes *et al.*, 2015; Machado *et al.*, 2015). The *x*

and y coordinates of the forelimb were manually tracked using MaxTRAQ 2D (Innovision Systems) and the following parameters were extrapolated and analyzed (Figure 8):

Cadence (s^{-1}): frequency of strides per second

Stance duration (ms): time taken during stance phase

Swing duration (ms): time taken during swing phase

x-displacement (mm): stride length (one stance position to the next stance position)

y-displacement (mm): average vertical distance between the limb and the walking platform during the swing phase from each individual frame

y max (mm): maximum vertical limb position

time to y max: time between the start of swing phase to the time when y max is reached

Each swing was further subdivided into a Swing-Flex and Swing- Extend phase based on flexion at the wrist joint observed as the deflection from a perpendicular line drawn from the paw to platform.

Figure 8 Limb positions during locomotion. (A) Schematic of a complete stride cycle illustrating different forelimb positions from the side view of the mouse (trajectory, blue line). (B) A single hindlimb using the side panel as a support during stance phase.

Statistics

Comparable numbers of strides per animal of each parameter in control and ablated animals were analyzed to avoid disproportionate effect on the results. Similarly, for

photostimulation experiments, equal numbers of strides from each animal during the on and off conditions were used for statistical comparison. Values are represented as Mean $\pm$ SEM unless otherwise noted. Comparisons were made by unpaired Mann-Whitney U test. Non-parametric statistical tests were primarily used for histological and locomotion behavioural studies. Parametric statistical analysis was used for electrophysiological analysis and staircase skilled reaching behavioural tests. Statistics were analyzed using GraphPad Prism v.7.00 (GraphPad, La Jolla, California, USA).

Analysis of recombination of Ucn3::Cre; Rosa26::ChR2-eYFP neurons, n = 3 mice (7 to 9 coronal sections per brain; 1764, 1088, 648 cells). Analysis of electrophysiological studies on IntA^{Ucn3} neurons, n = 7 mice (1 – 2 neurons per mouse, eYFP^{ON}, 7 – 15 neurons; eYFP^{OFF-L}, 6 – 9 neurons; eYFP^{OFF-S}, 6 – 9 neurons). Analysis of co-localization of IntA^{Ucn3} neurons with excitatory and inhibitory markers, n = 3 mice (7 – 9 coronal sections; 2615, 2176 and 4331 cells). Analysis of electrophysiological recordings of photostimulated IntA^{Ucn3} neurons, n = 8 mice (1-2 neurons per mouse). Analysis of *in vivo* photostimulation of IntA^{Ucn3} neurons during locomotion, n = 4 mice (7 – 10 strides per mouse, light-OFF, 29 – 37 strides; light-ON, 35 – 37 strides). Coronal sections from each mouse were stained with DAPI and imaged with fluorescent protein expressed within the DCN. Image analysis that showed fiber tracts that penetrated the cerebellar cortex and the tip is directly dorsal to the DCN of interest are grouped as correct implant placement while mice that did not show fiber tracts directly dorsal to the DCN of interest are grouped as incorrect implant placement. Mice that showed incorrect implant position are excluded from the analysis. Analysis of *in vivo* photostimulation of Med^{vGluT2} neurons, n = 1 mouse (iFL: light-OFF, 122 – 123 strides; light-ON, 150 – 154 strides. iHL: light-OFF, 124 –

128 strides; light-ON, 161 – 177 strides. cFL: light-OFF, 28 – 125; light-ON, 58 – 144 strides.
cHL: light-OFF, 25 – 121 strides; light-ON, 61 – 167 strides).

Chapter 2: A distinct neuronal subpopulation of the interposed anterior nucleus regulates precision of ipsilateral limb movements

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Title: Precision of Discrete and Rhythmic Forelimb Movements Requires a Distinct Neuronal Subpopulation in the Interposed Anterior Nucleus

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“Precision of discrete and rhythmic forelimb movements requires a distinct neuronal subpopulation in the interposed anterior nucleus.”

Introduction

Perturbation of neuronal activity in the Int nucleus is presumed to disrupt motor functions since photostimulation of PCs in the Sim lobule of the cerebellar cortex, which sends inhibitory inputs to both the Med and Int nuclei, evokes motor responses (Lee *et al.*, 2015; Sarnaik and Raman, 2018; Witter *et al.*, 2013). Consistently, manipulation of the activity of neurons in the Int nucleus disrupts motor performance (Wang *et al.*, 2017; Ekerot *et al.*, 1995; Schultz *et al.*, 1979). The contribution of the Int nucleus towards eyeblink motor response and learning is established by assessing the functional relevance of NC-MF that originates from the Int nucleus projection neurons (Figure 9) (Gao *et al.*, 2016). Photostimulation of NC-MF that expresses ChR2 after the mice have been trained showed higher conditioned eyeblink response while Arch-expressing NC-MF showed reduced eyeblink response during photostimulation (Figure 9C) (Gao *et al.*, 2016). Reversible inactivation of the Int nucleus through microinjection of muscimol in the Int nucleus has also been demonstrated to greatly reduce conditioned eyeblink response (Figure 9B) (Bracha *et al.*, 1999). Since unperturbed in the Int nucleus activity is imperative for conditioned eyeblink response, disruption of the Int nucleus may also alter locomotion.

Figure 9. Photostimulation of nucleo-cortical mossy fibers from DCN affects conditioned eyeblink response

(A) Scheme of eyeblink conditioning. (B) Schematic of viral injection into the Int nucleus and photostimulation of NC-MF that originate from labelled Int neurons. (C) Conditioned eyeblink response increased when paired with photostimulation of ChR2-expressing NC-MF that originate from the Int nucleus (top) while photostimulation of Arch-expressing NC-MF reduced the response (bottom) (Gao *et al.*, 2016). Arch, archaerhodopsin-3; ChR2, channelrhodopsin; CS, conditioned stimulus, hSyn, human synapsin; Sim, simple lobule; US, unconditioned stimulus.

Neurotransmitters added to the Int nucleus have been demonstrated to affect rota-rod and balance beam performance (He *et al.*, 2012; Song *et al.*, 2006). Microinjection of histamine increased stride length but not stride width during locomotion (Figure 10A) (He *et al.*, 2012; Song *et al.*, 2006). However, since PCs are able to respond to both histamine and serotonin (Kondoh *et al.*, 2004; Tian *et al.*, 2000), further studies have to be made to elucidate if the microinjection of both neurotransmitter could pharmacologically activate PCs through the synaptic terminals, which could result in inhibition of multiple cell types within the DCN.

Figure 10. Reversible inactivation of the Int nucleus reduces conditioned motor response and affects locomotion

(A) Schematic showing injection of muscimol into the Int nucleus (top) and schematic of behavioural conditioning setup (bottom). (B) Average of total amplitude of the conditioned response to CS tone (yellow) reduced after reversible inactivation of the Int nucleus. The average of the 25 trials before injection (thin line) and there are 65 trials analyzed after the injections, which is represented by the average of the first 35 trials immediately after injection (dotted line) and the average of the last 30 trials after injection (thick line). (C) Muscimol injection in the Int nucleus reduced average forelimb and hindlimb conditioned motor responses. Overlay representation is the same as (B). (D) Inactivation of the Int nucleus reduced precision of forelimb paw placement on the rung of the ladder. Saline-injected control trial (top) and muscimol-injected trial (bottom) (Bracha *et al.*, 1999). CS, conditioned stimulus; Int, interposed nucleus; Lat, lateral nucleus; Med, medial nucleus; US, unconditioned stimulus.

Addition of corticotropin-releasing factor (CRF) to the Int nucleus has been shown to excite only projection neurons but not interneurons (Wang *et al.*, 2017). Bilateral microinjection of CRF into the Int nucleus increased stride lengths of the hindlimb but not stride width and improved performance on accelerating rota-rod but reduced performance on balance beam

(Figure 11B & C) (Wang *et al.*, 2017). However, whether the CRF-mediated effects elicit any forelimb response has not been determined.

Figure 11. Histamine and CRF injection into the Int nucleus affect locomotion and motor behaviours

(A) Schematic showing injection of histamine into the Int nucleus (left), which increased the stride length of hindlimbs (He *et al.*, 2012). (B) Schematic showing double injection of CRF into the Int nucleus of both cerebellar hemispheres (top) and stride length of both forelimbs increased after CRF injection into the Int nucleus (bottom) (Wang *et al.*, 2017). (C) which increased rotarod performance but decreased balance beam performance (Wang *et al.*, 2017). CRF, corticotropin-releasing factor.

Muscimol injection into the Int nucleus greatly reduced the conditioned limb withdrawal response for both ipsilateral forelimb and hindlimb when cats are trained to respond to air puffs paired with electrical stimulation (Figure 10C) (Bracha *et al.*, 1999). Deficit in paw placement on the rung with frequent loss of balance and reduced gait as the muscimol-injected cat was walking on the ladder were also observed (Figure 10D) (Bracha *et al.*, 1999). Microinjection of muscimol into the Int nucleus also disrupt forelimb prehension movements in primates and cats (Monzee *et al.*, 2004; Goodkin and Thach, 2003; Milak *et al.*, 1997; Mason *et al.*, 1998). Reversible inactivation of the Int nucleus induce dynamic tremor and dysmetric arm movement during prehension (Monzee *et al.*, 2004). The microinjection of muscimol could mimic the artificial

amplification of PC inhibition on all neuronal subpopulations in the Int nucleus. However, consequences of acute activation of neurons in the Int nucleus on motor behavioural responses are not known. A prediction is that activation may result in an opposite effect from the artificial inhibition approach.

Electrical stimulation to artificially activate Int neurons elicits eyeblink response and evoke forelimb movements (Heiney *et al.*, 2014; Ekerot *et al.*, 1995; Schultz *et al.*, 1979). However, how microstimulation affects limb movements during locomotion is not examined. The use of microstimulation and muscimol on DCN have been demonstrated to affect motor functions but whether all the different neuronal subtypes in DCN are important for orchestrating the regulation of motor signals or within the heterogeneous population, only a limited subpopulation are required while the perturbation of other subpopulations will not confer any motor deficits is yet to be known. The use of a transgenic mouse line that allows the manipulation of individual neuronal subtypes in the DCN could reveal subtype-specific functions and improve our understanding of the functional diversity within the DCN.

The molecular distinctions between GABAergic neuronal subpopulations in the DCN suggest that glutamatergic neurons in the Int nucleus could also contain multiple distinct subpopulations in the Int nucleus (Figure 2). In order to explore potential distinctions between subpopulations of glutamatergic Int neurons, electrophysiological measurements and immunohistochemical analysis will be performed. The ability of neurons in the DCN to regulate limb movements suggests that manipulation of select subpopulations of glutamatergic neurons may perturb motor behaviours. To test whether DCN neuronal subpopulations contribute to

regulation of gait, optogenetic manipulation of discrete subsets of DCN neurons using a specific Cre-expressing transgenic mouse line on specific parameters of locomotion will be examined.

Results

Selective genetic labelling of a subpopulation of neurons within the IntA

Limited studies have addressed the functional significance of neuronal subpopulations in the DCN due to the lack of a cell type-specific transgenic mouse line. After screening public expression database (GENSAT and Allen Brain Atlas), we identified and acquired an *Urocortin 3* (*Ucn3*)::Cre transgenic mouse line which may recombine in neurons in the deep cerebellum (Mutant Mouse Regional Centre). We first carried out detailed characterization of the recombination profile of this mouse line and the electrophysiological and molecular properties of recombined neurons.

The *Ucn3*::Cre mice are crossed to either *Rosa26::lsf-eGFP* or *Rosa26::lsf-ChR2-eYFP*, and the location of recombined neurons in each of the nine subnuclei of the DCN were determined using histological distinctions (Franklin and Paxinos, 2013). Recombined neurons are highly concentrated within the IntA nucleus with limited recombination within other subnuclei (Figure 12A-E). To determine the extent of recombination across all DCN subnuclei, histological quantification was performed and the result shows that >70% of the recombined ChR2-YFP⁺ neurons are in the IntA nucleus while the remaining recombined neurons are in other DCN subnuclei (Figure 12F). As the DCN is comprised of a heterogeneous population of neurons, *Ucn3* recombined neurons could represent a specific subtype or multiple subtypes of DCN neurons (Chan-Palay, 1977; Uusisaari and Knopfel, 2008; Uusisaari and Knopfel, 2010; Chung *et al.*, 2009; Chen and Hillman, 1993).

