Bone Morphogenetic Protein signaling in structural plasticity of cerebellar mossy fibers

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Abstract

ABSTRACT

Establishment of precise neural wiring is crucial for proper functioning of the brain. Neuronal circuits are formed and refined during embryonic and postnatal development. However, mature circuits and their connections remain plastic and can change with learning and adaptation. Structural plasticity is one fundamental way in which neuroplasticity is achieved, and it involves changes in morphology and number of synapses. While is it well-described that structural plasticity can be elicited by specific patterns of neuronal activity, the molecular and cellular mechanisms underlying such structural changes are poorly understood.

The cerebellum is a brain structure best known for its role in coordinating skilled, fine tuned movements and motor learning. The cerebellar circuit is an ideal model to study structural plasticity because its cellular elements are simple and well characterized. Mossy fibers, arising from a collection of precerebellar nuclei, relay cortical and proprioceptive information to Purkinje cells indirectly via granule cell. Purkinje cells integrate mossy fiber information with inputs directly received from climbing fiber afferents emerging from the inferior olivary nucleus. Golgi cells, an inhibitory interneuron that is innervated by mossy fibers, control timing and distribution of mossy fiber-derived information, resulting in feedforward inhibition. Structural growth of this feedforward inhibition was implicated in motor learning. However, the molecular mechanism of such structural plasticity and most forms of structural plasticity remains largely unknown.

Here, I report that the canonical Bone Morphogenetic Protein (BMP) pathway is a positive regulator of structural plasticity of cerebellar mossy fibers. I demonstrate that structural plasticity of cerebellar mossy fibers can be elicited by mossy fiber activation. Furthermore, I present data that suggests that BMP signaling pathway is regulated during learning. In sum, this work highlights novel roles for BMP signaling pathway in cerebellar circuit, extending beyond early developmental functions.

Introduction

INTRODUCTION

General introduction

It is arguable that the human brain is one of the most complex organs known to mankind. The billions of neurons interconnected with each other form neural circuits. These circuits are the basic functional units of the brain. Emergent properties of a group of neurons firing in synchrony together gives rise to proper brain functions. This results in day-to-day behaviors such as respiration, movement and cognition. Any dysfunctions in neural circuits gives rise to disorders like Parkinson's disease, Autism Spectrum Disorder and Schizophrenia just to name a few. Trying to understand the brain from a circuit perspective allows one to retain an emergent view of brain function, while keeping a molecular resolution. Thus, is it a good entry point to gather mechanistic understanding of the brain at the molecular level.

An intriguing question is how the brain is not only an integral part that helps us to sense and process information but also to reliably and reproducibly drives behaviors. The system has to possess elements of both stability and plasticity to achieve that. Modification of behaviors in response to the environment is a form of learning. It is thought that the learning process relies on neuroplasticity. At a very fundamental level, plasticity can occur in at least two ways, namely, functional plasticity and structural plasticity. Functional plasticity is where the connection strength in one given synapse strengthens or diminishes. In contrast, structural plasticity is where numbers of synapses are increased or decreased. Although described separately, they do not happen in a mutually exclusive manner. Both forms of plasticity depend on neuronal activity, and have been

correlated with learning. Therefore, these processes form the basis of the prevailing theory in our understanding of learning and memory.

While cellular aspects of brain circuits and plasticity are relatively well characterized, the molecular aspects controlling these processes remain largely unknown. A broad aim of my graduate work is to understand the molecular mechanisms underlying structural plasticity.

Cellular aspects of structural plasticity

Plasticity in the nervous system

Functional plasticity involves changes in synaptic strength between two connecting neurons. This involves the idea that two connecting neurons that fire simultaneously, will have stronger connection, and vice versa. Donald Hebb first postulated this idea in 1949. Until now, the succinct paraphrase “Neurons that fire together, wire together” still hold true (Seung, 2000). Classical and contemporary work has firmly established the occurrence of functional plasticity in the form of long-term potentiation (LTP) and long-term depression (LTD). Bliss and Lomo (Bliss and Lomo, 1973) were the first to describe LTP in rabbit hippocampus, where they observed a sustained increase in synaptic strength in the same population of neurons that underwent a tetanic stimulation. LTD was observed in the hippocampus after sustained low frequency stimulation of the perforant pathway (Bramham and Srebro, 1987) and in the cerebellum after continuous simultaneous stimulation of parallel fibers and climbing fibers (Ito and Kano, 1982).

Evidence that functional plasticity is directly related to learning and memory came from work by Eric Kandel and colleagues in Aplysia. They have found that there are changes in synaptic efficacy between sensory and motor neurons upon a rigorous gill-withdrawal reflex training (Castellucci et al., 1970, Kandel, 2001). They also found the underlying neural circuit and molecular mechanism that drives this facilitation (Kandel, 2001). It turned out that a modulatory serotonergic input from the tail innervates the sensory and motor neuron pool in the Aplysia abdominal ganglia. The training results in an increase of PKA and

cAMP signaling levels in the sensory neurons, which sensitized its response to a tactile stimulus. This short-term facilitation led to an enhanced response of the motor neuron downstream of the sensory neuron (Kandel, 2001), which was read out as a gill withdrawal response. Long-term facilitation mechanisms included CREB signaling pathway and transcription of other genes.

Dendritic structural plasticity

Changes in connection strength during neuronal plasticity can be achieved by changing the properties of an existing set of synaptic connections. Alternatively, such plasticity can also be accompanied by structural changes. Structural plasticity adds on another literal dimension to the mechanisms that bring about physical changes in neural circuits. Dendritic spines are the postsynaptic ends of glutamatergic synapses and are hotspots for structural plasticity.

Early work by Fifkova and colleagues (Fifkova and Van Harreveld, 1977) reported a change in the number of dendritic spines in hippocampal tissues that were subjected to tetanic stimulation. Although is it one of the first studies drawing a link between LTP and structural changes in neural circuit, samples obtained were postmortem and unpaired, interpretation of the data is limited. The first time-lapsed imaging of dendritic spines after LTP induction in hippocampal slices were done by Hosokawa et al. (Hosokawa et al., 1995). They saw an increase in spine length and change in spine orientation after repeated imaging of hippocampal slices after chemical LTP induction. Following the advent of 2-photon microscopy, it was possible to observe the very same set of synapses in vivo and track their dynamics at a higher resolution. Engert et al. (Engert and Bonhoeffer, 1999) and Maletic-Savatic et al. (Maletic-Savatic et al.,

1999) independently reported local dendritic spinogenesis in response to LTP induction or focal activity stimulation. Their work established that dendritic spine growth could be induced following neuronal activity.

Recently, work by two independent groups, Xu et al. (Xu et al., 2009) and Yang et al. (Yang et al., 2009), has studied spine dynamics in the context of motor learning. Both groups trained animals on skilled motor tasks (accelerating rotarod and single-seed reaching task) and observed a dramatic change in initial spine formation, and followed by spine elimination. The rate of spine turnover positively correlated with behavioral performance, suggesting that dendritic spine plasticity is the structural basis of learning. Furthermore, their work has demonstrated that the phenomenon of dendritic plasticity is an underlying physical substrate for long-term memory.

Taken together, progress in the field of dendritic spine plasticity over the past four decades has firmly established that dendritic spine dynamics are altered by LTP, and this turnover of spines in a neural circuit is a structural basis for learning and memory.

Axonal structural plasticity

Although many work on structural plasticity focused on dendritic spines, work on axonal structural plasticity is catching up. An early work in this field is by De Paola et al (De Paola et al., 2003), where they characterized hippocampal mossy fiber terminals, large mossy fiber terminals (LMT), dynamics in organotypic culture using time-lapsed confocal imaging. Their work revealed that there are distinct types of axonal arbors in hippocampal mossy fibers: *en*

passant varicosities, LMT and secondary LMT. These morphologically distinct structures have different synaptic partners and rates of plasticity in mature hippocampal circuit. The secondary LMT is the most plastic among the three types of hippocampal mossy fiber axon terminals. Although this work has provided evidence for the existence of structural plasticity in a defined neuronal cell type, the observation nonetheless remains observed in an in vitro set up. Its relevance in in vivo neural circuits remained to be established.

The first reports of in vivo structural plasticity in the axon terminal in adult cortical circuits came from De Paola et al. (De Paola et al., 2006) and Stettler et al. (Stettler et al., 2006). It was shown that in axonal plasticity exist at baseline (i.e. without any sensory manipulations or learning paradigms). In addition, different subtypes of neurons in the cortex undergo different rates of baseline turnover (De Paola et al., 2006). Following the baseline characterization of axonal structural plasticity, Galimberti et al. (Galimberti et al., 2006) addressed the effects of experience on axonal structural plasticity. In their study, they observed the hippocampal LMT in animals of different ages, and with or without exposure to sensory enrichment in an enriched environment (EE). They found that the LMT displayed an increase in complexity with age and also animals exposed to EE display more complex LMT than the control counterparts.

More recently, Ruediger et al. (Ruediger et al., 2011) showed a correlation between learning of Morris water maze (a hippocampus-dependent task) and increase in hippocampal LMT complexity. They proposed that the increase in LMT complexity enhanced the feedforward inhibition in the local network,

thereby having an impact on output of the circuit to change behavior. They also had evidence suggesting that such learning-induced structural plasticity is not only limited to the hippocampus, but also observed in the cerebellar mossy fibers (Ruediger et al., 2011). Their work has implicated plastic structural changes in a network during the process of learning. Furthermore, axonal plasticity is also observed in two distinct populations of neurons in the cerebellum: climbing fibers (Nishiyama et al., 2007) and parallel fibers (Carrillo et al., 2013). These studies had correlatively linked axonal activity and motor learning paradigm to changes in axon structural dynamics, respectively.

In sum, these recent work has collectively established that axonal structural plasticity occurs at baseline, and this structural change in axon terminal seems to be a substrate for learning and memory. Axonal plasticity occurs in different populations of neuronal axon terminals, induced by a relevant learning paradigm. For instance, structural changes in hippocampal mossy fibers are induced by hippocampus-dependent task (Ruediger et al., 2011), structural changes in cerebellar mossy fibers required a cerebellum-dependent learning task (Nishiyama et al. 2007; Carrillo et al. 2013).

Cerebellum is involved in motor, cognitive and affective processing

In this study, cerebellum was used as a model system to understand mechanisms of structural plasticity. The cerebellum is an important site for regulating the learning process. It is a distinctive brain structure that lies in the hindbrain. It contains the bulk of the neurons in the brain, and has extensive foliation. Traditionally, the cerebellum is associated with motor processing. It is

not required for orchestrating motor programs per se, but they are crucial for real time feedback of ongoing movements, motor learning and adaptation. As such, having lesions in the cerebellum results in motor phenotypes like ataxia and loss of muscle coordination. The cerebellum receives about 40 times more inputs than outputs. Most of its inputs come from the peripheral sensory feedback, premotor and primary motor areas. Anatomically, the cerebellum can be divided into three distinct components: vermis, paravermis and hemisphere (Figure 1.1B).

Although the basic circuitry of the cerebellum is stereotypic over the whole cerebellum, different parts of the cerebellum receive different inputs from various parts of the brain and spinal cord. As such, different cerebellar regions process information related to different body parts and/or modalities. The vermis region receives inputs from the visual, auditory and vestibular systems, and thus has roles in maintaining balance, posture and gaze. The paravermis receives inputs from the somatosensory inputs of distal limbs, and thus have roles in controlling fine motor skills. Lastly, the hemisphere area receives extensive input from the cerebral cortex. Thus it has functions in motor planning, execution and mental rehearsal of complex movements.

The cerebellum receives afferent inputs from the inferior olive and a collection of precerebellar nuclei. Of these inputs, pontine gray nucleus is one of the major afferent inputs into the cerebellum (Figure 1.1A). It relays inputs from the cerebral cortex and project as mossy fibers into the cerebellum. The main projection area of pontine mossy fibers in the cerebellum is Crus I and II (Figure

1.1B), which is the cerebellar hemisphere lobules 6 and 7. Pontine mossy fibers are the major source of mossy fibers into the cerebellum. It is also a major site of cortical relays into the cerebellum (Figure 1.1A).