Figure 12. Selective genetic targeting of IntA neurons

(A) Expression of GFP in the Int nucleus of an *Ucn3::Cre; Rosa::eGFP* mouse. (B) Schematics of nine subdivisions of the three major subnuclei of the DCN. (C-E) Expression of eYFP is observed primarily within the IntA nucleus of *Ucn3::Cre; Rosa::ChR2-eYFP* mouse (C, D) but not in other subnuclei (C-E). (F) Quantification of the distribution of eYFP^{ON} neurons across different subdivisions of the DCN of *Rosa::ChR2-eYFP* mouse (Lat, 5 ± 1%; LatPC, 2 ± 1%; IntDL, 1 ± 0%; IntA, 71 ± 3%; IntP, 3 ± 1%; IntPPC, 3 ± 1%; MedM, 11 ± 3%; MedDL, 5 ± 1%; MedL, 1 ± 1%). IntA, interposed anterior nucleus; IntDL, interposed dorsolateral; IntP, interposed posterior; Lat, lateral; LatPC, lateral parvicellular; MedM, medial region of Med DCN; MedDL, medial dorsolateral; MedL, medial lateral. Mean SEM; n = 3 mice (7 – 9 coronal sections from each brain; 1764, 1088, 648 cells). (Liang *et al.*, 2012).

IntA^{Ucn3} neurons are a subpopulation of glutamatergic neurons in the IntA

The DCN has a heterogeneous population of neurons characterized by their electrophysiological and molecular profiles (Chan-Palay, 1977; Chen and Hillman, 1993; Chung *et al.*, 2009; Uusisaari and Knopfel, 2008; Uusisaari and Knopfel, 2010; Uusisaari *et al.*, 2007). To determine the electrophysiological properties of the neurons recombined within the IntA nucleus in the *Ucn3::Cre; Rosa::ChR2-eYFP* mice, patch clamp recordings were performed on neurons that express eYFP (YFP^{ON}) and neurons that do not express eYFP (YFP^{OFF}). In current clamp mode without any current injection, eYFP^{ON} neurons showed higher spontaneous firing rate than most of the eYFP^{OFF} neurons that look smaller (Figure 13A). Using the diameter size of the neurons, the YFP^{OFF} neurons can be subdivided into large (eYFP^{OFF-L}) (area, $430.4 \pm 46.71 \mu\text{m}^2$), which is similar to eYFP^{ON} (area, $344.2 \pm 37.15 \mu\text{m}^2$) neurons, and small (eYFP^{OFF-S}) (area, $91.86 \pm 6.91 \mu\text{m}^2$) neurons (Figure 13B, Table 1). eYFP^{OFF-L} neurons ($26.3 \pm 3.5 \text{ Hz}$) have similar action potential (AP) frequency to eYFP^{ON} neurons ($35.4 \pm 5.7 \text{ Hz}$), while eYFP^{OFF-S} ($8.1 \pm 1.4 \text{ Hz}$) has lower AP frequency (Figure 13C). eYFP^{ON} neurons also have very similar input resistance ($166 \pm 20.2 \text{ M}\Omega$) membrane capacitance ($161.0 \pm 16.2 \text{ pF}$) and AP half width ($0.72 \pm 0.04 \text{ ms}$) values with the eYFP^{OFF-L} neurons ($195 \pm 34.1 \text{ M}\Omega$; $160.8 \pm 19.9 \text{ pF}$; $0.79 \pm 0.07 \text{ ms}$) but different from the YFP^{OFF-S} neurons ($645 \pm 127.7 \text{ M}\Omega$; $45.1 \pm 9.7 \text{ pF}$; $1.71 \pm 0.27 \text{ ms}$) (Figure 13D-F). However, their slow afterhyperpolarization (AHP) peak are similar (eYFP^{ON}, $-14.3 \pm 0.6 \text{ mV}$; eYFP^{OFF-L}, $-13.4 \pm 0.8 \text{ mV}$; eYFP^{OFF-S}, $-17.4 \pm 1.4 \text{ mV}$) (Figure 13D-G). Previous studies have shown that large Gad⁻ neurons that are presumably glutamatergic have an area of $322 \pm 20 \mu\text{m}^2$, AP frequency of $30.6 \pm 5.8 \text{ Hz}$, input resistance of $220 \pm 22 \text{ M}\Omega$, membrane capacitance $\geq 100 \text{ pF}$ and AP half width of $0.53 \pm 0.03 \text{ ms}$ (Uusisaari *et al.*, 2007). Therefore, based on the measurements of these intrinsic electrical properties, the IntA^{Ucn3} neurons belong to a class of large glutamatergic neurons within the DCN. Since there are also

neurons that are not recombined but also exhibited glutamatergic neuronal properties, the recombination of *Ucn3::Cre* could occur within a subset of the glutamatergic neurons within IntA nucleus.

Figure 13. Functional characterization of a distinct subpopulation of glutamatergic neurons in the IntA nucleus

(A) Combination of patch-clamp recording and immunohistochemistry labeling of two IntA neurons of a P30 *Ucn3::Cre; Rosa::ChR2-eYFP* mouse. Neurobiotin (blue) was used to fill the patched neurons and are correlated with their AP firing. Top row: a neuron filled with neurobiotin (blue) and is eYFP⁺ (green)/Tbr1⁺ (red) that fires spontaneously at a higher frequency. Bottom row: another neuron filled with neurobiotin that appeared smaller in size, negative for eYFP and Tbr1, shows lower spontaneous firing frequency. (B, C) Action potential (AP) frequency of eYFP^{ON} neurons (area: 344.2 ± 37.15 μm²; AP: 35.4 ± 5.7 Hz) is similar to AP of eYFP^{OFF-L} (eYFP^{OFF-Large}) neurons (area: 430.4 ± 46.71 μm²; AP: 26.3 ± 3.5 Hz) but is different from eYFP^{OFF-S} (eYFP^{OFF-Small}) neurons (area: 91.81 ± 6.91 μm²; AP: 8.1 μm^{2 1.4} Hz). (D-H) eYFP^{ON} neurons have similar input resistance (166 ± 20.2 MΩ), membrane capacitance (161.0 ± 16.2 pF), AP half width (0.72 ± 0.04 ms), and frequency-current (F-I) plot (AP

threshold = -30.4 ± 1.4 mV; AP amplitude 46.0 ± 2.1 mV) profiles to eYFP^{ON-L} neurons (input resistance: 195 ± 34.1 ; membrane capacitance: 160.8 ± 19.9 pF; AP half width: 0.79 ± 0.07 ms; F-I plot: AP threshold, -30.4 ± 1.4 mV; AP amplitude: 46.7 ± 2.8 mV) but is different from eYFP^{ON-S} neurons input resistance: 645 ± 127.7 M Ω ; membrane capacitance: 45.1 ± 9.7 pF; AP half width: 1.71 ± 0.27 ms; F-I plot: AP threshold, -34.2 ± 1.3 mV; AP amplitude: 51.2 ± 3.8 mV). All three neuronal subtypes have similar slow afterhyperpolarization (AHP) peaks (eYFP^{ON}: -14.3 ± 0.6 mV; eYFP^{OFF-L}: -13.4 ± 0.8 mV; eYFP^{OFF-S}: -17.4 ± 1.4 mV). Significant difference compared with eYFP^{ON-L} (ANOVA with post hoc Tukey tests); Mean $\pm$ SEM; n = 7 mice (1 – 2 neurons from each mouse); n = 7 – 15 (eYFP^{ON}), 6–9 (eYFP^{OFF-L}), 6 – 9 (eYFP^{OFF-S}) neurons. *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, ****p $\leq$ 0.0001. AP, action potential.

Table 1. Electrophysiological parameters of IntA neurons in *Ucn3::Cre; Rosa::ChR2-eYFP* mice

	eYFP ^{ON}		eYFP ^{OFF-L}		eYFP ^{OFF-S}	
	Mean	SEM	Mean	SEM	Mean	SEM
area, μm^2	344.2	37.15	430.4	46.71	91.86	6.91
AP Frequency, Hz	35.4	5.7	26.3	3.5	8.1	1.4
input resistance, M Ω	166	20.2	195	34.1	645	127.7
membrane capacitance, pF	161	16.2	160.8	19.9	45.1	9.7
AP half width, ms	0.72	0.04	0.79	0.07	1.71	0.27
slow AHP peak, mV	-14.3	0.6	-13.4	0.8	-17.4	1.4
n = 6 – 8 mice (1 – 2 neurons from each mouse); n = 7 – 15 cells (eYFP ^{ON}); 6 – 9 cells (eYFP ^{OFF-L}); 6 – 9 cells (eYFP ^{OFF-S}). AP, action potential; AHP, afterhyperpolarization.						

Not all neurons that exhibited glutamatergic electrophysiological properties in the IntA nucleus were recombined in the *Ucn3::Cre; Rosa::ChR2-eYFP* mice (Figure 13B-H). To determine the molecular profiles of the IntA^{Ucn3} neurons and whether these neurons are a subset of the glutamatergic neurons in the DCN, the expression of vGluT2 and GABAergic molecular markers (Gad67, Calretinin or Tfap2A) in eYFP^{ON} neurons was assessed (Zainolabidin *et al.*, 2017; Chung *et al.*, 2009; Leto *et al.*, 2006). SMI32 was used to identify both excitatory and inhibitory subtypes (Leto *et al.*, 2006). The expression of eYFP on IntA^{Ucn3} neurons co-localizes

with 45% ($\pm 6\%$) of all vGluT2⁺ presumptive glutamatergic neurons in the IntA nucleus (Figure 14A-D). IntA^{Ucn3} neurons co-labelled with SMI32 but do not express Gad67, Calretinin or Tfap2A (Figure 14E-S). Therefore, *Ucn3::Cre* mice are capable of driving the expression of ChR2-eYFP within a subpopulation of glutamatergic neurons within the IntA nucleus. Because previous studies have shown that the activity of neurons in the Int nucleus increases during locomotion and that perturbation of these neurons disrupts limb movement (Armstrong and Edgley, 1984; Bracha *et al.*, 1999; Ekerot *et al.*, 1995; He *et al.*, 2012; Schultz *et al.*, 1979; Song *et al.*, 2006; Wang *et al.*, 2017), we set out to determine the consequences of photostimulating IntA^{Ucn3} neurons in *Ucn3::Cre; Rosa::ChR2-eYFP* mice on locomotor behaviours.

Figure 14. IntA^{Ucn3} neurons are a subpopulation of glutamatergic neurons in the DCN

(A-D) Almost half of the vGluT2⁺ cells are recombined in *Ucn3::Cre; Rosa::ChR2-eYFP* mouse (eYFP^{ON}: 45% ± 6%; eYFP^{OFF}: 55% ± 4%). (E-S) IntA^{Ucn3} neurons express SMI32 (blue, E-S) but not Gad67 (red, E-I), calretinin (red, J-N) and Tfap2A (red, O-S) (DAPI, grey). Colocalization is indicated by circles and cells without colocalization is indicated by arrows. n = 3 mice, total = 9122 cells; 2615, 2176 and 4331 cells.

Electrophysiological and behavioural responses to optogenetic stimulation of $\text{IntA}^{\text{Ucn3}}$ neurons

Through direct and indirect manipulation of Int neurons, microstimulation of IntA in cats and photostimulation of PCs in the simple lobule of the cerebellar cortex in mice has been shown to drive ipsilateral limb movements, respectively (Lee *et al.*, 2015; Witter *et al.*, 2013; Ekerot *et al.*, 1995). But how direct photostimulation of a specific subset of glutamatergic neurons within the IntA nucleus has not been clearly examined. Before *in vivo* photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons in $\text{Ucn3}::\text{Cre}; \text{Rosa}::\text{ChR2-eYFP}$ mice, whole cell recording using cerebellar slices was performed to detect change in firing frequency during photostimulation. Photostimulation increased their firing frequency by more than two-fold (light-OFF, 35.1 ± 6.5 Hz; light-ON, 75.3 ± 6.3 Hz) and presumptive GABAergic neurons do not respond to the light (light-OFF, 12.0 ± 2.2 Hz; light-ON, 12.3 ± 2.5 Hz) (Figure 15). Photoactivation of $\text{IntA}^{\text{Ucn3}}$ neurons in cerebellar slices provided some confidence that we could activate this specific subpopulation of glutamatergic neurons in the DCN using *in vivo* optogenetics.

Figure 15. $\text{IntA}^{\text{Ucn3}}$ neurons in response to photostimulation

(A-D) Whole cell recording of $\text{IntA}^{\text{Ucn3}}$ neurons (light-OFF, 35.1 ± 6.5 Hz; light-ON, 75.3 ± 6.3 Hz) shows photostimulation evoke increase of firing frequency but was not observed in cells that do not express ChR2 (light-OFF, 12.0 ± 2.2 Hz; light-ON, 12.3 ± 2.5 Hz) in $\text{Ucn3}::\text{Cre}; \text{Rosa}::\text{ChR2-eYFP}$ mice. Blue bar indicates photostimulation. Graphs in B and D are plotted as normalized frequency (Norm FR) in order to better reflect any changes in frequency. Baseline frequency is normalized as 1. $n = 8$ mice (1 – 2 neurons from each mouse); eYFP^{ON} , 15 neurons; $\text{eYFP}^{\text{OFF-L}}$, 9 neurons; $\text{eYFP}^{\text{OFF-S}}$, 9 neurons.

To assess the behavioural consequence of stimulating IntA^{Ucn3} neurons, light was delivered through optical fibers that were unilaterally implanted into the cerebellum (Figure 15A-C). A walkway chamber compatible for concurrent testing with optogenetics was designed and constructed in order to assess the ability of mice to walk freely and define relevant locomotor parameters (Figure 16D). Photostimulation was also given at random trials as the mice perform the locomotor task within the walkway and limb movements were captured using the infrared high-speed camera (300 fps, 1648 x 360 pixels) (Figure 16D). All four limbs were manually tracked using the side view of the mouse and also the ventral view that was observed through a mirror placed ventrally across the bottom of the transparent walking platform (Figure 16D-F). A single stride for each limb can be separated into the stance phase, when the setup is supporting the limb, and the swing phase, when the limb is not in support (Figure 16G). Photostimulation of IntA^{Ucn3} neurons at 1, 5 and 20 Hz (1-10 mW) did not result in any forelimb movement, indicating that IntA^{Ucn3} neurons may not be able to initiate any forelimb or hindlimb movement (data not shown).

Figure 16. Behavioural system used to analyse locomotion during photostimulation of IntA^{Ucn3} neurons

(A) Schematic of the strategy used to deliver light pulse to perturb Int^{Ucn3} neurons of an *Ucn3::Cre; Rosa::ChR2-eYFP* mouse. (B) Optic fiber implant dorsal to the IntA nucleus (white dotted tract). (C) Timeline of the experimental paradigm from the start of fiber implantation. (D) Schematic of the behavioural apparatus used that allows limb positions to be captured using a high-speed infrared camera (300 fps, 1648 x 360 pixels). (D) MaxTRAQ 2D software is used to manually track the position of the limbs from the and each limb is indicated with a coloured marker (iFL, blue; iHL, light blue; cFL, red; cHL, pink) (dotted line demarcates the side view of the mouse at the top and ventral view observed from the mirror). Homologous limbs are referring to either be both fore or hindlimbs and diagonal limbs are referring to a single forelimb and the contralateral hindlimb. (E) Example of the limb positions tracked from the side view and the ventral view through the mirror. (F) Strides are divided into the stance phase and the swing phase for further analysis. iFL, ipsilateral forelimb; iHL, ipsilateral hindlimb; cFL, contralateral forelimb; cHL, contralateral hindlimb.

Photostimulation of *Rosa::ChR2* control mice that have implants in the correct position or in *Ucn3::Cre; Rosa::ChR2-eYFP* mice that have implants in the incorrect position do not show

any observable effects of ipsilateral limb trajectory (data not shown). However, photostimulation of *Ucn3::Cre; Rosa::ChR2-eYFP* with implants dorsal to IntA nucleus shows perturbed iFL and iHL movement but no observable effects on contralateral forelimb (cFL) and contralateral hindlimb (cHL) during locomotion (Figure 17). Alternation of homologous limb and synchrony of diagonal limbs are preserved during photostimulation of IntA^{Ucn3} neurons, indicating activation of the neurons do not cause the gait to switch from either walk or trot to bound (Figure 17) (Bellardita and Kiehn, 2015).

Figure 17. Alternation of homologous limbs is preserved during photostimulation of Int^{Ucn3} neurons

(A, B) Continuous forward trajectory (A) and vertical trajectory (B) of a non-photostimulated trial. Homologous limb alternates while diagonal limbs move in synchrony. (C) Photostimulation of Int^{Ucn3} neurons neither impede movement nor perturb the alternation of homologous limbs and synchrony of diagonal limbs. Moment of pause is observed but the mouse is able to regain the natural pattern of locomotion even when the photostimulation is still ongoing. (D) Positioning of the iFL and iHL is raised higher and there are brief moments of tremors during the swing phase during photostimulation of IntA^{Ucn3} neurons. Blue bar indicates the period of photostimulation across the trial. Blue bar indicates photostimulation. iFL (blue), ipsilateral forelimb; iHL (light blue), ipsilateral hindlimb; cFL, (red), contralateral forelimb; cHL (pink), contralateral hindlimb. n = 1 animal (representative for the other 3 animals).

Grouping of traces from strides that are performed during photostimulated trials and trials without photostimulation shows that photostimulation disrupts limb trajectory (Figure 18A). Limb kinematics of photostimulated strides and non-photostimulated strides are analysed. Even though cadence is reduced while stance duration and swing duration are increased for iFL and iHL during photostimulation, the values did not reach statistical significance (Figure 18B-D, Table 2). Since there are no significant difference in the timing of limb positioning duration photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons, disruption could result in the change in distance of stride kinematics.

Figure 18. Photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons do not disrupt the rate of movement and the timing of limb positioning during locomotion

(A) Traces of the trajectory of iFL (top) and iHL (bottom) tracked from the side view of the mouse during photostimulated (cyan, 15 representative strides) and non-photostimulated trials (grey, 15 representative strides) of $\text{Ucn3::Cre}; \text{Rosa::ChR2-eYFP}$ mice. (B-D) Photostimulation of Int^{Ucn3} neurons did not cause significant change to cadence (B), stance duration (C) and swing duration (D) (Cyan bar indicates parameters obtained from strides that were recorded during photostimulation of Int^{Ucn3} neurons). Each point indicates parameter obtained from a single stride. $n = 4$ animals, 7-10 strides/animal, total: light-OFF, 37 strides; light-ON, 37 strides. See Table 2.

IntA^{Ucn3} neurons regulate the vertical positioning of ipsilateral limbs during locomotion

Even though photostimulation of IntA^{Ucn3} neurons showed observable difference in the traces of the limb trajectory for iFL and iHL, detailed kinematic analysis of the trajectory distance travelled during the swing phase did not reveal any significant change (Figure 18A, 19A). Similarly, the trajectory speed of both limbs also does not show any significant difference (Figure 19B). To identify the observable limb positioning difference during photostimulation of IntA^{Ucn3} neurons, the trajectory is further analysed based on the horizontal and vertical distance reached. Photostimulation of IntA^{Ucn3} neurons did not disrupt the stride length but increased the average vertical distance travelled across the entire swing phase (y-displacement) and increased the maximum vertical height reached (y-max) of iFL (Figure 19C-E). Despite the increase of y-max, the duration taken to reach the maximum height shows no significant change (Figure 19E-F).

Figure 19. Photostimulation of IntA^{Ucn3} neurons increases vertical limb positioning of iFL and iHL during swing phase

(A, B) Perturbation of IntA^{Ucn3} neurons during locomotion do not change the trajectory length (A) of traces from the side view of the *Ucn3::Cre; Rosa::ChR2-eYFP* mice and trajectory speed (B) during the swing phase of iFL (blue) and iHL (light blue) (C) Analysis of x-displacement for iFL and iHL do not show changes upon photostimulation. (D) Average vertical distance across each limb position from the platform during the swing phase for iFL increased during photostimulation of IntA^{Ucn3} neurons. (E) Photostimulation of IntA^{Ucn3} neurons also increases the maximum vertical limb position that iFL and iHL could reach during locomotion. (F) Despite the increase of y-max, the time taken from the start of swing phase to the time point when y-max was reached do not increase when IntA^{Ucn3} neurons were optogenetically activated. Each point indicates parameter obtained from a single stride. Cyan bar indicates parameters obtained from strides that were recorded during photostimulation of IntA^{Ucn3} neurons. n = 4 animals, 7-10 strides/animal, and total: light-OFF, 37 strides; light-ON, 37 strides. * $P \leq 0.05$. See Table 2.

Increase of average vertical displacement could be due to disruption of IntA^{Ucn3} neurons during initial phase or late phase of limb movement during the swing phase. Using the classification of flexion and extension of the wrist joint reported in cats, the swing phase is divided into swing-flex and swing-extend and the average of the vertical distance for both phases are compared (Figure 20A) (Engberg and Lundberg, 1969). Average vertical distance during both swing-flex and swing extend phases are disrupted during photostimulation of IntA^{Ucn3} neurons (Figure 20B, C). These results indicate that regulation of limb positioning during swing phase is controlled by a specific subpopulation of glutamatergic neurons in the IntA nucleus and suggest that the loss of IntA^{Ucn3} neurons could result in uncoordinated movement.

Figure 20. IntA^{Ucn3} neurons regulate the vertical positioning throughout swing phase

(A) Swing phase can be separated into swing-flex and swing-extend (Engberg and Lundberg, 1969). (B, C)

Photostimulation of IntA^{Ucn3} neurons increase average vertical distance of iFL during Δy (flex) (B) and Δy (extend) (C). Blue bar indicates photostimulation. n = 4 animals, light-OFF: 7-10 strides/ animal (total: 37 strides); light-ON: 7-10 strides/ animal (total: 37 strides), Δy (extend): light-OFF: 7-10 strides/animal (total: 37 strides), light-ON: 7-10 strides/animal (total: 37 strides); Mean $\pm$ SEM; Mann-Whitney test was used to compare the samples. * $P \leq 0.05$.

Table 2. Stride parameters of Ucn3::Cre; Rosa;;Chr2-eYFP mice

	iFL			iHL		
	light-OFF	light-ON	<i>P</i> value	light-OFF	light-ON	<i>P</i> value
cadence, s⁻¹	3.175±0.3099	3.03±0.2699	0.6857	2.273±0.3756	2.107±0.1439	0.4857
stance duration, ms	214.4±28.32	256.6±39.02	0.6857	350.1±66.4	400.6±58.81	0.8857
swing duration, ms	128.5±12.66	145.9±15.9	0.6857	166.8±24.08	187.7±34.67	0.4857
trajectory, mm	52.38±3.949	54.81±2.205	0.6857	53.63±3.683	53.21±3.828	0.8857
trajectory speed, mm/ms	0.4482±0.0622	0.4249±0.0503	0.8857	0.3554±0.0549	0.3131±0.0427	0.6857
x-displacement, mm	53.61±1.784	50.94±2.245	0.5615	52.64±1.907	50.48±2.371	0.4654
y-displacement, mm	2.93±0.1987	4.775±0.3906	0.0286*	4.679±0.3671	6.014±0.4429	0.1143
y-max, mm	4.159±0.2898	6.918±0.6173	0.0286*	6.649±0.7034	8.284±0.8425	0.4857
time to y-max, ms	75.25±6.529	77.12±6.03	0.8857	57.25±8.3	66.48±8.646	0.3429
Δy (flex), mm	1.821±0.1402	3.102±0.3739	0.0286*	na	na	na
Δy (extend), mm	3.534±0.2917	5.58±0.4939	0.0286*	na	na	na

n = 4 animals, light-OFF: 7 - 10 strides/ animal (total: 37 strides); light-ON: 7 - 10 strides/animal (total: 37 strides); Δy (flex), light-OFF: 7-10 strides/ animal (total: 37 strides); light-ON: 7-10 strides/ animal (total: 37 strides), Δy (extend): light-OFF: 7-10 strides/animal (total 37 strides), light-ON: 7-10 strides/animal (total: 37 strides); Mean ± SEM; Mann-Whitney test was used to compare the samples; *, significant difference. na, not applicable

Discussion

Int nucleus regulates horizontal and vertical limb positioning

In this chapter, we show that photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons affect limb trajectory during locomotion. The recombination capability of $\text{Ucn3}::\text{Cre}$ mice is not entirely restricted to the Int nucleus (Figure 12F). The behavioural response upon *in vivo* optogenetic stimulation could also be caused by manipulation of $\text{Ucn3}::\text{Cre}$ -recombined neurons in the other nuclei within the DCN. A possible method to resolve whether the observed motor behavioural response is solely due to manipulation of Int^{Ucn3} neurons in the Int nucleus is by injecting Cre-dependent channelrhodopsin virus into each of the other two nuclei (medial and lateral) followed by *in vivo* optogenetic stimulation to determine whether a similar motor response can also be elicited.