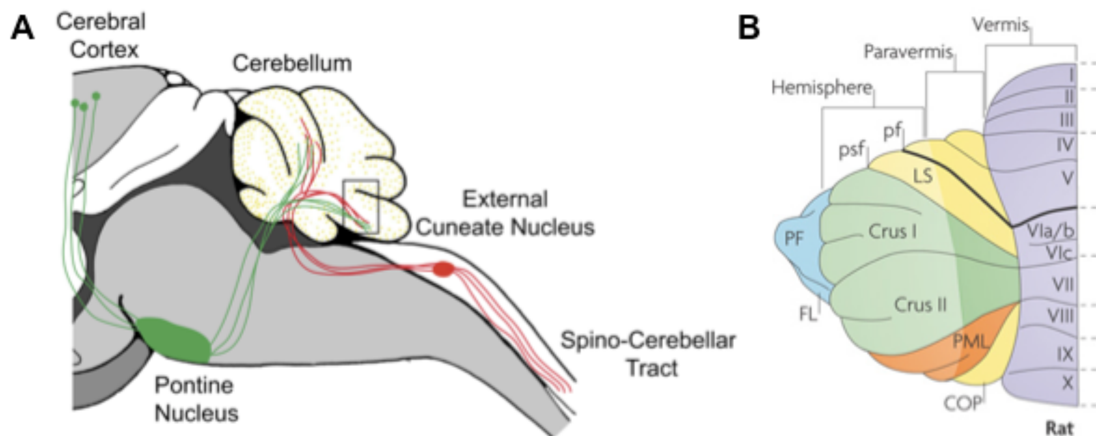

Figure 1.1 Schematic diagrams of the cerebellum. **A** Parasagittal view of the hindbrain. There are two classes of mossy fibers that project into the cerebellum – pontine mossy fibers and external cuneate mossy fibers (adapted from (Hatten and Lisberger, 2013). **B** Posterior view of the cerebellum. The cerebellum has 10 lobules and 3 lateral regions: vermis, paravermis and hemisphere (adapted from (Apps and Hawkes, 2009).

Besides motor functions, contemporary findings has also implicated cerebellum in cognitive and affective functions. Evidences came from neuroanatomical tracing studies, functional neuroimaging and clinical observations. Anatomical tracing studies using transneuronally transported viruses have shown a reciprocal connection between cerebellum and area 46 of prefrontal cortex in monkeys (Kelly and Strick, 2003). The physical connectivity between the cerebellum and area of the brain associated with cognitive functions suggests that cerebellum is involved in higher cognitive functions. Furthermore, functional

Magnetic Resonance Imaging in healthy individuals performing cognitive tasks such as Wisconsin Card Sorting Test (WCST) showed activation of cerebellum in addition to the frontal cognitive regions of the brain (Lie et al., 2006). As the WCST is used clinically to assess executive functions, this is direct evidence that the cerebellum is involvement in cognitive tasks. Lastly, the Cerebellar Cognitive Affective Syndrome, a condition where patients with cerebellar lesions have deficits in language, spatial processing, altered emotional processing, further implicated cerebellum in cognitive and affective functions (Schmahmann and Sherman, 1998).

Recent functional imaging studies has showed that cerebellum function are modular in nature: specific network loops of the cerebellum are involved in different types of behaviors, be it motor or cognitive (Salmi et al., 2010, Stoodley et al., 2012). The anterior lobules of the cerebellum (connecting with the sensorimotor cortex) has been associated with motor functions whilst posterior lobules of the cerebellum (connecting with frontal association cortices) has been linked to cognitive and affective processing (Stoodley et al., 2012). Taken altogether, the cerebellum functions more than motor computation unit, it also has roles in cognitive and affective processing. These functions are carried out by discrete parts of the cerebellum in parallel; hence the cerebellum displays modular compartmentalization of various computations it performs.

An entry point to understanding how the cerebellum might function is to study it at a circuit level. This perspective allows room for emerging properties of brain function to be considered, while not losing cellular and molecular resolution

during analysis. Below, I will describe the core components of the cerebellar circuit, with focus on cerebellar mossy fibers and its local inhibitory circuitry. The pontine mossy fibers are the focus of this study as they represent the bulk of mossy fiber afferents input and also it is the major cortical relay into the cerebellum.

Cerebellum as an model circuit

The cerebellum is an ideal model to study structural plasticity due to its relative simplicity and stereotypy. The cerebellar circuit receives two afferent inputs: climbing fibers and mossy fibers.

Climbing fibers, from the inferior olive, innervates Purkinje cells directly, while mossy fibers, arising from a collection of pre-cerebellar nuclei, connect indirectly with Purkinje cells via granule cells. Purkinje cells are the sole output neuron of the cerebellar cortex. It connects with the deep cerebellar nuclei to project to other areas of the brain (1 1.2A). The pontine gray nucleus is the main source of mossy fiber inputs, relaying cortical inputs, into the cerebellum.

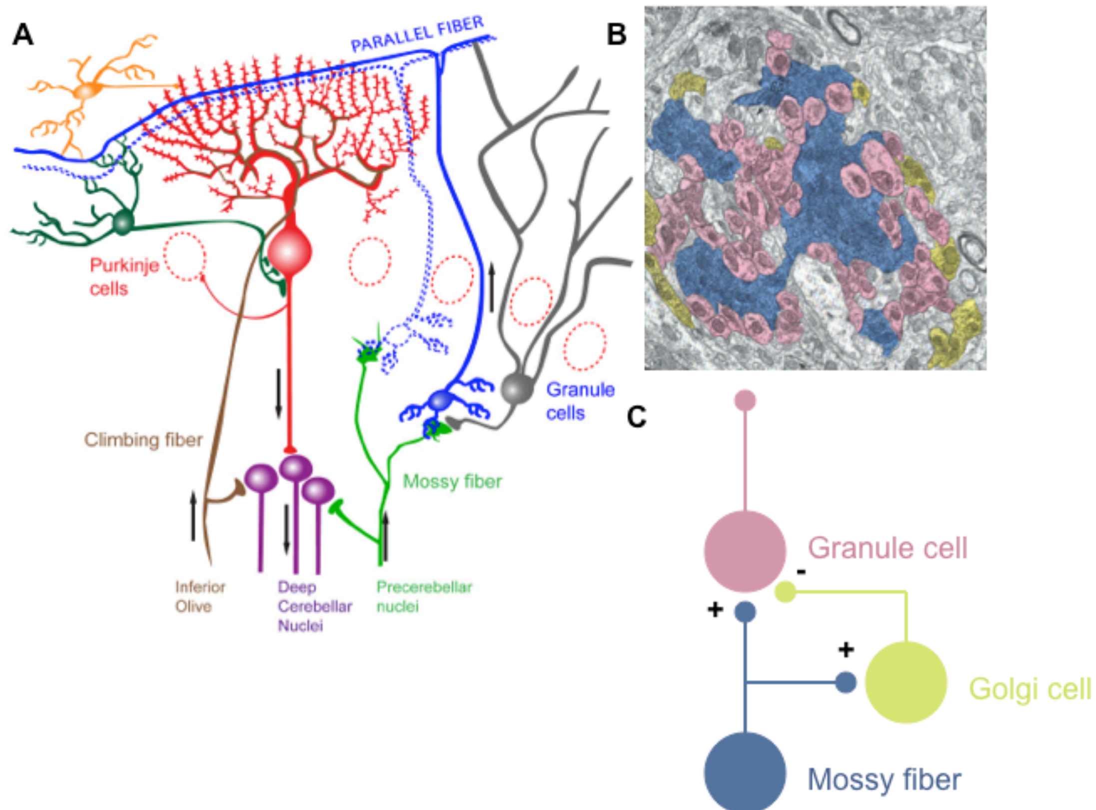

Figure 1.2 The cerebellar circuit. **A** Cerebellar circuit **B** Electron micrograph of the cerebellar glomerulus and **C** Connectivity logic of cerebellar glomerulus.

Besides connecting with granule cells, mossy fibers also sends and receives inputs from Golgi cell, an inhibitory interneuron. Together, the granule cell, mossy fiber and Golgi cell form the cerebellar glomerulus, a large multi-synaptic terminal (Figure 1.2B). The connectivity logic of the glomerulus is such that the mossy fibers send excitatory inputs to both the granule cell and Golgi cell (Figure 1.2C). Golgi cell in turn relays forward an inhibitory signal to the granule cell layer. This local connection is the feedforward inhibition (FFI). Recently, Ruediger and colleagues (2011) have shown that mossy fibers are display learning-induced structural plasticity, and suggested that growth of FFI has implications in learning and memory in the hippocampus and cerebellum. Enhancement in the local FFI changes the way an incoming afferent input is

processed, thus potentially changing the input computation of afferent signals in the cerebellum. Although much is known about the cellular aspects of structural plasticity, the molecular mechanisms underlying these processes remain largely obscure. The aim of my graduate work is to understand the molecular mechanisms that are involved in the regulation of structural plasticity of cerebellar mossy fibers.

Molecular mechanisms underlying structural plasticity

Morphogens in postnatal neural development

Morphogens are important patterning molecules that work in a gradient-dependent manner to change transcriptional profile of cells. An emerging theme in the field of neurodevelopment is the recurrence of various morphogens in later stages of development and circuit function (Poon et al., 2013, Salie et al., 2005, Sanchez-Camacho and Bovolenta, 2009). They are good candidate molecules to investigate if they are involved in later circuit functions, such as structural plasticity.

Wnts

Wnts are secreted proteins that have prominent roles in development. They are identified in Drosophila for having key role in wing development, hence it is named Wingless (Wg) in Drosophila. In the mammalian system, *Int-1* was found because it promoted breast tumor proliferation. The name Wnt came from the contraction of Wg and *Int-1.* In the canonical pathway, Wnt protein binds to its transmembrane receptors, Frizzled and Lipoprotein-related Receptor Protein (LRP), which activates Disheveled (Dsh), a downstream intracellular signaling target. The activation of Dsh causes the inactivation of glycogen synthase kinase (GSK)-3β, a kinase that targets β-catenin for proteasomal degradation. The stabilization, thus accumulation, of β-catenin results in its nuclear translocation. Together with LEF-1/TCF protein in the nucleus, β-catenin exerts its effects on its target genes in response to Wnt signaling cascade. Its

extracellular antagonists, such as Dkk, add another layer of complexity to the canonical Wnt signaling pathway.

Wg is crucial in the assembly of pre- and post-synaptic structures in the Drosophila neuromuscular junction (NMJ) (Packard et al., 2002). A loss of function in Wg secretion by presynaptic neurons results in not only aberrant NMJ structures, it also reduced the number of new synaptic boutons formed in response to muscle growth during larval development (Packard et al., 2002). In the mammalian spinal cord, Wnt3, secreted by motoneurons, was also involved in regulating Neurotrophin-3-responsive spinal sensory neuron axonal remodeling (Krylova et al., 2002). In the mammalian cerebellum, Wnt-GSK-3β signaling has also been implicated in mossy fiber axonal remodeling and maturation (Hall et al., 2000). It was shown that Wnt7, expressed by postsynaptic partner of mossy fibers – granule cells, is critical in axonal maturation of mossy fiber terminals in the cerebellum (Hall et al., 2000). Taken together, it appears that Wnt signaling work in an anterograde and/or retrograde fashion in various systems to induce axonal growth and terminal arborization during later stages of circuit maturation.

Besides circuit maturation, Wnt signaling has implications in adult circuit function. For example, Gogolla and colleagues (Gogolla et al., 2009) showed that Wnt7 expression is up-regulated in the hippocampus CA3 in response to exposure to an enriched environment. They also found that administration of Wnt antagonist and agonist has a negative and positive effect on hippocampal mossy fiber complexity, respectively (Gogolla et al., 2009). Their work provided

evidence that Wnt signaling is also engaged in structural plasticity in the adult hippocampal mossy fibers in response to a novel sensory environment. Recently, it was reported that in the amygdala, Wnt signaling is dynamically regulated during fear learning (Maguschak and Ressler, 2011). Infusion of Wnt antagonist, Dkk-1, or supplementation Wnt ligand into the amygdala interfered with fear memory consolidation (Maguschak and Ressler, 2011). In sum, Wnt signaling is not just involved in circuit maturation; it has also been linked to proper functioning of an adult circuit, such as during the process of learning and memory. However, how exactly Wnt signaling works in the adult system to elicit learning and memory remains to be elucidated.