Even though cadence decreased while stance and swing duration were increased during photostimulation, these values did not reach statistical significance. Despite no significant changes in trajectory distance and x -displacement, photostimulation significantly increased the y -displacement and y -max values during locomotion. Pharmacological activation of glutamatergic neurons in the Int nucleus has been shown to affect horizontal stride lengths (He *et al.*, 2012; Wang *et al.*, 2017). Glutamatergic neurons in the Int nucleus have been shown to express receptors that bind to corticotropin-releasing factor (CRF) (Wang *et al.*, 2017). Addition of CRF resulted in excitation of these neurons and microinjection of CRF into the Int nucleus significantly increase the x -displacement of mice during locomotion (Wang *et al.*, 2017). Excitation of glutamatergic neurons through histamine also resulted in increase of x -displacement (He *et al.*, 2012). However, our study did not show any significant increment of x -displacement upon photostimulation of $\text{IntA}^{\text{Ucn3}}$ neurons.

Since it is not clear whether all or only a subset of glutamatergic neurons within the Int nucleus express receptors for CRF and histamine, addition of CRF or histamine could acutely stimulate all or only a subset of glutamatergic neurons in the Int nucleus. Moreover, the studies did not show whether CRF and histamine could disrupt the GABAergic and glycinergic neurons in the DCN, which could result in increase of x -displacement. Another possible reason is that Int nuclei were pharmacologically activated in both cerebellar hemispheres in the other studies (He *et al.*, 2012; Wang *et al.*, 2017). The bilateral activation may have caused profound limb positioning effects that unilateral manipulation is unable to evoke. This limitation can be solved by bilateral photostimulation of IntA^{Ucn3} neurons of the *Ucn3::Cre; Rosa::ChR2-eYFP* mice. We show in this chapter that the recombination of *Ucn3::Cre* is restricted to a subset of glutamatergic neurons within the Int nucleus. Therefore, the remaining subset of glutamatergic neurons that are not recombined in *Ucn3::Cre* mice maybe neurons that regulate ipsilateral horizontal limb movements.

IntA mediated internal cerebellar feedback loop is required for proper locomotor movement

Photostimulation of PCs in the Sim lobule of the cerebellar cortex reduces DCN neuronal firing frequency and disrupts ipsilateral limb movement during locomotion (Sarnaik and Raman, 2018). If PCs' activity is increased during locomotion, the firing frequency in DCN neurons will be expected to be reduced. However, *in vivo* recordings in mice have detected spontaneous activity in both PCs and DCN neurons during resting states and an increase of firing frequency in both the PCs and DCN neurons when mice are engaged in running behaviours (Sarnaik and Raman, 2018). The increase in firing rate in DCN could be generated from the rebound firing elicited after termination of optogenetic activation of PCs (Lee *et al.*, 2015; Sarnaik and Raman, 2018; Witter *et al.*, 2013).

Rebound firing in DCN neurons has been shown to be controlled by the increase of simple spikes (SSs) and complex spikes (CSs) during activation of PCs (Witter *et al.*, 2013; Hoebeek *et al.*, 2010). SSs and CSs responses in PCs are generated by excitatory PFs and CFs, respectively (Hoebeek *et al.*, 2010; Sudhakar *et al.*, 2015). After activation by SS-like train stimuli or CS-like train stimuli, PCs firing rate will pause for a brief period, allowing the DCN neurons to be disinhibited and facilitate a rebound firing (Hoebeek *et al.*, 2010). Therefore, despite directly inhibiting the DCN neurons, an increase of SSs and CSs through photostimulation of PCs could indirectly induce large amounts of rebound firing within the DCN neuron, which may disrupt the well-timed firing pattern that is required for regulated limb movement. Using a transgenic mouse line able to drive ChR2 expression to a specific cell type can elucidate the consequence of direct manipulation of DCN neurons.

Regulation of limb movement through cerebellar internal feedback and extra-cerebellar network

Through photostimulation of the $\text{IntA}^{\text{Ucn3}}$ neurons of *Ucn3::Cre; Rosa::ChR2-eYFP* mice, I provide evidence that the direct perturbation these neurons disrupts limb positioning during locomotion (Figure 19, 20). Stimulation of the $\text{IntA}^{\text{Ucn3}}$ neurons may elicit a feedback response to PCs through the NC fibers (Low *et al.*, 2018). NC fibers could indirectly perturb PCs by exciting granule cells (Houck and Person, 2015). Alternatively, SSs response in PCs could be disrupted through the activation PFs of granule cells upon activation of the $\text{IntA}^{\text{Ucn3}}$ neurons. Perturbation of PCs will result in a cascade of uncoordinated inhibition of the $\text{IntA}^{\text{Ucn3}}$ neurons or all DCN subtypes, thus disrupting the spontaneous activity of the cerebellar internal feedback circuitry required for regulation of limb movement (De Zeeuw and Berrebi, 1995; Han *et al.*, 2014). *In vivo* electrophysiological recordings could be performed to examine how direct

manipulation perturbs the change in spontaneous activity between resting and running behaviours. As glutamatergic projection neurons of the Int nucleus are known to send projections to multiple brain regions (Houck and Person, 2015; Teune *et al.*, 2000), photostimulation of IntA^{Ucn3} neurons could disrupt modulated cerebellar output sent to one or more motor control centres in the brain.

Disrupted limb movements during locomotion could be a result of perturbed network signaling between glutamatergic projection neurons in the Int nucleus and thalamus and magnocellular region of RN (RMC), which are target regions of IntA^{Ucn3} neurons (Ilg *et al.*, 2008; Ruigrok *et al.*, 1996; McCrea *et al.*, 1978; Houck and Person, 2015; Teune *et al.*, 2000; Low *et al.*, 2018). Electrical stimulation of Int neurons evokes excitatory post-synaptic potential responses in motor cortex through the VL thalamus (Yamamoto *et al.*, 1979). Both forelimb and hindlimb regions of the RMC receive projections from the IntA nucleus (Courville, 1966; Robinson *et al.*, 1987). RN nucleus influence muscles activity through the rubrospinal tract that send motor input to the motor neurons in the spinal cord (Liang *et al.*, 2012). Therefore, by directly photostimulating the IntA^{Ucn3} neurons, motor input towards the VL thalamus and red nucleus is disrupted and could interfere motor commands generated within the motor cortex and motor input sent to the spinal cord, resulting incorrect limb positioning during locomotion (Marlinski *et al.*, 2012).

IntA^{Ucn3} neurons regulate limb positioning during prehension

Patients with surgical lesions in the Int nucleus have lower reaching velocity and increase in lift-off duration (Kuper *et al.*, 2011b). Multiple studies have demonstrated that inactivation of the Int nucleus impairs prehension (Goodkin and Thach, 2003; Milak *et al.*, 1997; Mason *et al.*,

1998). Muscimol microinjection results in overshoot and increased limb trajectory when the animal was reaching for the object (Goodkin and Thach, 2003; Milak *et al.*, 1997). However, whether each or all of the neuronal subpopulations in the Int is required for regulating the reaching and grasping behavior has not been examined. We have performed unilateral ablation of IntA^{Ucn3} neurons by microinjection of AAV2-EF1a::double-floxed-DTR-eGFP into the IntA nucleus and administration of diphtheria toxin in the *Ucn3::Cre* mice (Low *et al.*, 2018). Ablation of IntA^{Ucn3} neurons caused the mice to be less successful in collecting the food pellets in the staircase reaching task (Low *et al.*, 2018). Detailed analysis showed that IntA^{Ucn3}-ablated mice have two-fold increase in reaching errors but grasping and retrieval of food pellets were not disrupted (Low *et al.*, 2018). Ablation also resulted in ~25° deviation from the food pellet in the direction of overreaching (Low *et al.*, 2018). These results indicate that IntA^{Ucn3} is required for the accuracy of reaching during prehension.

Investigating the function of non-IntA^{Ucn3} glutamatergic projection neurons

As the main excitatory output neurons linking the cerebellum with extra-cerebellar regions, whether glutamatergic projection neurons in the Int nucleus are comprised of multiple subpopulations that are molecularly distinct have not been demonstrated. Through our studies, we discovered that IntA^{Ucn3} neurons constitute a subpopulation of glutamatergic neurons within the IntA nucleus, and that photostimulation of IntA^{Ucn3} neurons can directly influence vertical limb positioning of iFL and iHL (Figure 19, 20). An examination of how the remaining glutamatergic projection neurons that do not express the ChR2 will facilitate different limb movement will resolve whether different subpopulations of neurons in the IntA nucleus could control distinct aspects of limb movement. Significance of the remaining glutamatergic projection neurons in the IntA nucleus that do not express ChR2 in *Ucn3::Cre* mice can be

examined through Cre^{OFF} expression construct that has the ChR2-eYFP gene inserted (Saunders *et al.*, 2012; Saunders and Sabatini, 2015).

Instead of targeting the expression of ChR2 in neurons that express Cre within the IntA nucleus of *Ucn3::Cre* mice, the Cre^{OFF} will permit the targeting of ChR2 expression only within neurons that do not express Cre (Cre⁻). Neurons that express Cre will invert lox-P sites that will prevent the expression of downstream genes. By packaging the Cre^{OFF} construct into a retrograde viral vector and injecting the virus in either the thalamus or RN, glutamatergic projection neurons in the IntA nucleus that are Cre⁻ will be retrogradely labelled and express ChR2. Direct manipulation of these Cre⁻ neurons could elucidate whether distinct subpopulations of glutamatergic neurons in the IntA nucleus send motor input through similar pathways to modulate different aspects of limb movement. Since glutamatergic projection neurons in the IntA nucleus are not the only source of DCN input into the thalamus and RN, glutamatergic projections in other DCN subnuclei could in principle regulate other aspects of limb positioning.

Figure 21. Selective genetic targeting of non-IntA Ucn3 neurons

(A) Microinjection of rAAVretro-*EF1α*::DO-ChR2-mCherry into the thalamus to retrogradely label and permit expression of ChR2-mCherry in non-IntA^{Ucn3} neurons of a *Ucn3::Cre; Rosa::GFP* mouse. Photostimulation of the IntA will selectively activate neurons that are non-IntA^{Ucn3} and not the IntA^{Ucn3} neurons.

In this chapter, we found that a distinct genetically-defined subset of neurons in the IntA nucleus exhibits glutamatergic electrophysiological properties and expresses excitatory molecular markers but not inhibitory molecular markers. *In vivo* photostimulation of these ChR2-expressing IntA^{Ucn3} neurons during locomotion perturbed both ipsilateral forelimb and hindlimb kinematics. In a study conducted by a colleague, IntA^{Ucn3} neurons reduced activity in extensor muscles during reaching movements (unpublished data, A. Thanawalla). These results suggest that IntA^{Ucn3} neurons may control limb muscles that result in increased flexion of the elbow during the swing phase when the neurons are experimentally activated. Other subset of the glutamatergic neurons in the DCN may modulate limb positioning through different types of limb muscles.

**Chapter 3: Glutamatergic neurons in the medial
nucleus regulate speed and precision of ipsilateral
forelimb and hindlimb placement**

Introduction

From the previous chapter, we uncovered that manipulation of Int^{Ucn3} neurons disrupts ipsilateral vertical limb positioning. Glutamatergic neurons in other DCN may modulate different aspects of limb movement during locomotion. PCs from a single lobule can differentially inhibit multiple neuronal subpopulations within the DCN and can also selectively inhibit distinct subnuclei of the DCN (De Zeeuw and Berrebi, 1995; Lee *et al.*, 2015). A direct manipulation of a specific DCN subnuclei could elicit either similar or different phenotypic effects on motor performances. The Med nucleus has been shown to regulate immune response and the spiking activity has been correlated to respiratory and orofacial behaviours (Lu *et al.*, 2013; Cao *et al.*, 2015). Inhibition of glutamate synthesis in the Med nucleus for both hemispheres has been shown to reduce the number of natural killer cells in the blood (Cao *et al.*, 2015). Spike firing pattern of subpopulation of Med neurons responded distinctly to respiration, whisking and licking behaviours (Lu *et al.*, 2013). These results indicate that Med nucleus could regulate diverse functions through different subpopulation of Med neurons.

Very few studies have shown how limb movement could be regulated by the Med nucleus (McCall *et al.*, 2015; He *et al.*, 2012; Zhang *et al.*, 2014). Increased firing rates were observed in the Med nucleus when the hindlimb shifts between extension and flexion positions (McCall *et al.*, 2015). Pharmacological manipulations of the Med nucleus have been shown to disrupt various motor function (He *et al.*, 2012; Zhang *et al.*, 2014). Pharmacological activation of serotonergic receptor 5-HT_{2A} in Med improved rota-rod performance but reduced balance beam performance (Figure 22A-C) (Zhang *et al.*, 2014). Decrease in stride width of hindlimb was also observed but no observable difference in stride length was observed for both ipsilateral and contralateral hindlimb upon addition of the agonist for 5-HT_{2A} (Figure 22D) (Zhang *et al.*,

2014). However, whether the 5-HT_{2A} receptors are expressed in all neuronal subtypes or only in a specific population of neurons in the Med nucleus was not determined and the behavioural effects upon 5-HT application on other DCN subnuclei were not examined. The effects on stride parameters during pharmacological activation of neurons that express 5-HT_{2A} receptors suggests that photostimulation of glutamatergic neurons within the Med nucleus might disrupt the limb movements kinematics.

Figure 22. Activation of 5-HT_{2A} receptors in the Med nucleus affect motor behaviours

(A) Schematic showing injection of agonist for 5-HT_{2A} receptor (TCB-2) in Med. (B, C) Activation of 5-HT_{2A} receptor improves rotarod performance and reduce balance beam performance. (D) Stride width of hindlimb decreased upon activation of 5-HT_{2A} (Zhang *et al.*, 2014).

Investigating a subset of neurons in the Med nucleus during locomotion can help discern whether each nucleus of the DCN has different control over limb positioning. To manipulate glutamatergic neurons in the Med nucleus, Cre-dependent ChR2-expressing virus will be stereotactically injected into the Med nucleus of vGluT2::Cre mouse. To determine the effects of the Med nucleus on control of locomotion, glutamatergic neurons in the Med nucleus will be perturbed through *in vivo* photostimulation while the mouse walks through the walkway.

Results

Targeted recombination within glutamatergic neurons of the Med Nucleus

Many studies have aimed to investigate the contributions of the cerebellum towards regulation of locomotion (Machado *et al.*, 2015; Sarnaik and Raman, 2018; Armstrong and Edgley, 1984; Song *et al.*, 2006; Hoogland *et al.*, 2015). However, majority of these studies manipulated neuronal subtypes in the cerebellar cortex which in turn indirectly manipulate DCN neurons (Machado *et al.*, 2015; Sarnaik and Raman, 2018; Hoogland *et al.*, 2015). A previous study has shown that BAC-*vGluT2::Cre* transgenic mouse line has the ability to recombine glutamatergic neurons within the DCN, but does not recombine in the glutamatergic neurons in the cerebellar (Borgius *et al.*, 2010). *In situ* hybridization results show that *vGluT2* mRNA expression is within all the DCN subnuclei but not the cerebellar cortex (Figure 23A, B). However, *vGluT2* protein is highly expressed within the cerebellar cortex, which are axonal terminals of MFs, CFs and NC fibers from the DCN (Figure 23C) (Hisano *et al.*, 2002; Sillitoe and Joyner, 2007; Houck and Person, 2015; Hioki *et al.*, 2003). Thus, crossing the BAC-*vGluT2::Cre* mouse with *Rosa::ChR2-eYFP* will cause expression of ChR2 within the axonal terminals that are collaterals of MFs and CFs and photostimulation may cause undesirable activation of these fibers. To directly manipulate specifically the glutamatergic neurons in a specific region of the DCN, AAV1.CAGGS.Flex-ChR2-tdTOMATO is unilaterally microinjected into the Med nucleus of BAC-*vGluT2::Cre* mouse (Figure 24A). However, the transduction site in this experiment includes the medial part of the Int nucleus. Therefore, the motor response that was observed during photostimulation may also be attributed to partial activation of the Int. Further studies will be required to elucidate the distinct aspects of limb movement regulated by each of the three cerebellar nuclei.

Figure 23. Expression of *vGluT2* in the cerebellum

(A, B) In situ hybridization shows *vGluT2* mRNA is expressed in the DCN but not in the cerebellar cortex. (B) Magnified region from the white box in (A) showing expression of *vGluT2* mRNA in all DCN subnuclei. (C) *vGluT2* (green) and Gad67 (red) protein expression is observed in the Sim lobule of the cerebellar cortex. Expression of *vGluT2* is high in the IGL and ML. Dotted line delineates the IGL, PL and ML (multiple Z-stack images were compressed to identify the PL boundary). IGL, internal granular layer; IntA, anterior interposed nucleus; Lat, lateral nucleus; MedM, medial region of Med DCN; ML, molecular layer; PL, Purkinje layer; Sim, simple lobule.

Med^{vGluT2} neurons give rise to NC fibers in the cerebellar cortex

Glutamatergic neurons in all subnuclei of the Med nucleus (medial part of the Med nucleus, MedM; lateral part of the Med nucleus, MedL; dorsolateral part of the Med nucleus, MedDL) have been transduced (Figure 24C-D). Limited transduction is also observed in the medial regions of the IntA and IntP nuclei (Figure 24E). Consistent with the intra- and extra-cerebellar targeting of neurons in the Med nucleus, labelled puncta were observed within the cerebellar cortex and several key motor centres within the extra-cerebellar region (Figure 24F, 24) (Houck and Person, 2015; Kuramoto *et al.*, 2011; Teune *et al.*, 2000). Glutamatergic NC projections from the Lat nucleus has been reported to send excitatory projections to simple lobule (Sim), Crus 1, Crus 2 and paramedian lobule in the cerebellar cortex (Houck and Person, 2015). In addition, lobules 3, 4 and 5, and simple lobule receive glutamatergic NC projections from the Int nucleus (Houck and Person, 2015; Gao *et al.*, 2016). The ChR2-tdTomato-labelled NC projections from the Med nucleus were observed in lobule 3, 4 and 5 are similar to the

pattern of NC projections from the Int nucleus (Figure 24F) (Houck and Person, 2015; Gao *et al.*, 2016). As the viral transduction is not restricted to the Med nucleus, labelled NC projections could be from both Med and Int nuclei.

Figure 24. Expression of ChR2 within Med^{vGluT2} neurons and nucleocortical projections within the cerebellar cortex

(A) Schematic showing the microinjection of AAV1.CAGGS.Flex-ChR2-tdTomato into Med. (B) Timeline of the experimental paradigm from the start of viral injection. (C, D) Glutamatergic neurons mainly within the MedM and MedL, and to a limited extent the IntP nucleus, are successfully transduced and express ChR2-tdTomato. (E) Analysis of the spread of viral transduction within the DCN. Transduction was observed mainly within the MedM, MedL, MedDL and medial parts of IntA and IntP nuclei. (F) Nucleocortical projections within Lobule 3, 4 and 5 of the cerebellar cortices were labelled with ChR2-tdTomato.

Med^{vGluT2} neurons project to multiple extra-cerebellar regions

In addition to NC projections, glutamatergic neurons within the DCN send excitatory projections to multiple motor centres outside the cerebellum (Houck and Person, 2015; Kuramoto *et al.*, 2011). To explore whether known motor centres indeed receive the glutamatergic projections, sections from the thalamus and red nucleus were analyzed. Puncta expressing ChR2-tdTomato and co-labelled with vGluT2, a marker for labelling excitatory terminals, were observed in multiple thalamic regions (Figure 25A). Labelled puncta can be observed within the ventrolateral (VL), centromedial (CM) and ventromedial (VM) parts of the thalamus (Figure 25A). Int neurons has been shown by others to selectively project to the VL thalamus (Houck and Person, 2015; Kuramoto *et al.*, 2011). Even though in our experiment, transduction of the viral reporter is observed in the Int nucleus, the observed labelled projections in CM and VM thalamus likely originate from the Med nucleus. Therefore, this result suggests that the glutamatergic neurons from the Med nucleus send inputs to a distinct region of thalamus from the Int nucleus and that multiple pathways exist between excitatory DCN neurons and the thalamus.

Other non-thalamic forebrain regions also receive projections from neurons in the Med nucleus (Figure 56B-D). Axonal projections from Med^{vGluT2} are also observed in zona incerta (ZI), red nucleus (RN), vestibular nuclei (VN) and reticular nucleus, which is consistent with previous mapping studies (Figure 25) (Teune *et al.*, 2000). These results demonstrate that Med^{vGluT2} neurons send excitatory inputs to multiple motor centers and the selective expression of ChR2 using the viral injected BAC-*vGluT2*::Cre transgenic mouse can provide direct manipulation of these projection neurons to examine the consequence when the input has been disrupted.

Figure 25. Extra-cerebellar projections from glutamatergic neurons in the DCN

(A) Puncta labelled with ChR2-tdTomato (red) and colabelled with vGluT2 (green) has been observed in VL, CM and VM regions of the thalamus. (B-D) Excitatory puncta expressing ChR2-tdtomato was also observed in zona incerta (B), red nucleus (C) and reticular nucleus (D). Circles indicate colocalization of tdTomato and vGluT2. CM, centromedial; VL, ventrolateral; VM, ventromedial. n = 1 animal.

Photostimulation of Med^{vGluT2} neurons reduces the speed of the mouse

A walkway chamber compatible for concurrent optogenetics testing is used to test the limb kinematics during locomotion (Figure 26A) (Machado *et al.*, 2015). A mirror is placed below the transparent walking platform to deflect the ventral side of the mouse during each trial. After habituating a mouse to the setup, the effects of photostimulation while the mouse was allowed to walk freely along the walkway were tested (Figure 26A). A trial consists of when a mouse walks from one end of the platform to the other. Photostimulation is given at random trials and trajectory of limb movements from the side view and ventral view of the mouse are recorded using a high-speed infrared camera (300 fps, 1648 x 360 pixels) (Figure 26A).

Through video recordings of the side view of the mouse and the ventral view that is deflected through a mirror, details of the limb movements are tracked (Figure 26A, B). A single stride cycle consists of the stance phase, which is considered as the time point when the limb is supported by either the walking platform or the side panel, and the swing phase (Figure 26B) (Machado *et al.*, 2015). With the recordings of different trials, limb movements were manually tracked using MaxTRAQ 2D and limb trajectories were compared between non-photostimulated trials and photostimulated trials. Forward trajectory and vertical trajectory of all limbs are plotted against time (Figure 26C, D). The forward trajectory of the limbs during non-photostimulated trial demonstrates alternating stride phases between both homologous limbs and the diagonal limbs have a synchronized walking pattern (Figure 26C). Since analysis of forward trajectories in non-photostimulated trials revealed regulated and coordinated limb movements, we were in position to start examining the consequences of perturbing Med^{vGluT2} neurons.