Neuronal activity has been shown to regulate Wnt secretion and expression in various types of synapses and neuronal populations (Gogolla et al., 2009, Chen et al., 2006, Ataman et al., 2008). In the hippocampal circuit, Wnt3a is secreted in the dentate gyrus molecular layer upon tetanic stimulation (Chen et al., 2006). Blocking Wnt signaling affects late phase LTP in the hippocampal circuit (Chen et al., 2006). On the other hand, Wnt 7a/b was expressed in the CA3 hippocampal principal neuron after bicuculline treatment in organotypic culture (Gogolla et al., 2009). The authors (Gogolla et al., 2009) also found an increase or reduction in mossy fiber complexity when Wnt signaling is up- or down-regulated in the hippocampus, respectively. In the Drosophila NMJ, Ataman and colleagues (Ataman et al., 2008) showed that Wg is secreted from the presynaptic site upon acute stimulation, this is accompanied by a presynaptic motoneuron growth (Ataman et al., 2008). In sum, Wnt expression can be regulated by neuronal activity to orchestrate synaptic development and circuit

function in response to changes in environmental cues. As such, Wnt is an important molecular cascade downstream of neuronal activity to bring about functional or structural changes to neural circuits.

BMP

Bone Morphogenetic Proteins (BMPs) belong to the Transforming Growth Factor-β (TGF-β) superfamily of secreted growth factors. As its name suggests, it was first identified to have roles in bone development (Chen et al., 2004). But later it was found that BMP signaling pathway also has critical roles in neural development (Liu and Niswander, 2005).

BMP, secreted by the roof plate, was found to be critical in regulating commissural axon guidance in the spinal cord (Augsburger et al., 1999). BMP acts as a diffusible chemorepellent, after specification of spinal cord progenitor identity, to orientate commissural axon growth toward to the floor plate (Phan et al., 2010, Perron and Dodd, 2012, Perron and Dodd, 2011, Augsburger et al., 1999). It was found that the non-canonical, Smad-independent pathways are crucial in the guidance roles of BMP signaling (Phan et al., 2010, Perron and Dodd, 2011). In particular, the Limk1 cascade was found to be crucial for the rate of commissural axon growth (Phan et al., 2010) while the PI3K pathway was found to be important for axon repulsion and growth cone collapse (Perron and Dodd, 2011).

Studies in the Drosophila neuromuscular junction implicated BMP signaling in the regulation of presynaptic function and morphology. Glass bottom boat (Gbb)

– a BMP homologue – is secreted by muscle cells to induce presynaptic growth during larval development (McCabe et al., 2003). On the presynaptic motoneuron, Type I and II BMP receptors, Wishful thinking and Saxophone, are recruited to mediate the Gbb-induced presynaptic growth (Rawson et al., 2003, Marques et al., 2002, Aberle et al., 2002). Activation of the presynaptic BMP receptors leads to phosphorylation of Mad, a downstream effector of BMP signaling cascade (McCabe et al., 2003, Marques et al., 2002). Trio, a Rac GEF, which modulates actin cytoskeleton, is a direct transcriptional target of BMP-Smad signaling activation to promote NMJ presynaptic growth during development (Ball et al., 2010). Target of Wit (Twit), a Ly6 family of neurotoxin-like molecule, is a target gene of BMP-Smad signaling pathway. It is involved in presynaptic functional maturation of Drosophila NMJ (Kim and Marques, 2012). Taken together, the canonical BMP signaling pathway orchestrates Drosophila NMJ growth and maturation.

In the mammalian system, BMP is also involved in later stages of presynaptic development and maturation in various systems. In the trigeminal system, retrograde BMP signaling is crucial in the establishment of specific identities of various facial sensory neurons (Hodge et al., 2007). In the calyx of Held, the large relay neurons in the auditory system, BMP receptor expression is crucial for proper synaptic innervation and maturation of synapse firing properties (Xiao et al., 2013). In the cerebellum, BMP4, secreted by Purkinje cell, is implicated in the contact elimination of immature mossy fibers from Purkinje cell layer at early post-natal stage (Kalinovsky et al., 2011). In the mammalian system, Inhibitor of DNA-binding proteins (Id proteins) are one of the known BMP downstream

target genes (Ying et al., 2003). Id proteins are dominant negative basic helix-loop-helix transcription (bHLH) repressor proteins (Ruzinova and Benezra, 2003). With respect to later aspects of neurodevelopment and synaptogenesis, some interesting bHLH proteins are Npas4, TCF4 and ATOH1 (or MATH1). Interestingly, BMP expression in the cerebellum (in Purkinje cells and Golgi neurons) persists throughout adulthood (Kalinovsky et al., 2011), alluding to an unexplored role for BMP signaling in the adult cerebellar circuit.

Taken altogether, growth factors are reused in later stages of development to orchestrate various maturation and functional processes in neural circuits. Wnt and BMP ligands, secreted at the synapses, engage the pre-synaptic site to develop or mature at an appropriate time and place in various types of synapses, such as NMJ, cerebellum, spinal cord etc. Beyond the assembly of circuits, Wnts, being regulated by neuronal activity, are also involved in normal functioning of adult circuits, such as novel sensory experience and fear memory consolidation. An open question remains for the potential role for BMP signaling in adult circuit function.

Aims of the project

Even though important roles for BMP signaling during circuit development and maturation has been established, it remains to be explored if BMP signaling plays a role in regulating adult brain function of a given circuit. As BMP ligand expression persists well into adulthood (Kalinovsky et al., 2011), it alludes to an unexplored potential for BMP signaling in regulating aspect(s) of adult cerebellar circuit function (such as learning and memory). In particular, BMP signaling has been implicated in positively regulating cerebellar mossy fiber rosette complexity (unpublished data) and increased structural complexity of mossy fiber rosettes have been correlated to learning of a motor task (Ruediger et al., 2011). As such, I would like to address if BMP signaling is the underlying molecular pathway regulating learning-induced structural plasticity. Another unexplored aspect of BMP signaling is whether it might be regulated by neuronal activity. In the context of exploring adult circuit function, neuronal activity is an important physiological cue to bring about appropriate adaptive circuit changes. If BMP signaling can indeed be regulated by activity, it could be a bridging pathway, like Wnts, as a molecular signal encoding structural changes in neural circuits upon changes in environmental cues, such as learning.

Some specific outstanding questions are:

i. Is the canonical BMP signaling pathway involved in regulating structural plasticity of pontine mossy fibers?
ii. Does neuronal activity or learning induce structural plasticity in pontine mossy fibers?
iii. Is BMP signaling pathway regulated by neuronal activity?

Results

RESULTS

Preface

This project is a collaborative effort of Fatiha Boukhtouche, Caroline Bornmann and I. Fatiha spearheaded this project, and gathered initial data on the canonical BMP signaling pathway in the regulation of mossy fiber structural plasticity. Caroline generated the IRX1-cre line with the Transgenic Mouse Core Facility of the Biozentrum, where she did an initial screening of the generated lines. When I joined the project, Fatiha and I jointly characterized further the role of canonical BMP signaling pathway in the regulation of mossy fiber structural plasticity. In an attempt to search for a biological context for the BMP phenotype, I went on to do a series of neuronal activity manipulation and motor learning experiments to induce structural plasticity in the mossy fibers. Lastly, I also explored the possibility of neuronal activity interacting with BMP signaling pathway.

The canonical BMP signaling pathway positively regulates cerebellar mossy fiber structural plasticity

Since BMP4 ligand expression persists in the adult mouse cerebellum (Kalinovsky et al., 2011; Figure 2.0), we initially sought to find out if BMP signaling plays a role in the mature cerebellum. To investigate a potential role for BMP signaling function in postnatal stages of cerebellar circuit development, the levels of BMP signaling in pontine mossy fibers were manipulated.

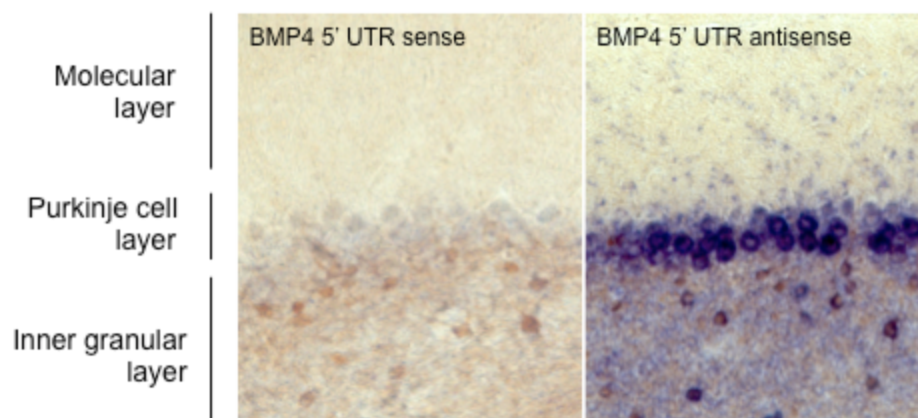

Figure 2.0 Double in situ of BMP4 and GFP in adult cerebellum of GlyT2::GFP animals (where GFP is expressed in Golgi cells of the cerebellum). Strong expression of BMP4 ligand was detected with antisense probe in the Purkinje cell layer and Golgi cell of the cerebellar cortex.

The full-length BMP type II (BMPR2-fl) receptors in the pontine mossy fibers were expressed by in utero electroporation to elevate BMP signaling levels. BMPR2-fl was expressed, as it is the constitutively active receptor in the signaling pathway. Animals were brought to term and analyzed at postnatal day 21 (P21) as the focus of this work is on the mature cerebellar circuit function. To quantify rosette morphology, surface areas of rosettes were re-constructed on

Imaris and the filopodia-like protrusions from the rosette were scored. The filopodia-like protrusions (magenta), terminal rosettes (yellow) and axons (cyan) were distinguished on Imaris by their morphology (see Figure 2.1A' reconstruction). As there is heterogeneity in the rosette morphology, populations of rosettes were categorized into simple (0 or 1 filopodia) and complex (2 or more filopodia) (Figure 2.1B). In BMPR2-fl overexpression, a strong increase of proportion of rosettes with complex morphology was observed (Figure 2.1A and 2.1B). In this same manipulation, there was a corresponding reduction in rosettes with a simple morphology compared to GFP overexpression controls.