Figure 26. Photostimulation of Med^{vGluT2} neurons alters trajectory during locomotion

(A) Schematic and dimensions of the locomotor behavioural setup. (B) All four limbs can be tracked through the ventral side of the mouse using the mirror (iFL, blue; iHL, light blue; cFL, red; cHL, pink). Stride can be divided into stance phase and the swing phase. (C) Representative forward trajectory of the horizontal distance travelled across time for all four limbs during non-photostimulated trials. (D) Representative vertical trajectory during non-photostimulated trial of iFL and iHL. (E) Photostimulation of Med^{vGluT2} neurons showed perturbed limb movement, which is clearly observable at the iHL. (F) Continuous forward trajectory of all four limbs during photostimulation (2 seconds, 30 Hz). (G) Vertical trajectory of iHL showed observable increase in vertical distance from the walking

platform during photostimulation. (H) Both cFL and cHL did not show any observable change during photostimulation. cFL, contralateral forelimb; cHL, contralateral hindlimb; iFL, ipsilateral forelimb; iHL, ipsilateral hindlimb. n = 1 animal.

To determine the effects of optogenetic photostimulation of Med^{vGluT2} neurons, photostimulation of 2000 ms at 30 Hz (16.7 ms pulse duration) is delivered at the start of the trial. Photostimulation of Med^{vGluT2} neurons do not affect the alternation of homologous and synchronization of diagonal limbs during locomotion but phenotypic changes of the ipsilateral limb movements is observed throughout the laser stimulation period (Figure 26E-G). Moreover, disruption to the locomotor movement returned to normal after photostimulation is terminated (Figure 26E-G). iHL is prominently perturbed during photostimulation and is often observed to be raised above the usual height while cFL and cHL do not show observable changes (Figure 25E, G). Instead of returning to the stance position at the walking platform, the side panel is used as a support (Figure 26E). Reduction of stride cycles is also observed, which indicates that disruption of Med^{vGluT2} neurons is sufficient to reduce the speed of the mouse (Figure 26C, F). The reduction of stride cycles can be due to increase stance or swing duration of either one or multiple limbs during locomotion.

To examine more closely how stride kinematics are affected by disrupting Med^{vGluT2} neurons, individual strides are pooled according to strides that are collected during non-photostimulated trials or strides that are prior to photostimulation during photostimulated trials. The data is compared to stride parameters of strides that were made after the photostimulation duration within a single trial to examine if there are possible post-photostimulation effects on locomotion (Figure 27).

After comparison between stride parameters of non-photostimulated strides and photostimulated strides, photostimulation of Med^{vGluT2} neurons reduce the cadence of all four limbs (Figure 27B, Table 3). As a single stride is comprised of a stance and swing phase, the reduction of cadence could either be due to increased amount of time taken during stance phase, swing phase or both phases (Figure 26B). Photostimulation result in a significant increase of the stance duration of cHL but result in a decrease in stance duration of iHL (Figure 27C). Photostimulation of Med^{vGluT2} neurons increase stance duration of both iFL and cFL but the values did not reach significance (Figure 27C, Table 3). As for swing duration, photostimulation result in an increase for both iFL and iHL and decrease in cHL (Figure 27C, D). These results suggest that the reduction of cadence during photostimulation of Med^{vGluT2} neurons could be due to the overall increase in the swing phase of the ipsilateral limbs and increase in stance duration of the cHL as there are observable difference between the traces of limb trajectory during photostimulation, we next look at how disruption of Med^{vGluT2} neurons changed the trajectory distance of the stride kinematics for each limb.

Figure 27. Disruption of Med^{vGluT2} neurons reduce step frequency

(A) Representative stride trajectories of all four limbs. (B) Optogenetic activation of Med^{vGluT2} neurons reduce the cadence of all four limbs. (C) Stance duration of iHL was reduced and increased for cHL during photostimulation. (D) Swing duration of iFL and iHL were increased and cHL had reduced swing duration. $n = 1$ animal; iFL: light-OFF, 122-123 strides; light-ON, 150 – 154 strides. iHL: light-OFF, 124 – 128 strides; light-ON, 161 – 177 strides. cFL: light-OFF, 125 strides; light-ON, 144 strides. cHL: light-OFF, 121 strides; light-ON, 167 strides. Mean $\pm$ SEM; Mann-Whitney test was used to compare the samples. See Table 3.

Photostimulation of Med^{vGluT2} neurons disrupts the vertical positioning of iFL and iHL

The increase in swing duration implies that the ipsilateral limbs either travel a longer distance during locomotion or the limbs could be generally locked in a fixed position during the swing phase during photostimulation (Figure 28D). To examine whether there is an increase in trajectory distance, the Euclidean distance travelled by each limb during swing phase is

measured. Both ipsilateral limbs indeed show increase in trajectory distance and decrease in trajectory speed (Figure 28). These results indicate that photostimulation of Med^{vGluT2} neurons induce prolonged limb movement of ipsilateral limbs.

Figure 28. Both iFL and iHL are continuously engaged during photostimulation of Med^{vGluT2} neurons

(A) Both iFL and iHL travelled significantly longer trajectory distance upon photostimulation. (B) Photostimulation significantly reduced the trajectory speed of both iFL and iHL. $n = 1$ animal; iFL: light-OFF, 122-123 strides; light-ON, 150 – 154 strides. iHL: light-OFF, 124 – 128 strides; light-ON, 161 – 177 strides. cFL: light-OFF, 28 strides; light-ON, 58 strides. cHL: light-OFF, 25 strides; light-ON, 61 strides. Mean $\pm$ SEM; Mann-Whitney test was used to compare the samples. See Table 3.

To determine if photostimulation of Med^{vGluT2} neurons increases the stride length or vertical positioning of the mouse, the x -displacement, average vertical distance travelled across the swing phase (y -displacement) and maximum vertical position reached (y -max) are analyzed. The x -displacement of all four limbs shows no significant changes but both iFL and iHL show an increase in y -max while both contralateral limbs showed decreased in y -max (Figure 29A-C). The time taken for both iFL and iHL to reach y -max also show an increase during photostimulation (time to y -max) (Figure 29D). From these results, photostimulation may induce ipsilateral limbs to be lifted higher while the contralateral limbs will engage stance phase earlier than normal.

Figure 29. Med^{vGluT2} neurons controls vertical ipsilateral limb placement but not horizontal positioning

(A) The stride length of all four limbs were not affected during photostimulation (iFL, blue; iHL, light blue; cFL, red; cHL, pink). (B) The average vertical displacement of the limb from the walking platform across the swing phase is significantly increased for iHL. Both cFL and cHL showed significant decrease in the y-displacement. (C) Activation of Med^{vGluT2} neurons caused both iFL and iHL to reach a higher vertical maximum distance across the swing phase and resulted in lower y-max distance for cFL and cHL. (D) The time to reach maximum vertical position is significantly longer for iFL and cFL. n = 1 animal; iFL: light-OFF, 122-123 strides; light-ON, 150 – 154 strides. iHL: light-OFF, 124 – 128 strides; light-ON, 161 – 177 strides. cFL: light-OFF, 28 strides; light-ON, 58 strides. cHL: light-OFF, 25 strides; light-ON, 61 strides. Mean ± SEM; Mann-Whitney test was used to compare the samples. See Table 3.

The photostimulation parameter used (2000 ms) continuously disrupts Med^{vGluT2} neurons while the mouse is performing the locomotion task. The temporal specificity of optogenetics provide desired photostimulation to be turned on at precise time points and duration. To examine whether perturbing Med during either stance phase or swing phase can affect the limb trajectory, short duration (100ms) of photostimulation was given to Med at random time points and trials that have shown perturbation specifically during stance phase or swing phase were grouped separately and compared. Photostimulation provided during the stance phase only has shown

reduced y-max for iHL while photostimulation at swing phase only has shown increased y-max for both iFL and iHL (Figure 30). Thus, these results suggest that manipulation of Med^{vGluT2} neurons differentially disrupt the stance and swing phases, and that distinct mechanisms are utilized for Med^{vGluT2} neurons to control locomotor movement.

Figure 30. Vertical limb positioning is state-dependent

(A) Short steps of light (100 ms) during the stance phase of iHL significantly reduced its maximum vertical positioning. (B) Photostimulation of either iFL or iHL during swing phase significantly increased y-max. n = 1 animal, iFL: light-OFF, 122 strides; light-ON, 13 strides. iHL: light-OFF 128 strides; light-ON, 11.

Table 3. Stride parameters of all four limbs viral injected BAC-*vGluT2::Cre* mouse

	light-OFF	light-ON	<i>P</i> value	light-OFF	light-ON	<i>P</i> value
	iFL			iHL		
cadence, s ⁻¹	3.319±0.06268	3.144±0.1075	0.0009*	3.422±0.08838	3.045±0.07191	<0.0001*
stance duration, ms	187.4±5.781	207.2±7.702	0.0718	191.1±5.418	183.6±7.122	0.0129
swing duration, ms	129.6±2.835	143.6±3.554	0.0055*	118.3±2.245	171.6±4.492	<0.0001*
trajectory, mm	60.78±0.8377	62.05±1.116	0.0225*	57.92±1.015	66.55±1.21	<0.0001*
trajectory speed, mm/ms	0.4882±0.01004	0.456±0.01099	0.0378*	0.501±0.009234	0.415±0.00932	<0.0001*
<i>x</i> -displacement, mm	57.35±0.8043	55.46±1.223	0.8600	55.63±1.074	53.6±1.393	0.7880
<i>y</i> -displacement, mm	3.342±0.05666	3.402±0.05682	0.4913	4.211±0.0937	6.606±0.1312	<0.0001*
<i>y</i> -max, mm	4.934±0.07747	5.349±0.09171	0.0006*	5.295±0.1331	9.467±0.2157	<0.0001*
time to <i>y</i> -max, ms	77.92±1.698	89.59±2.725	0.0019*	42.4±2.29	103.8±3.823	<0.0001*
	cFL			cHL		
cadence, s ⁻¹	3.222±0.05888	3.061±0.07476	0.0137*	3.289±0.05922	2.998±0.06651	0.0002*
stance duration, ms	187.6±5.4	208.9±7.901	0.0513	194.4±6.576	247.8±10.33	<0.0001*
swing duration, ms	137±3.115	146.4±4.942	0.3235	123.9±2.011	120.5±3.221	0.0264*
trajectory, mm	61.92±2.342	59.5±1.524	0.2064	62.91±1.912	57.84±1.388	0.0835
trajectory speed, mm/ms	0.4826±0.0223	0.4504±0.01415	0.1301	0.5368±0.02017	0.5193±0.01154	0.3945
<i>x</i> -displacement, mm	56.54±0.9665	55.77±1.267	0.5426	57.22±0.8541	57.3±0.9454	0.3961
<i>y</i> -displacement, mm	3.448±0.1263	2.792±0.06625	<0.0001*	3.999±0.1538	3.163±0.101	<0.0001*
<i>y</i> -max, mm	4.989±0.1792	4.281±0.1021	0.0009*	5.251±0.1963	4.357±0.179	<0.0001*
time to <i>y</i> -max, ms	83.33±4.1	83.22±4.48	0.7017	36.93±4.546	43.28±3.097	0.2950

n = 1 animal, iFL: light-OFF, 122 – 123 strides; light-ON, 150 – 154 strides. iHL: light-OFF, 124 – 128 strides; light-ON, 161 – 177 strides. cFL: (cadence; stance duration; swing duration; *x*-displacement) light-OFF, 125 strides; light-ON, 144 strides. (trajectory, trajectory speed, *y*-max, time to *y*-max) light-OFF, 28 strides; light-ON, 58 strides. cHL: (cadence; stance duration; swing duration; *x*-displacement) light-OFF, 121 strides, light-ON, 167 strides. (trajectory, trajectory speed, *y*-max, time to *y*-max) light-OFF, 25 strides; light-ON, 61 strides. Mean ± SEM; Mann-Whitney test was used to compare the samples; *, significant difference.

Discussion

Med^{vGluT2} neurons regulate balance and limb movement during locomotion

The cerebellum plays a critical role in coordinating movements, and cerebellar dysfunction has been linked to myriad of motor disorders (Eccles, 1967; Takahashi and Linden, 2000; Manto *et al.*, 2012; Flament and Hore, 1986; Goodkin *et al.*, 1993). Being the main output sub-structure of the cerebellum, the DCN is presumed to provide regulatory input to other motor centres of the brain (Armstrong and Edgley, 1984; Schultz *et al.*, 1979; Wang *et al.*, 2017; Houck and Person, 2015; Teune *et al.*, 2000; Yamamoto *et al.*, 1979). However, very few studies have examined the locomotor behavioural consequence after direct manipulation of the DCN. Furthermore, how a specific subpopulation of neurons in DCN or subregion of the DCN contributes to motor regulation during locomotion is not clearly defined.

Vesicular glutamate transporter 2 (vGluT2) has been shown to be specifically expressed in DCN but not in the cerebellar cortex (Figure 23) (Hisano *et al.*, 2002). Using viral mediated approach, I targeted the expression of ChR2 specifically within glutamatergic neurons of the Med nucleus. Through this approach, I investigated the effects of photostimulation of Med^{vGluT2} neurons on locomotion. Photostimulation of Med^{vGluT2} neurons during locomotion showed reduced cadence of all four limbs and increased swing duration of both ipsilateral limbs (Figure 26). The increase in swing duration has been shown to be due to increased trajectory length and an increase in vertical limb movement (Figure 29). The results obtained in this chapter provides preliminary findings to the effects of perturbing MedvGluT2 neurons on locomotion. More replicates will be required to provide a definitive function of MedvGluT2 neurons on control of limb positioning.

The lack of significant change of x -displacement during photostimulation of Med^{vGluT2} neurons is consistent with the effects of activating Med neurons through the addition of histamine to the Med nucleus (Figure 29) (Zhang *et al.*, 2014; He *et al.*, 2012). The neurons that were excited by histamine showed a high firing frequency, which is indicative of glutamatergic neurons within the DCN (Zhang *et al.*, 2014). Therefore, Med^{vGluT2} neurons do not mediate the regulation of horizontal limb placement. Interestingly, there were no reports of whether addition of histamine would affect the vertical limb placement of the mice. The acute addition of histamine may not be able to provide a precise temporal perturbation of Med neurons during locomotion and compensatory mechanisms may have been implemented, showing unperturbed vertical limb placement.

Pharmacological activation of Med neurons by histamine in both cerebellar hemispheres showed a decrease in stride width of the hindlimb, indicating that perturbation of the Med nucleus can result in narrower strides in the mediolateral axis. However, in our study, optogenetic activation of Med^{vGluT2} neurons often resulted in iHL positioned on the side panel of the walkway instead of the walking platform, indicating that disruption of Med^{vGluT2} neurons will increase the stride width. These results suggest that disruption Med^{vGluT2} neurons can cause the limb to be laterally positioned and bilateral activation of the Med nucleus, possibly all the neurons subpopulation, could reverse the effects. The increase in stance duration and decrease in y -displacement of contralateral limbs may not be a direct consequence of photostimulation of Med^{vGluT2} neurons but a response to stabilize the position of the body after the ipsilateral limb positioning has been disrupted.

Optogenetic activation of both IntA^{Ucn3} neurons and Med^{vGluT2} neurons resulted in increase of vertical limb positioning (Figure 19, 29). However, we have shown that photostimulation of Med^{vGluT2} neurons reduced the cadence, disrupted the stance duration and swing duration, and these effects were not observed during photostimulation of IntA^{Ucn3} neurons. Lesion in the Med nucleus in humans and rodents has shown deficits in postural control and balance (Modianos and Pfaff, 1976; Ilg *et al.*, 2008). Disruption of Med^{vGluT2} neurons could cause imbalance during locomotion and iFL, cFL and cHL are engaged in a longer stance phase to compensate and maintain balance during locomotion.

Cerebellar regulation and initiation of limb movement

Indirect manipulation of DCN through the photostimulation of PCs has been shown to elicit limb movements when the mice are stationary (Lee *et al.*, 2015). Electrophysiological and mapping studies have shown that DCN neurons could indirectly influence the motor cortex via the thalamus or spinal cord neurons through the RN and reticular nucleus (Kuchler *et al.*, 2002; Liang *et al.*, 2012; Yamamoto *et al.*, 1979; Proville *et al.*, 2014; Teune *et al.*, 2000). In our current study, photostimulation of IntA^{Ucn3} and Med^{vGluT2} neurons have not been shown to elicit an immediate limb movement. The observation that limb movement is not induced upon photostimulation of IntA^{Ucn3} and Med^{vGluT2} neurons could indicate that neurons in the Int and Med nucleus control different motor process not assessed with our behavioural setup. The behavioural setup used in this study allows the mice to walk freely across the platform and photostimulation is only applied while the mice were performing the locomotion task. As compared to the other studies, photostimulation is given when the mice are stationary (Lee *et al.*, 2015; Witter *et al.*, 2013). Different motor centres could be engaged during stationary and locomotion and perturbation during different behaviours may affect limb movements differently.

As PCs are providing inhibitory inputs to all cell types within the DCN, photostimulation of PCs indirectly inhibits all DCN neuronal subtypes and whether non-glutamatergic neuronal subtypes are responsible for eliciting limb movement has not been investigated (De Zeeuw and Berrebi, 1995; Lee *et al.*, 2015; Witter *et al.*, 2013). However, it is noteworthy that photostimulation of Med^{vGluT2} neurons during locomotion resulted in significant reduction of stance duration for iHL but not during photostimulation of IntA^{Ucn3} neurons (Figure 18, 27). These results suggest that continuous photostimulation of Med^{vGluT2} neurons could promote iHL to engage into the swing phase. To examine whether photostimulation of IntA^{Ucn3} or Med^{vGluT2} neurons can evoke movement, photostimulating optically implanted mice head-fixed to the treadmill that locks the mice in the stationary position can be performed. This approach could determine if IntA^{Ucn3} or Med^{vGluT2} neurons can have separate modes of motor function, regulating limb movement during locomotion and generating limb movement when the mice are stationary.

Function of the Lat Nucleus in control of limb movements

The perturbed limb kinematics observed during photostimulation of Int^{Ucn3} and Med^{vGluT2} neurons during locomotion suggest that perturbation of Lat nucleus could elicit similar disrupted limb positioning response. However, the Lat nucleus has been associated to both motor and non-motor functions (Locke *et al.*, 2018; Kuper *et al.*, 2011a). Selective inhibition of D1R⁺ neurons in the Lat nucleus reduced spatial navigation memory, working memory and prepulse inhibition (Kuper *et al.*, 2011a). In fMRI studies, healthy participants who performed verbal working memory tasks showed higher activation levels in the ventro-caudal region of the Lat nucleus.

Bilateral ablation of the Lat nucleus reduced synchronization of licking and bar pressing as compared to control rats (Vajnerova *et al.*, 2000).

The rostral region of the Lat nucleus has been shown to be associated to motor function (Schultz *et al.*, 1979; Kuper *et al.*, 2011a). In monkeys, microstimulation of the rostral region of the Lat nucleus resulted in ipsilateral flexion of the elbow and dorsiflexion of the ankle in the hindlimb (Schultz *et al.*, 1979). Dorso-rostral of the Lat nucleus in humans showed higher levels of activity when simple finger movements were made (Kuper *et al.*, 2011a). These results suggest that glutamatergic neurons in the rostral region of the Lat nucleus control limb movement and disruption of glutamatergic neurons in the caudal region of the Lat nucleus will reduce cognitive performance.

In this chapter, we expressed ChR2 in glutamatergic projection neurons in the Med nucleus of *vGluT2::Cre* mice using a Cre-dependent viral construct. We found that Med^{vGluT2} neurons project to thalamic nuclei, red nucleus, zona incerta and reticular nucleus. Photostimulation of Med^{vGluT2} neurons during locomotion reduced the cadence and the increased vertical limb positioning. These results indicate that Med^{vGluT2} neurons are important for proper gait, and that a more general manipulation of Med^{vGluT2} neurons in both cerebellar hemispheres impedes locomotion. As the experiment is still in the exploratory phase and only one mouse was used in the analysis, conclusion made on the function of Med^{vGluT2} neurons is preliminary.

Chapter 4: Significance and Future Directions

Summary of Significance and Future Directions

The cerebellum regulates a multitude of motor functions (Eccles, 1967; Takahashi and Linden, 2000; Manto *et al.*, 2012). Imaging studies have shown that damage either to the cerebellar cortex or the deep cerebellar nuclei (DCN) is associated to aberrant and impaired limb movements (Flament and Hore, 1986; Gilman, 1969; Goodkin *et al.*, 1993; Ilg *et al.*, 2007). Despite ample evidence associating cerebellum to numerous motor disorders, these imaging studies lack the resolution to determine the exact cell types involved in regulation of motor movement. Thus, this project aimed to take a multi-disciplinary approach to examine the organization of cerebellar output circuits and to define the functional relevance of these circuits in control of motor behaviors.

Reversible and non-reversible lesion studies in animals have begun to reveal the relevance of components of the cerebellum in motor behaviours, but these techniques lack specificity as these techniques can affect beyond the intended cerebellar region due to afferent connections that originate from extra-cerebellar regions terminating in the cerebellum. By using optogenetics, with high level of spatial and temporal specificity, studies by others have investigated the functional contribution of cerebellum towards motor and non-motor functions (Proville *et al.*, 2014; Sarnaik and Raman, 2018; Witter *et al.*, 2013; Lee *et al.*, 2015). However, majority of the optogenetic studies performed on animals focused on the cerebellar cortex, which integrate afferent inputs and converge to the DCN, and how the direct perturbation of the DCN affects motor functions is not well established. My project represents one of the first attempts to dissect the connectivity and function of select neuronal populations in the DCN, and how they control locomotion.

The performance of locomotion of rodents have been studied using several behavioural tests (Mendes *et al.*, 2015; Brooks and Dunnett, 2009; Deacon, 2013). Rotarod, balance beams and footprint analysis are able to assess motor deficits when the cerebellar activity is perturbed . However, most of these behavioural tests are unable to reveal subtle changes in the limb kinematics and kinetics which requires high-speed video recording. A suitable commercially available behavioural analysis equipment capable of analyzing the gait parameters has been developed, but the high cost of the equipment and inability to customize individual components lead us to develop a different strategy (Appendix I) (Bellardita and Kiehn, 2015). The locomotor behavioral setup developed and described in this thesis, thus provides an exciting alternative for analyzing mouse locomotor behavior at a low cost and permits changing different parts of the walkway for optogenetics circuit manipulation, and examination of a wide range of limb kinematics.

In order to examine a specific subpopulation of neurons in the DCN, one of the most important contributions of this project to the cerebellar field is our identification of the *Ucn3::Cre* transgenic mouse line. Results from my project provided the initial characterization of the localization of targeted neurons as well as the electrophysiological and histological profiles of these neurons. Assessment of the recombined neurons using the *Ucn3::Cre* transgenic mice showed that the neurons are the excitatory glutamatergic neurons. To examine if manipulation of $\text{IntA}^{\text{Ucn3}}$ neurons and their related circuits disrupts locomotion, I have expressed channelrhodopsin within the neurons and established an optogenetics setup. In addition, I have constructed a behavioural setup that is compatible to optogenetics to investigate the effects of perturbing $\text{IntA}^{\text{Ucn3}}$ neurons in locomotion. Multiple prototypes were constructed before finalizing a cost-effective design that enables the mice to walk freely along the walkway without

the need of intervention from the experimenter. Furthermore, the setup can be disassembled to be transported, allow thorough washing of the components to prevent traces of odour left by the mice, which may interfere with subsequent behavioural experiments, and can be readily set up in less than 3 minutes. Importantly, the characterization of *Ucn3::Cre* recombined neurons and optogenetic manipulation of these neurons, as well as the development of the walkway provides necessary groundwork for future analysis of the upstream and downstream circuits of this important subset of DCN output neurons.

Figure 31. Different configurations of the mouse locomotor walkway

A) A walkway with openings on the top and a single side of the walkway. B) A narrower walkway with openings on the ends and a slit on the top to allow only the optic fiber cable to access.

Figure 32. Blueprints of the finalised walkway

Walkway that was constructed based on previous prototypes that include improvements required for the experiments. The walkway was designed to allow the mice to walk freely without intervention from the experimenter and is compatible to optogenetics setup. The walkway can be disassembled for complete removal of animal odour and transported.