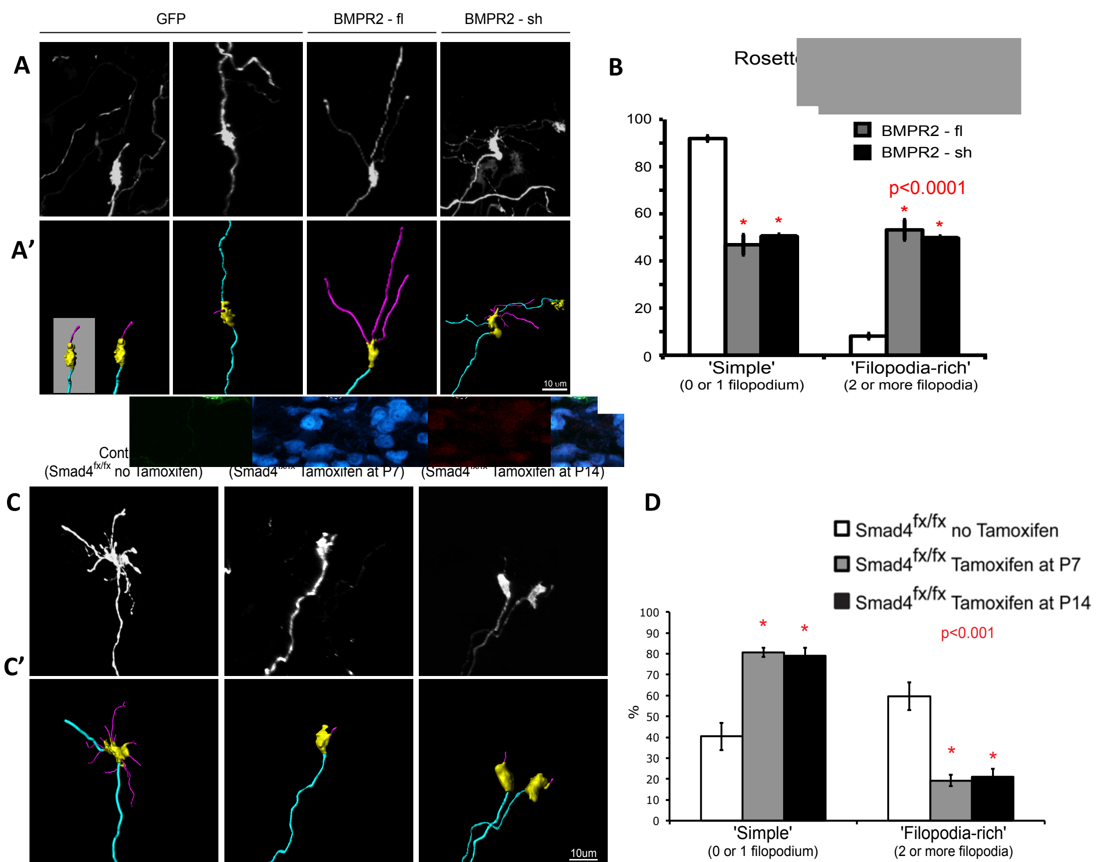

Figure 2.1 BMP signaling pathway positively regulates structural plasticity of cerebellar mossy fiber complexity. **A** Gain of BMP receptor expression increases the number of filopodia-like protrusions from rosette terminal

compared to GFP expressing controls. **A'** 3-dimensional reconstruction of BMPR2 overexpressing mossy fiber terminals using Imaris. Magenta, filopodia. Yellow, rosette. Cyan, Axon. **B** Quantification of BMPR2 gain of function phenotype. The population of rosettes is categorized into 'Simple' (0 or 1 filopodia) or 'Filopodia-rich' (2 or more filopodia) rosettes. Two-way ANOVA, p<0.0001. **C** Loss of Smad4 results in a reduction of rosette surface complexity. **C'** 3-dimensional reconstruction of Smad4 cKO mossy fiber terminals using Imaris. Magenta, filopodia. Yellow, rosette. Cyan, Axon. **D** Quantification of Smad4 loss of function phenotype. The population of rosettes is categorized into 'Simple' (0 or 1 filopodia) or 'Filopodia-rich' (2 or more filopodia) rosettes. Two-way ANOVA, p<0.001. Error bars are SEM. (Work by Fatiha Boukhtouche)

Since there are the canonical and non-canonical wings of BMP signaling, it prompted us to address if the canonical or non-canonical BMP signaling pathway was involved in regulating mossy fiber rosette complexity. To address that, the truncated form of BMPR2 (BMPR2-short, or BMPR2-sh) was overexpressed. This version of the receptor lacks in intracellular domain, which interacts with signaling cascades of the non-canonical BMP signaling (like LIMK and MAPK pathway) but is still able to activate the canonical BMP signaling pathway. In this overexpression, we observed a similar increase in rosette complexity like the BMPR2-fl overexpression (Figure 2.1A and 2.1B). Taken together, these overexpression data suggests that an elevation in the canonical, not the non-canonical, BMP signaling pathway is sufficient to increase mossy fiber rosette morphology.

To further address necessity of the canonical BMP signaling pathway in the regulation of mossy fiber rosette complexity, conditional knockout (cKO) of Smad4 in pontine mossy fibers by in utero electroporation was performed. Smad4, or common Smad, is a key mediator of nuclear translocation of Smad1, 5, 8 complexes in the BMP signaling cascade. The Cre-lox system was used to mediate genome recombination to attain spatial restriction of Smad4 knockout in the pontine mossy fibers. This is achieved by electroporating Cre in to pons of Smad4 floxed animals (Figure 2.1D). In addition, a modified Cre combinase, which is fused to an estrogen receptor (CreERT2), was used. This form of Cre is only active upon administration of Tamoxifen (its steroid ligand). The combination of Cre-lox system and CreERT2 allowed spatial and temporal ablation of Smad4 in the pontine mossy fibers. In this manipulation, a reduction of rosette surface complexity compared to control animals was observed. The ablation was conducted at two different stages – P7 and P14. Both time points showed a similar simplification of rosette complexity (Figure 2.1C and C'). In short, these data strongly suggest that the canonical BMP signaling pathway is active during postnatal stages of mossy fiber complexity maturation, and positively regulates structural plasticity of rosettes.

In order to further characterize the filopodia-like structural growth (complexity of rosettes), we next examined whether the filopodia-like structures contact Golgi cells, a known postsynaptic partner of mossy fibers. We used Neurogranin, a marker of Golgi cell, to label a subset of Golgi cell, and looked at whether or not filopodia-like structures in BMPR2 overexpression samples contacted them. Indeed, the filopodia-like protrusions came into close proximity with Golgi cell

dendrites (Figure 2.2A). At the tip of the filopodia-like structures, there are synaptic bouton-like swellings that contain vGlut1, a presynaptic marker (Figure 2.2B). Together, these suggest that BMP-induced filopodia growth forms putative synaptic connections with Golgi cells. BMP signaling regulates growth of FFI (mossy fiber-Golgi cell connection) in the cerebellar circuit.

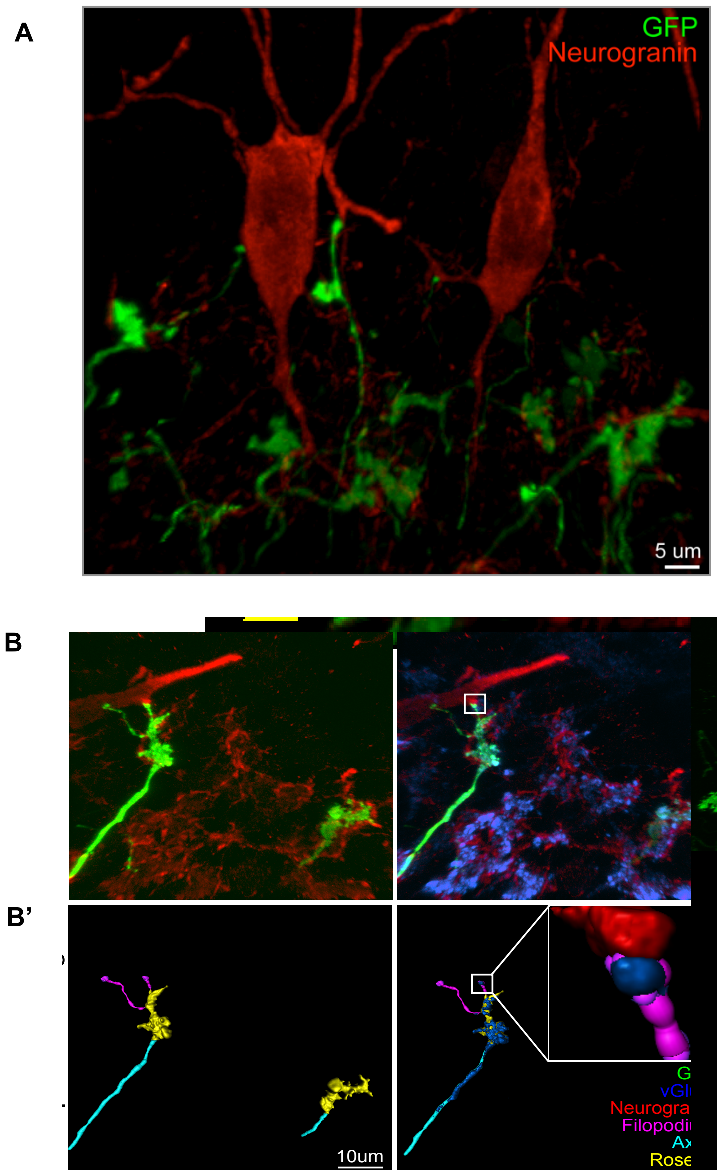

Figure 2.2 BMP signaling gain of function-induced filopodia-like protrusions from mossy fiber rosettes forms putative synaptic contacts with Neurogranin-positive Golgi cells. **A** Filopodia-like protrusions contacts Golgi cell dendrites. **B**

The tips of filopodia-like structure display synaptic bouton-like swellings that contain vGlut1, a presynaptic marker. **B'** 3-dimensional reconstruction of **B**. Insert: magnification of a representative filopodia tip. (Work by Fatiha Boukhtouche)

Next, we were interested to find out if Smad1, a downstream effector of the canonical BMP signaling pathway, was sufficient to induce similar structural changes in the mossy fiber rosette morphology as well. Smad1 was overexpressed in pontine mossy fibers by in utero electroporation. In this experiment, there was no significant change (Figure 2.3B) in the rosette complexity between the overexpression mutant and GFP controls (Figure 2.3A). There was only a trend in an increase in complex rosettes and decrease in simple rosettes (Figure 2.3C). Furthermore, the distribution of population of rosettes did not have a significant change as well.

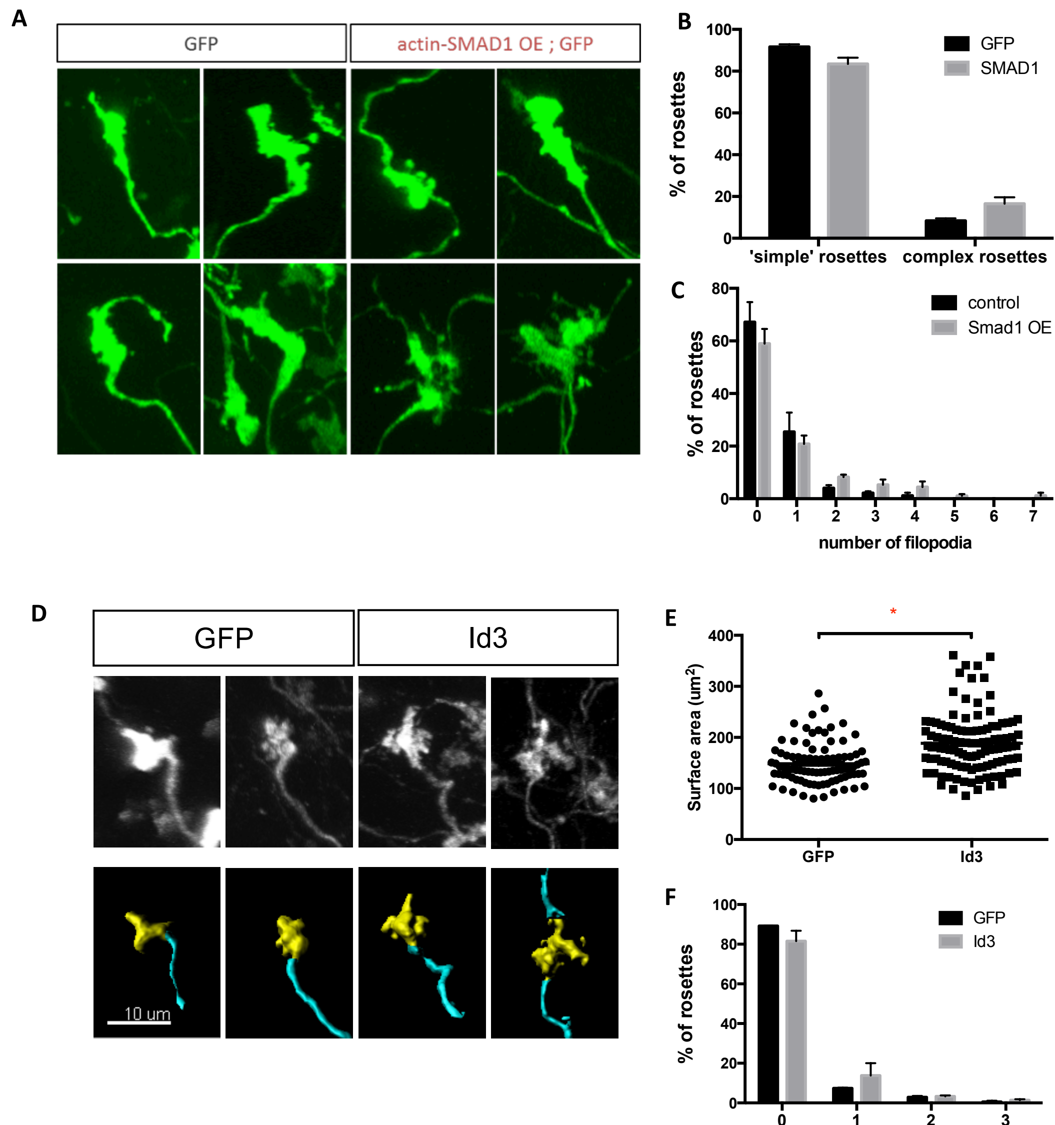

Figure 2.3 The canonical BMP signaling pathway regulates mossy fiber rosette morphology. **A** Smad1 overexpression was not sufficient to induce in change in rosette morphology. **B** Quantification of Smad1 overexpression phenotype. Rosettes were classified in 'Simple' (0 or 1 filipodia) or 'Complex' (2 or more filopodia) rosettes. The two populations were not significantly different. (n = 3 controls animals, n = 5 mutants animals). **C** The distribution of Smad1 overexpressing and GFP overexpressing rosettes according to their number of filopodia. **D** Id3 overexpression increases surface area of rosette but not filopodia distribution of rosette phenotypes. Yellow, rosette. Cyan, Axon. **E**

Quantification of Id3 mutant phenotype. Surface areas of Id3 overexpression rosettes are significantly larger than GFP controls (Student's t-test, $p < 0.0001$). (n = 2 animals, 148 rosettes for controls, n = 2 animals, 163 rosettes for mutants) **F** The distribution of Id3 overexpressing and GFP overexpressing rosettes according to their number of filopodia. Error bars are SEM.

Next, we wanted to find a downstream target of BMP signaling pathway in the regulation of cerebellar mossy fiber elaboration. Id proteins, or inhibitor of DNA-binding, are known downstream targets of BMP signaling pathway in stem cell biology (Ying et al., 2003). To test if Id proteins are involved in regulating structural plasticity of mossy fiber rosette morphology, Id3 was overexpressed in the pontine mossy fibers by stereotaxic AAV-Id3 virus injection. To this end, there is an increase in the proportion of rosettes with larger surface area of the rosettes compared to the GFP controls but not in the number of filopodia-like protrusions (Figure 2.3D). These rosettes have a more complex surface convolution. (Figure 2.3C). It is interesting to note that is has a slightly different phenotype than the BMPR2 receptor overexpression phenotype (Figure 2.1A).

Taken together, BMPR2 overexpression was sufficient to increase mossy fiber elaboration and Smad4 was necessary for maintaining mossy fiber elaboration. Furthermore, Smad1 was not the limiting factor in this pathway, and Id3 might be one of the downstream targets genes of BMP signaling in regulating an aspect of mossy fiber rosette surface elaboration.

Neuronal activity dependent regulation of structural plasticity

In a previous report by Ruediger et al. (2011), they observed an increase in cerebellar mossy fiber rosette complexity upon rotarod training. However, the molecular mechanism underlying this process is largely unknown and also the source(s) of mossy fibers that undergo structural changes are not known from their study. From an independent molecular approach, we have observed that BMP signaling gain of function has resulted in an increase in pontine mossy fiber complexity (see results in previous section). As such, we were interested to address if BMP signaling is the underlying pathway for learning-induced structural plasticity in pontine mossy fibers.

To address that, wildtype animals were trained on a modified accelerating rotarod protocol and examined if training activates pontine mossy fibers. Upon eight days of consecutive training, the animals displayed a significant improvement of performance over time (Figure 2.4A). Using c-Fos as a marker for recent neuronal activity in the pontine gray nucleus, there was an increase in proportion of pontine neurons expressing c-Fos by immunostaining and RT-qPCR (Figure 2.4B, C, D). This suggests that upon rotarod training, the pontine neurons are engaged during the task.

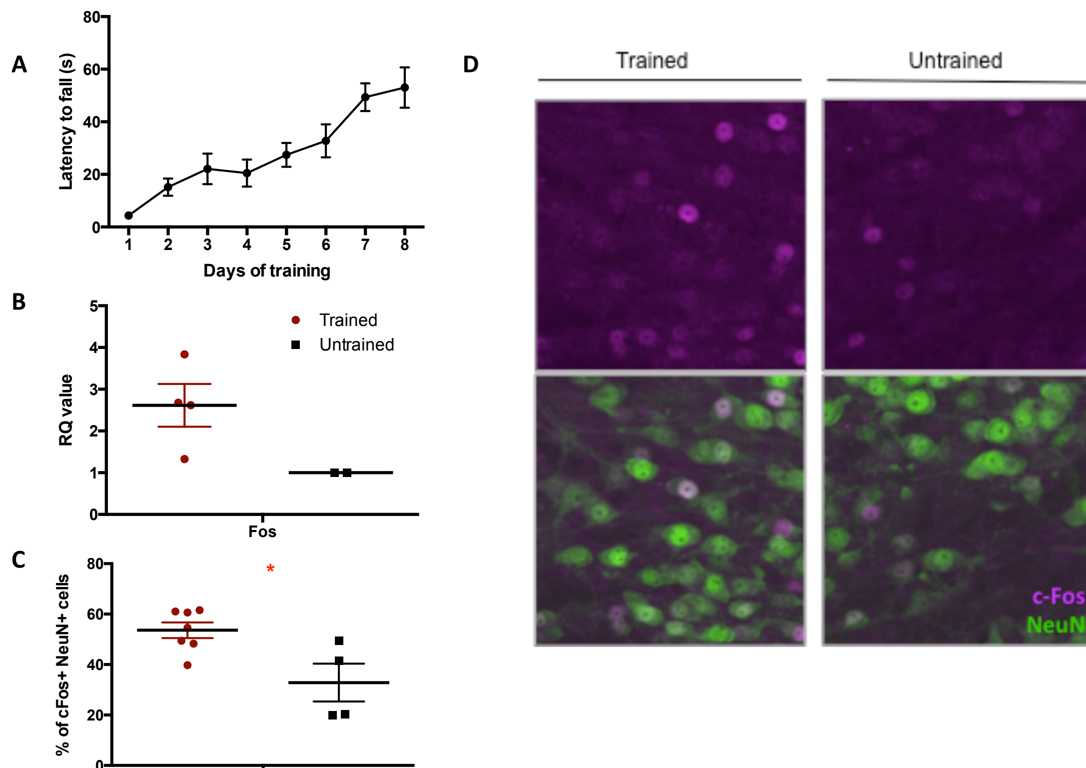

Figure 2.4 Rotarod training activates and engages pontine neurons. **A** Learning curve of wildtype animals over eight consecutive days, quantified by latency to fall in seconds. **B** There was elevation of c-Fos mRNA, detected by RT-qPCR (n = 7 trained, 4 untrained)**. C** Quantification of percentage of c-Fos expressing neurons by immunostaining in trained and untrained animals. (n = 4 trained, n = 2 untrained) Red, trained animals. Black, untrained animals. Student's t-test $p < 0.05$. **D** Representative images of c-Fos immunolabeling in pontine gray nucleus of trained and untrained animals. More pontine neurons express c-Fos in trained animals. Error bars are SEM.

Since there was an increase in neuronal activity of pontine mossy fibers upon rotarod training, an open question for investigation is if neuronal activity per se is sufficient to increase morphological complexity of the mossy fiber terminals. To address that, the DREADD (Designer Receptor Exclusively Activated by

Designer Drug) system was employed to manipulate neuronal activity. DREADD receptors are a class of evolved G-protein coupled receptor (GPCR) that is designed to respond only to an exogenous ligand, Clozapine-N-Oxide (CNO) (Armbruster et al., 2007). There are two versions of the DREADD receptors, hM3Dq and hM4Di. The hM3Dq version was used because activation of these receptors by its ligand will engage downstream signaling pathways such as IP_3, DAG pathway which results in depolarization of the neuron (Wulff and Arenkiel, 2012) Figure 2.5A). DREADD receptors were overexpressed in the pontine neurons by in utero electroporation (Figure 2.5C) and animals were administered CNO daily for five days at early postnatal days (P14-P18). In the CNO treated pontine neurons, there was a strong increase in c-Fos upon ligand administration compared to saline controls and surrounding untargeted pontine neurons (Figure 2.5B). This suggests that the DREADD receptors are useful tools to remotely and reversibly manipulate neuronal activity.

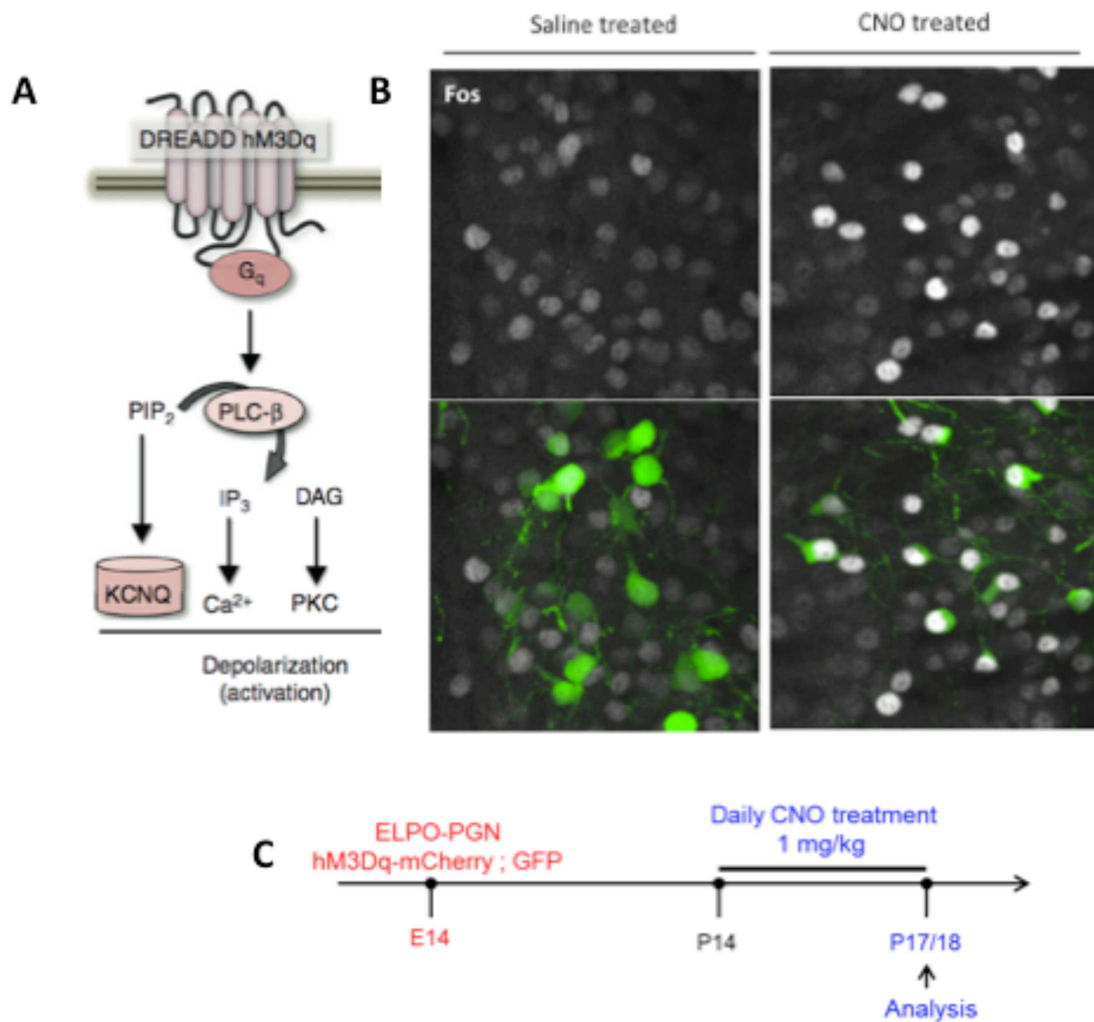

Figure 2.5 Using DREADD as a pharmacogenetic tool to manipulate neuronal activity exclusively in the pontine neurons. **A** Schematic of downstream signaling cascades engaged upon activation of DREADDs (adapted from (Wulff and Arenkiel, 2012). **B** Pontine neurons of saline and CNO treated animals expressing GFP and hM3Dq-mCherry. There was elevation of c-Fos expression in CNO treated animals compared to saline treated and untargeted cells. **C** The experimental timeline. Animals were electroporated at E14 to overexpress hM3Dq-mCherry and GFP. Animals were brought to term and given daily CNO or saline i.p. injections for 4 to 5 days.