An important question we set out to address is whether the Ucn3-subset of DCN neurons selectively regulate discrete movement such as skilled reach or rhythmic movement such as locomotion, or perhaps these neurons regulate both. Perturbation of $\text{IntA}^{\text{Ucn3}}$ neurons did not affect the stride length resulted in increased in vertical positioning of the ipsilateral forelimb. In collaboration with a colleague, using targeted-ablation strategy, diphtheria-induced ablation of $\text{IntA}^{\text{Ucn3}}$ neurons did not affect the spatial but disrupted the speed of the ipsilateral forelimb

movements. These results suggest that the IntA nucleus may control specific muscles that drives flexion movements and may regulate skilled movements. To elucidate whether the IntA nucleus regulate skilled movements, IntA^{Ucn3} neurons were perturbed during skilled reaching tasks and resulted in hypermetric movements (Low *et al.*, 2018). Together, our results demonstrate that IntA^{Ucn3} neurons are capable of regulating both discrete and rhythmic forelimb movements, and raises the important question of whether distinctions in their target selectivity or sources of input innervations distinguishes these two important limb behaviors

In summary, because of the lack of specific tools to manipulate neuronal subpopulations within the DCN, this study provides an important transgenic mouse line that can be used to investigate other motor or non-motor functions regulated by discrete subsets of neurons in the IntA nucleus. Using the newly identified *Ucn3::Cre* mouse line, I examined the functional significance of genetically-accessible subset of cerebellar nuclear neurons through the optogenetic manipulation and chemogenetic ablation and found that Ucn3- positive neurons regulate the vertical positioning of the limb during locomotion and end-point targeting of the forelimb during skilled reaching movements. In addition, the walkway that I developed can be used to investigate walking abnormalities caused by perturbation of different regions of the cerebellum at a fraction of the cost of commercially available locomotor analysis equipment.

Disruptions in the cerebellum has been associated to motor disorders like ataxia and dysmetria, and complex cognitive disorders like autism spectrum disorder and schizophrenia (Yeganeh-Doost *et al.*, 2011; Wang *et al.*, 2014; Schmahmann, 2004; Bauman and Kemper, 2005). The overarching goal of our research is to elucidate the functional contributions of discrete cerebellar output pathways to motor and non-motor functions. A systematic examination

of individual identifiable pathways originating from the cerebellar nuclei will aid in unravelling the pathophysiological contributions of the cerebellum towards motor and non-motor behavioural deficits and may serve as a compelling target for future treatment strategies. For example, aberrant neuronal activity within neuronal subpopulations in the cerebellar nuclei can be modulated to normal physiological levels through electrical or optogenetic stimulation and pharmacological manipulation to selectively ameliorate motor or non-motor symptoms.

An important question which has not been resolved in this study is whether targeted inactivation of neuronal subpopulations in the DCN is capable of disrupting locomotor kinematics. Even though we have provided some evidence that the ablation of neurons in the interposed nucleus perturbed the accuracy of both skilled reach and locomotion, we cannot rule out the possibility that destruction of these neurons caused non-specific disruptions to input and output pathways, and whether compensatory mechanisms occurred. Thus, an important experiment which we hope to conduct is to selectively express, Archaeorhodopsin (Arch) or soma-targeted *Guillardia theta* anion-conducting channelrhodopsin 2 (stGtACR2) within neuronal subpopulations in the DCN (Mahn *et al.*, 2018; Chow *et al.*, 2010a). Using the optogenetics setup that was established, deficits in motor functions will be examined upon photoinhibition of the neurons. In addition, the electrophysiological properties of the cells within the DCN can be examined using recording tetrodes when the mice perform the motor tasks to observe a change in neuronal firing pattern.

The regulation of discrete and rhythmic motor behaviours through the Int nucleus generates a hypothesis that the Int nucleus could regulate motor adaptation. In addition, to determine if DCN is involved in motor adaptation, we will assess the consequence of

photoinactivation of the DCN neurons when the mice perform joystick push-pull task and knob-turn task. Conditional AAV expressing Arch or stGtACR2 will be injected into the DCN of the Cre-expressing transgenic mice. Photoinhibition of the neurons will be assessed through multielectrode electrophysiological recordings (NeuroNexus) in the Int. The projections of IntA^{Ucn3} neurons to the VA-VL thalamus, which is linked to the sensory cortex, generates the hypothesis that silencing of IntA^{Ucn3} neurons will affect motor adaptation.

Markerless tracking of limb and tail movements through transfer learning with deep neural networks

Our custom walkway provides the ability to analyze a mouse from the side view and the ventral view, which is deflected from the mirror positioned at the bottom of the walking platform. Video that was captured using the 300 fps high-speed infrared camera permits tracking of four limbs from the ventral view of the mice and tracking of the limbs of the forelimb and hindlimb that is in view with the camera. Manual tracking of all six points for each trial is time consuming and subjective. High-throughput of the analysis can be achieved using an automated tracking software. A highly efficient tracking system has been developed that is capable of markerless tracking (Mathis *et al.*, 2018). Training the feature detector, based on deep neural networks, from DeeperLabCut with approximately 200 images is sufficient to train the network to reach high level of accuracy during the automated tracking of limb positions. Using this automated markerless tracking system, analysis could be completed 12 times faster as compared to manual tracking and permit high-throughput for the analysis of limb kinematics without experimenter bias.

Summary

By using the *Ucn3::Cre* transgenic mouse line, we identified a genetically defined subpopulation of neurons in the IntA nucleus (IntA^{Ucn3}). Analysis of the electrophysiological and molecular properties of these neurons revealed that IntA^{Ucn3} neurons are a subset of glutamatergic neurons within the IntA nucleus. In order to determine whether and how IntA^{Ucn3} neurons regulate locomotion, we developed a walkway that allows mice to walk freely and concurrent optogenetic manipulation. We generated the *Ucn3::Cre; Rosa::ChR2-eYFP* mice which selectively express ChR2 in IntA^{Ucn3} neurons. We show that photostimulation of IntA^{Ucn3} neurons increased vertical limb positioning of ipsilateral forelimb and hindlimb. We examined consequences of optogenetic manipulation of glutamatergic neurons in the Med nucleus by microinjecting AAV1.*CAGGS.Flex.ChR2-tdTomato* virus into the Med nucleus of BAC-*vGluT2::Cre* mice. Photostimulation of Med^{vGluT2} neurons resulted in drastic changes in limb kinematics for ipsilateral forelimbs and hindlimbs, which is very different from the phenotype observed through manipulating IntA^{Ucn3} neurons. In short, despite the heterogeneity of neurons located across the different subnuclei of the DCN, we believe that there are anatomical, molecular and functionally distinct neuronal subpopulations in each subnuclei that regulates distinct aspects of limb positioning during locomotion. Disrupting the Med^{vGluT2} neurons will result in greater locomotor irregularity as compared to the effects of disrupting IntA^{Ucn3}. It would be intriguing to investigate if IntA^{Ucn3} neurons indeed control only a specific aspect of locomotion or these distinct subpopulation of glutamatergic projection neurons regulate other non-motor behaviours that is yet to be clearly examined.

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Appendix

Appendix I

Bronjo Medi

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email: bronjo@singnet.com.sg web: www.bronjo.com

20 February 2018

Billing and delivery address:

To be received by Albert Chen/Alaric Yip
Nanyang Technological University
School of Biological Sciences
Clinical Sciences Building
11 Mandalay Road, Level 12-02-01
Singapore 308232

Dear **Albert Chen/Alaric Yip**,

QUOTATION – Q/ NTU-LKCSom/200218a-MotoRater For the supply of Laboratory Articles

We are pleased to provide the price information of the MotoRater from TSE as follow:

Description	Qty	Unit Price Eur	Total Price Eur
E-303030-RM MotoRater Mouse / Rat Kinematic Analysis System Complete innovative system for standardized quantitative and qualitative evaluation of rodent locomotor function and coordination. The modular system allows to investigate kinematic parameters of different types of locomotion: <ul style="list-style-type: none">• skilled walking on a ladder with regular or variable spacing• over-ground walking• wading• swimming Main hard- and software components: 1. Arena construction / parts: <ul style="list-style-type: none">• High optical quality water basin including modular inserts for• ladder running test mouse• ladder running test rat• over-ground walking• wading• swimming• space restriction unit for mouse• exit ladder• home & shelter cage• basin mounted on dedicated mobile rack system including 4 castors (2 with brakes) 2. High performance lighting system for optimized illumination ensuring optimal high-speed video monitoring of test animal from 3 sides (below / left / right) 3. Exchangeable top background frame ensuring optimal optical contrast for video monitoring 4. Mirror system: complete with two dedicated mirrors	1	99,375.00	99,375.00

Bronjo / Q/ NTU-LKCSom/200218a-MotoRater

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<p>5. Optical system consisting of:</p> <ul style="list-style-type: none"> • high-speed color video-camera • mini video camera • automated feed-back controlled camera driving system for automated animal recognition and tracking <p>6. Pump and pipeline for water</p> <p>7. Data Acquisition & Automated Experiment Control System</p> <ul style="list-style-type: none"> • Industrial Computer System with Monitor and minimum 1TB SSD Hard-Drive <p>8. Dedicated High-Speed Video Analysis software package. Kinematic, image based motion analysis software supporting several DV or high-speed video cameras. "TSE Motion" analysis software integrates TSE MotoRater high-speed videos and other common video formats into a user-friendly, intuitive graphical user interface which also includes functions such as reports, algorithms, filters and smoothers, phases and events and extensive export options. The automatically tracked data of selected anatomical markers of the body can be illustrated in diagrams, synchronized with the video and calculated for unlimited parameters useful for the kinematic description of animals walking, swimming or any other movement.</p>	
<p>(Optional) E-303030-M MotoRater Mouse Kinematic Analysis System Complete innovative system for standardized quantitative and qualitative evaluation of rodent locomotor function and coordination. The modular system allows to investigate kinematic parameters of different types of locomotion:</p> <ul style="list-style-type: none"> • skilled walking on a ladder with regular or variable spacing • over-ground walking • wading • swimming <p>Main hard- and software components:</p> <p>1. Arena construction / parts:</p> <p>High optical quality water basin including modular inserts for</p> <ul style="list-style-type: none"> • ladder running test mouse • over-ground walking • wading • swimming • exit ladder • home & shelter cage • basin mounted on dedicated mobile rack system including 4 castors (2 with brakes) <p>2. High performance lighting system for optimized illumination ensuring optimal high-speed video monitoring of test animal from 3 sides (below / left / right)</p> <p>3. Exchangeable top background frame ensuring optimal optical contrast for video monitoring</p> <p>4. Mirror system: complete with two dedicated mirrors</p> <p>5. Optical system consisting of:</p> <ul style="list-style-type: none"> • high-speed color video-camera • mini video camera • automated feed-back controlled camera driving system for automated animal recognition and tracking 	<p>1 93,125.00 --</p>

6. Pump and pipeline for water 7. Data Acquisition & Automated Experiment Control System <ul style="list-style-type: none"> Industrial Computer System with Monitor and minimum 1TB SSD Hard-Drive 8. Dedicated High-Speed Video Analysis software package. Kinematic, image based motion analysis software supporting several DV or high-speed video cameras. "TSE Motion" analysis software integrates TSE MotoRater high-speed videos and other common video formats into a user-friendly, intuitive graphical user interface which also includes functions such as reports, algorithms, filters and smoothers, phases and events and extensive export options. The automatically tracked data of selected anatomical markers of the body can be illustrated in diagrams, synchronized with the video and calculated for unlimited parameters useful for the kinematic description of animals walking, swimming or any other movement.			
E-999901-12-30 Application Training On-Site First day User oriented training / follow-up training. Including all travel costs.	1	3,500.00	3,500.00
E-999901-12-31 Application Training On-Site Following Consecutive 2nd Day User oriented training / follow-up training. Including all travel costs.	1	2,000.00	2,000.00
E-999901-12-25 Application Training Remote 4 hours User oriented training / follow-up training. Requirements: <ul style="list-style-type: none"> Fast internet connection with access to your system Microphone & speakers or a headset 	1	490.00	490.00
E-999901-12-06 Installation / Instruction / initial Training on-site, including all travel costs <ul style="list-style-type: none"> System installation System instruction Initial training Application training (remote or on-site) can be ordered separate.	1	6,250.00	6,250.00
Insurance/ packing / shipping / customs:			5,228.64
Total system:			116,843.64

Price excludes GST.

- Specifications and prices are subject to change.
- All banking charges and fees are to be paid by the purchaser.
- Positions marked with "Optional" are not included in the TOTAL.
- If fees and charges for freight, insurance and packing are shown, they do not refer to the positions marked with "Optional".

Warranty Information:

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