In the CNO treated rosettes, there was a significant increase in proportion of rosettes with a complex morphology compared to the saline treated controls (Figure 2.6A). There were a higher proportion of rosettes with 2 or more filopodia protrusions from the rosettes. There is also a slight right hand shift in the percentage of rosettes with increased number of filopodia-like protrusions (Figure 2.6B).

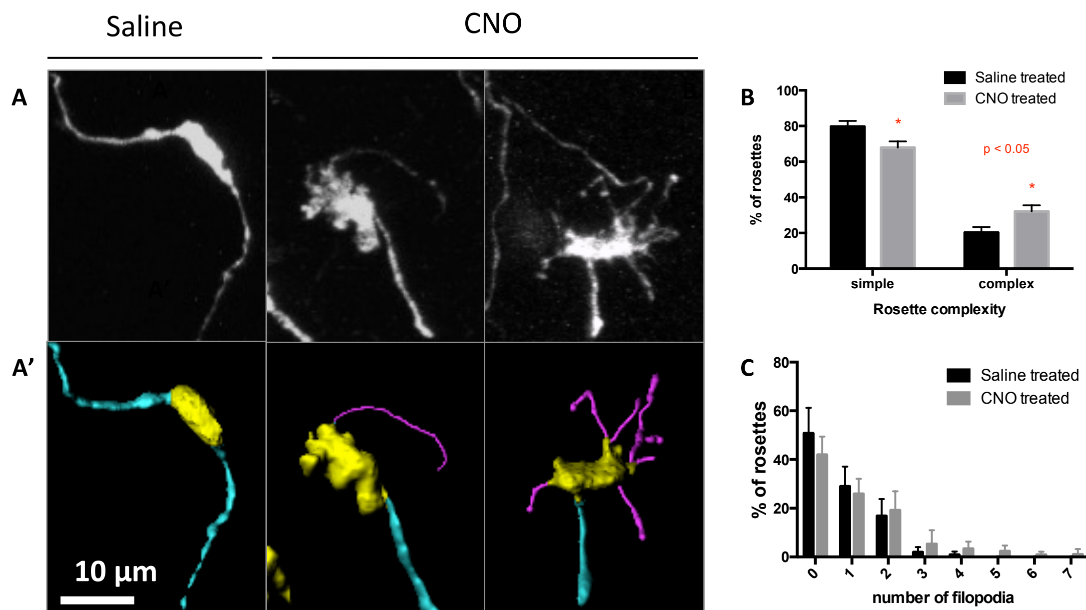

Figure 2.6 Neuronal activity is sufficient to enhance rosette elaboration in juvenile rosettes. **A** Elevation of neuronal activity increases structural complexity of pontine mossy fibers from P14-P18. **A'** 3-dimensional reconstruction of mossy fiber rosettes. Magenta, filopodia. Yellow, rosette. Cyan, axon. **B** Quantification of rosettes in saline and CNO treated conditions. Population of rosettes was categorized into 'Simple' (0 or 1 filopodia) and 'Complex' (2 or more filopodia). There were significantly more complex rosettes with a complex morphology in CNO treated condition than saline treated conditions. (n = 5 animals, 191 rosettes for saline treated and n = 5 animals, 189 rosettes for CNO treated, Student's t-test, $p < 0.05$). **C** The distribution of rosettes according to

their number of filopodia. There are more rosettes with increased number of filopodia.

Moving forward, we sought to examine if neuronal activity is sufficient to induce structural plasticity in the mature cerebellar mossy fibers. As such, hM3Dq was expressed in pontine mossy fibers but this time treated the animals with CNO at a later stage (P24-P28). There was no difference in the distribution and proportion of rosette morphology in the control saline treated and CNO treated animals (Figure 2.7A-C). This suggests that there is a time window where mossy fiber rosettes are amenable to activity-induced structural plasticity

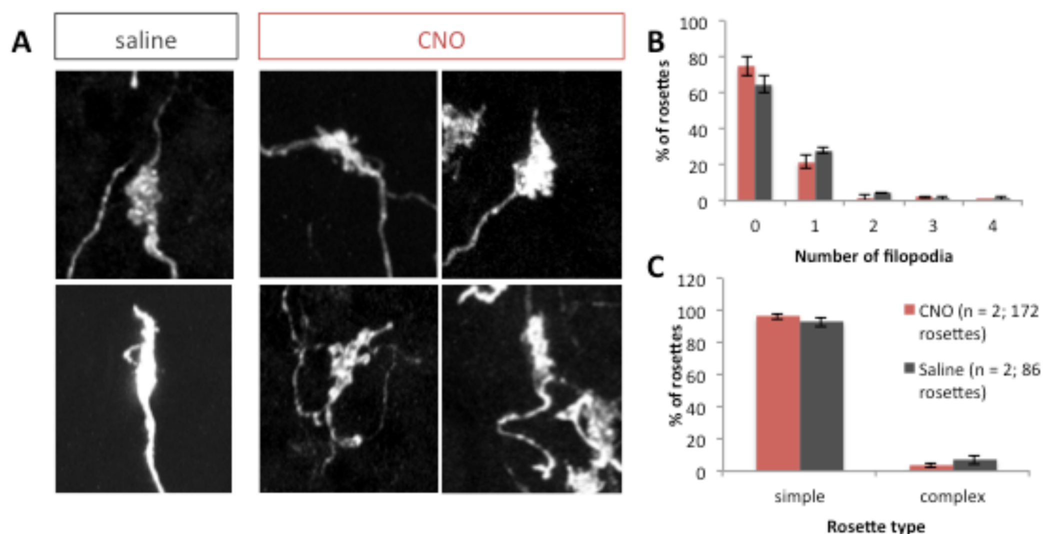

Figure 2.7 Neuronal activity is not sufficient to enhance complexity of mature rosettes. **A** Elevation of neuronal activity does not change morphology of rosettes at a mature stage P24-P28. **B** Quantification of rosette distribution according to their number of filopodia. **C** Quantification of rosette type proportion revealed no significant difference between controls and treated rosettes (n = 2 animals, 172 rosettes for CNO treated, n = 2 animals, 86 rosettes for WT).

To further test the hypothesis that neuronal activity is involved in regulating structural plasticity of mossy fibers, synaptic activity in the mossy fibers was silenced and examined if there were any change in rosette morphology. To do that, tetanus toxin (TeNT) was expressed to silence presynaptic transmission. TeNT is a naturally occurring toxin that cleaves VAMP2, a core exocytotic machinery of presynaptic transmission (Figure 2.8A). Expression of TeNT effectively halts neurotransmission. To achieve specific expression of TeNT in the pontine mossy fibers, we expressed Cre combinase in an R26$^{flox\text{-}stop\text{-}TeNT}$ transgenic mouse line (Figure 2.8B). To target early postnatal stages, the virus was injected into the pontine gray nucleus between P11-13 and analyzed 2 weeks later. In the Cre expressing mossy fibers, there was a loss of VAMP2 in the TeNT expressing mossy fibers as assessed by immunostaining (Figure 2.8C).

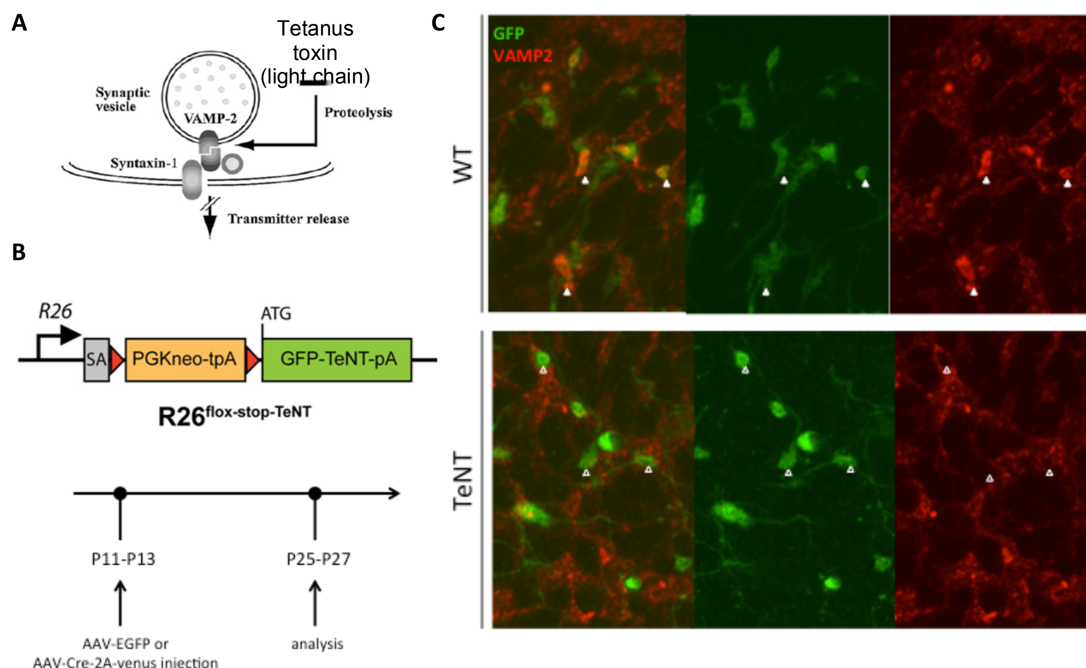

Figure 2.8 Tetanus toxin as a tool to manipulate synaptic activity. **A** Schematic diagram showing TeNT cleaving VAMP2, a core part of the exocytotic

machinery in neurotransmission at the presynaptic active zone. **B** R26$^{flox\text{-}stop\text{-}flox\text{-}TeNT}$ schematic diagram and experimental set up. Animals were injected at P11-P13 with AAV-GFP or AAV-Cre-2A-venus and analyzed two weeks later. **C** VAMP2 staining of cerebellar cortex. Open white arrowheads indicate loss of VAMP2 in TeNT expressing mossy fibers compared to GFP expressing rosettes (solid white arrowheads).

When the rosette morphology was examined, there was a simplification of rosette morphology. Instead of the regular convoluted rosettes, a simple balloon-shaped rosette was observed (Figure 2.9A and A'). In addition to the simplified rosette surface, there was also a loss of filopodia-like protrusions from more than 99% of the rosettes (Figure 2.9B). Furthermore, there is an increase in the sphericity of the rosettes as quantified by Imaris (Figure 2.9B).

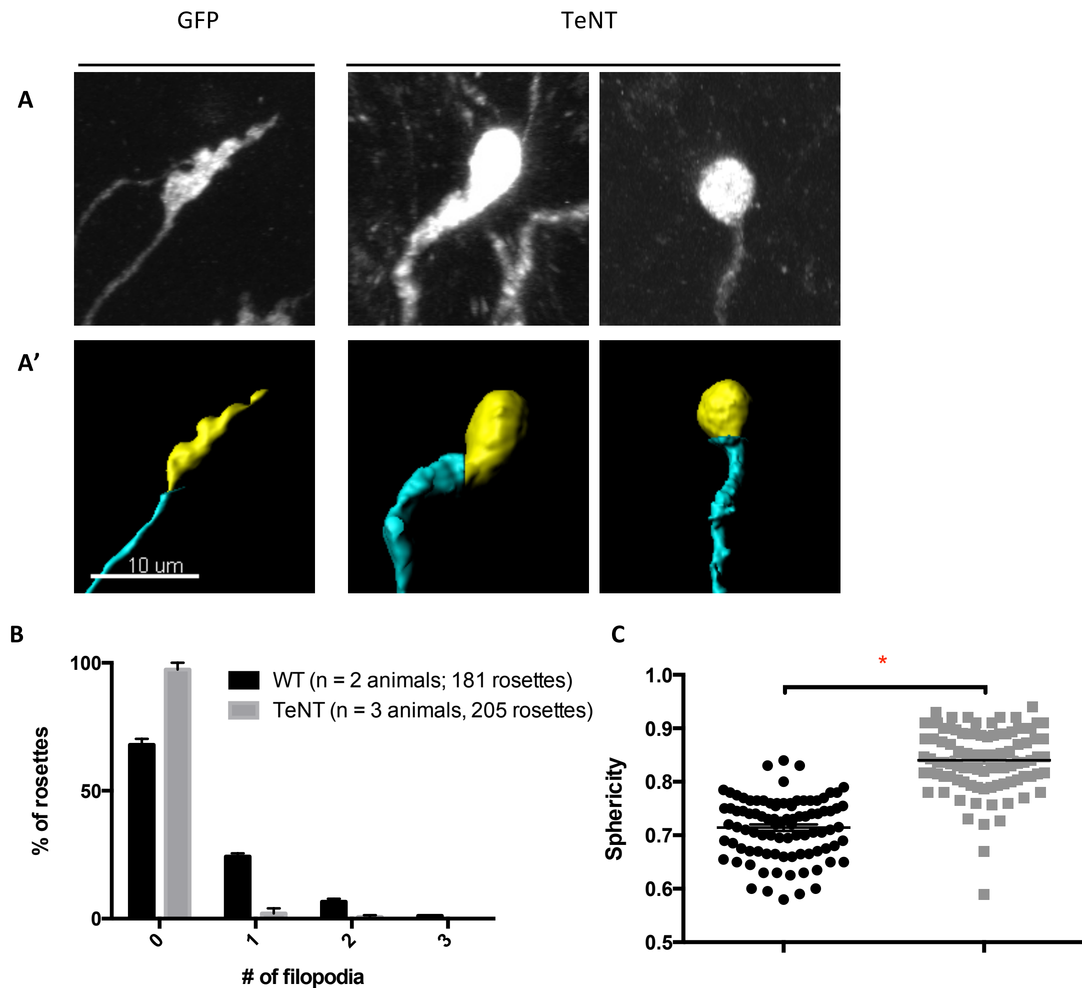

Figure 2.9 Abolishment of synaptic activity reduced rosette complexity. **A** TeNT expressing rosettes show a loss of surface convolution compared to GFP expressing rosettes. **A'** 3-dimensional reconstruction of GFP and TeNT expressing rosettes using Imaris. Yellow, rosette. Cyan, axon. **B** Quantification of filopodia distribution of TeNT and GFP expressing rosettes (n = 2 animals, 181 rosettes for WT and n = 3 animals, 205 rosettes for TeNT). **C** Sphericity quantification of TeNT and GFP expressing rosettes shows a significant increase in TeNT condition (Student's t-test $p < 0.0001$).

As the hM3Dq manipulation increased structural complexity of mossy fibers, while TeNT silencing of synaptic activity reduced structural complexity of mossy

fibers, these data suggests that structural plasticity of cerebellar mossy fibers may by subjected to activity-dependent regulation.

The use of TeNT is a versatile tool to shut down neurotransmission effectively, but it may also result in cell death (Yu et al., 2004). To investigate if there might be potential cell death in the pontocerebellar system upon expression of TeNT, GFAP (Glial Fibrillary Acidic Protein) expression was examined in the GFP and TeNT expressing pontine gray nucleus and cerebellum. GFAP is a marker for glial cells, which is expressed during cell inflammation and cell death. In the TeNT samples, there was a strong up-regulation of GFAP compared to the GFP expressing samples (Figure 2.10).

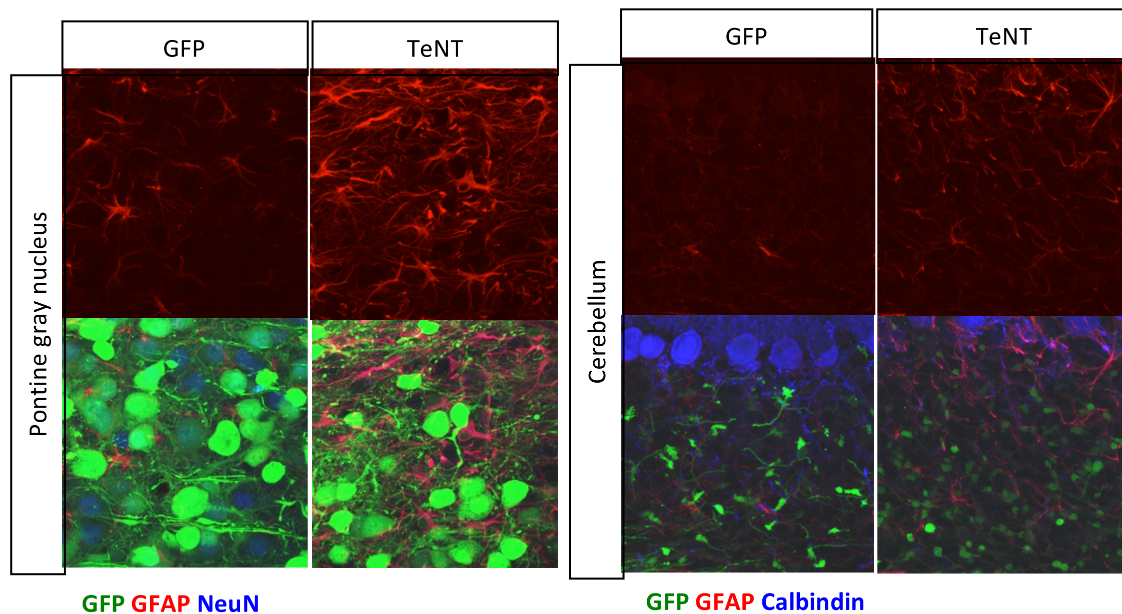

Figure 2.10 Expression of TeNT in the pontine gray nucleus to silence neuronal activity induced gliosis in the pontocerebellar system. **A** Up-regulation of GFAP in TeNT expressing samples in the pontine gray nucleus. **B** Cerebellar cortex

shows increase of GFAP expression for TeNT condition. GFAP, Glial Fribillary Acidic Protein.

This data suggests that while TeNT is a versatile tool to irreversibly shut down neurotransmission specifically, there might be cell death occurring in such manipulation. This calls for a careful interpretation of data available. In this light of this situation, hM4Di (the silencing form of DREADD, Figure 2.11A) was used to manipulation neuronal activity mossy fibers. hM4Di inhibits the adenylate cyclase pathway and activates GIRK channels, thus hyperpolarizing neurons when expressed and activated (Wulff and Arenkiel 2009).

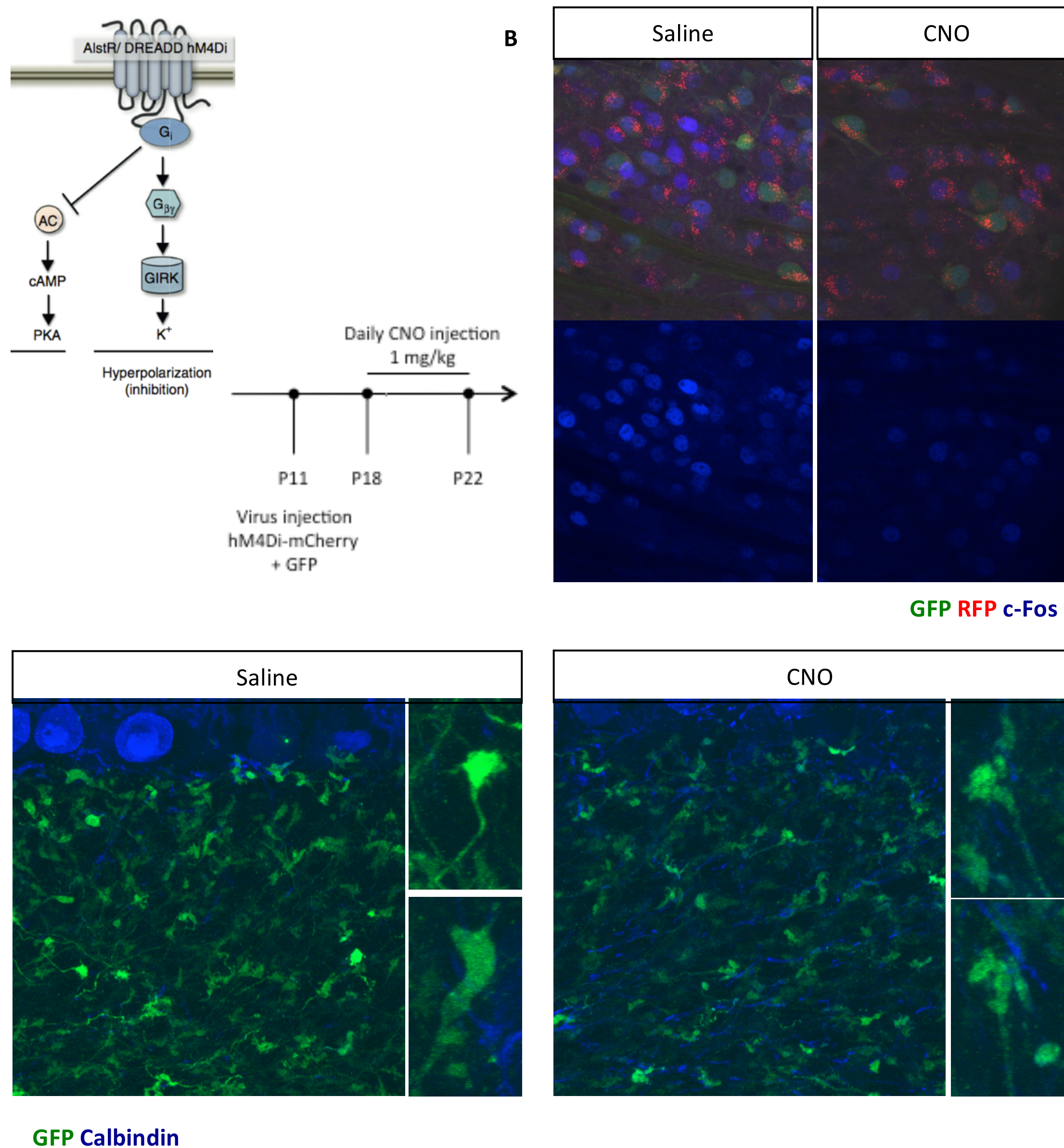

Figure 2.11 Dampening neuronal activity using hM4Di did not change morphology of rosettes. **A** Schematic diagram of pathways involved activation of hM4Di (adapted from (Wulff and Arenkiel, 2012). Experimental timeline. Animals were injected with AAV-hM4Di-mCherry and AAV-GFP at P11. CNO administered daily from P18 to P22. **B** Pontine gray nucleus of saline and CNO treated samples. Application of CNO in hM4Di expressing pontine gray nucleus reduces cFos levels. RFP, red fluroscence protein. **C** Rosette morphology of

saline and CNO treated samples. There was no difference in rosette morphology by qualitative assessment.

When hM4Di was overexpressed in the pontine gray nucleus and CNO was administered, there was a down-regulation of c-Fos (Figure 2.11B) compared to the saline treated controls. This indicated that hM4Di expression and ligand administration is sufficient to dampen neuronal activity in the pontine gray nucleus. However, when the rosettes were analyzed, there seem to be no difference between saline and CNO treated rosettes. This suggests that reducing neuronal activity by hM4Di was not sufficient to induce structural plasticity change in the rosettes by qualitative assessment.

Taken altogether, hM3Dq manipulation and TeNT expression in pontine mossy fibers results in a bi-directional change in rosette morphology. The former increases rosette complexity while the latter reduces rosette complexity. The use of TeNT may cause neuronal cell death due to up-regulation of GFAP marker in the TeNT samples. Hence, results have to be interpreted with caution. hM4Di is an alternative tool to silence neuronal activity its effects may be too subtle to change rosette morphology.

Learning induced structural plasticity in cerebellar mossy fibers

From a behavioral training entry point, Ruediger et al. (Ruediger et al., 2011) and Boele et al. (Boele et al., 2013) have observed that cerebellar mossy fibers display structural remodeling upon motor learning. Ruediger et al. (Ruediger et al., 2011) observed an increase in rosette terminal complexity upon rotarod training, while Boele et al. saw an increase in pontine axonal collateral sprouting during eye blink conditioning. These studies suggest that cerebellar mossy fibers are amenable to plastic structural changes after learning a specific behavioral paradigm. With this, the issue of whether pontine mossy fibers are also subjected to learning-induced structural plasticity was addressed next.

To do so, an inducible CreERT2 line under the IRX1 enhancer (generated in the lab) was used for behavioral experiments. When crossed to a reporter line (Figure 2.12A), this transgenic mouse line shows marker expression only at specific parts of the brain. These areas include olfactory bulb, colliculus, deep cerebellar nuclei, pontine gray nucleus cerebellar cortex and external cuneate nucleus (Figure 2.12B). Within the pontocerebellar system, the expression is largely restricted to mossy fibers. Thus, this is a pontine-enriched Cre line that allows us to perform behavioral experiments, where large cohorts of animals were needed.

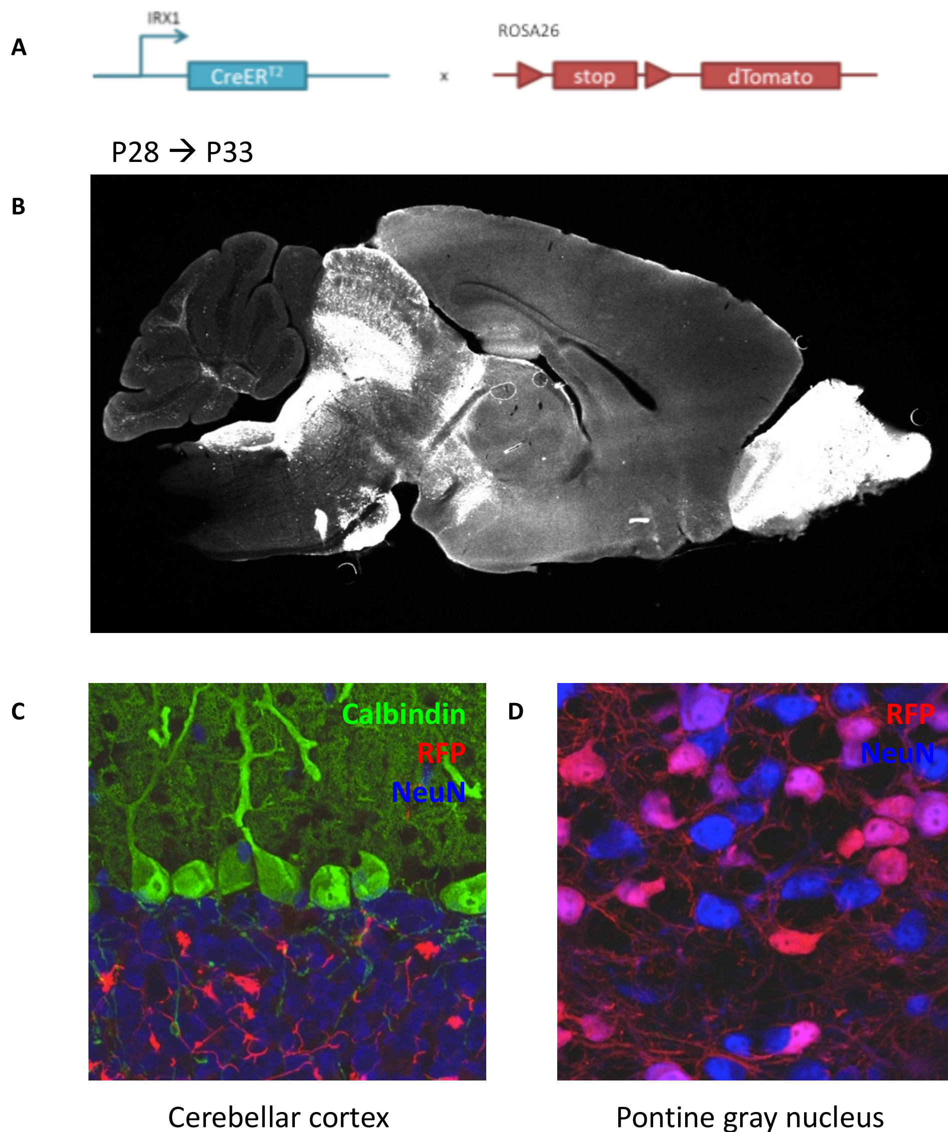

Figure 2.12 Characterization of IRX1-CreERT2 line. **A** Schematic representation of IRX1-CreERT2 ; tdTomato line. **B** Parasagittal section of IRX1-CreERT2 ; tdTomato line. Expression of reporter was found in olfactory bulb, colliculus, external cuneate nucleus, vestibular nucleus, pontine gray nuelcus and cerebellum. Animal induced with Tamoxifen postnatally at P28 and sacrificed at P33. **C** Expression of reporter in the cerebellar cortex is largely restricted to mossy fibers. **D** About 50% of pontine gray nucleus neurons undergo recombination in the IRX1-CreERT2 line.

The transgenic adult mice (8 weeks) were subjected to a modified accelerating rotarod training paradigm over a period of 8 days (after Ruediger et al., 2011). These animals learn the task sufficiently well as quantified by the learning curve (Figure 2.4A). When the rosettes were quantified, there were no differences between the morphology of trained and untrained animals in lobule 6 and 7 of the cerebellum (Figure 2.13). Lobules 6 and 7 are the main projection sites for pontine mossy fibers.

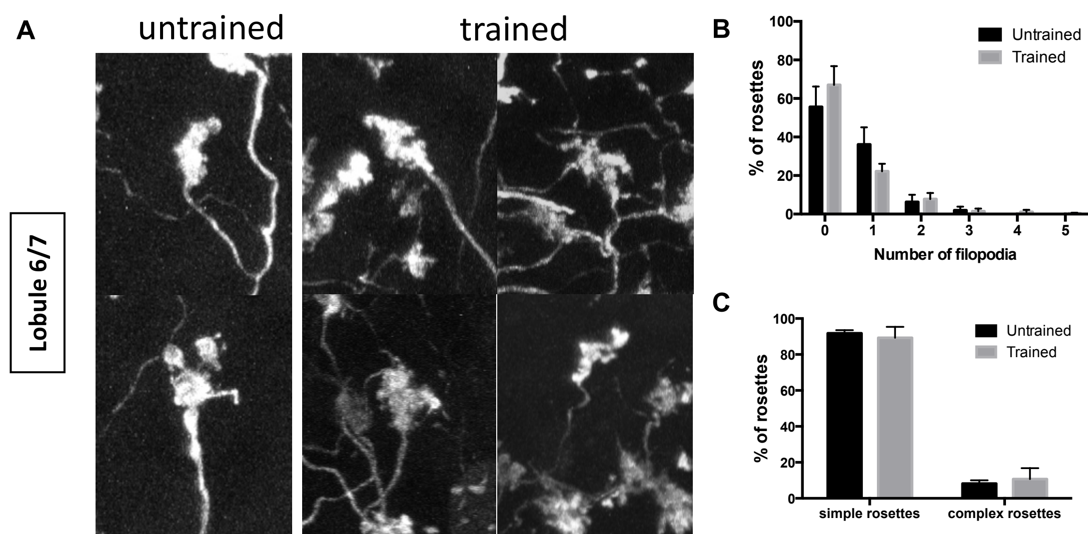

Figure 2.13 Rotarod training did not induce structural plasticity in cerebellar lobule 6/7 mossy fiber rosettes. **A** Representative rosettes from lobule 6/7 of cerebellum of rotarod trained and untrained animals. **B** Quantification of rosette morphology by distribution according to their number of filopodia. **C** Quantification of ‘Simple’ (0 or 1 filopodia) and ‘Complex’ (2 or more filopodia) rosette types in trained and untrained animals. (n = 3 animals, 178 rosettes for trained animals, n = 2 animals, 97 rosettes for untrained animals).

As a change in pontine mossy fibers upon rotarod training was not seen, we turned our attention to lobule 9 mossy fibers. Lobule 9 is the main vestibular

input of the cerebellum and the site where Ruediger et al. (Ruediger et al., 2011) has first analyzed their mossy fibers. There was also no change in the rosette morphology upon rotarod training (Figure 2.14).

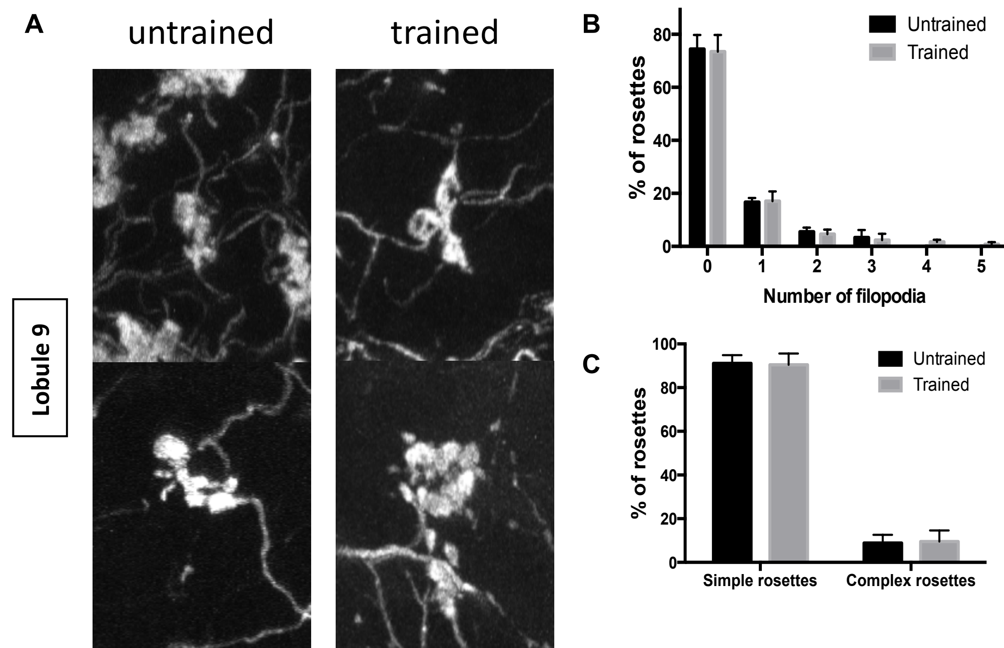

Figure 2.14 Rotarod training did not induce structural plasticity in cerebellar lobule 9 mossy fiber rosettes. **A** Representative rosettes from lobule 9 of cerebellum of rotarod trained and untrained animals. **B** Quantification of rosette morphology by distribution of filopodia (n = 3 animals, 188 rosettes for trained animals, and n = 3 animals, 184 rosettes for untrained animals). **C** Quantification of rosette morphology by 'Simple' (0 or 1 filopodia) and 'Complex' (2 or more filopodia) shows no difference between trained and untrained rosettes.

Taken together, the structural parameters of mossy fiber rosettes examined here did not show significant changes upon rotarod training. In our training experiments, the pontine gray nucleus is activated and thus engaged during learning (Figure 2.4B, C, D). Furthermore, a series of experiments described below shows that there are changes in the transcriptional profile of rotarod-

trained animals (Figures 2.16, 2.17 and 2.18). Since we could not induce filopodia-like growth from mossy fiber rosettes after rotarod training in wildtype animals, we did not go on to pursue if the canonical BMP signaling is involved in learning-induced structural plasticity in the pontine mossy fibers.

Activity-dependent regulation of BMP signaling pathway

As the activity manipulation experiments phenocopies the BMP manipulation phenotype, we wondered if there was an intersection between activity and BMP signaling in the regulation of structural plasticity of cerebellar mossy fibers. First, we wanted to ask if BMP signaling is subjected to regulation by neuronal activity. To address this issue, the transcription of ligands and some known downstream target genes of BMP signaling were examined. This is because it is an approachable entry point. Besides, it is the canonical BMP signaling pathway, which changes transcriptional profile of a given cell that positively regulates structural plasticity of pontine mossy fibers.

To start manipulating activity levels in the cerebellar circuit, cerebellar organotypic cultures were cultured to more easily access and manipulate activity levels in relatively intact cerebellar circuit. In the organotypic cultures, the cerebellar circuit is relatively intact with discernable Purkinje cell layer, Golgi cells and inner granular layers (Figure 2.15B). To manipulate activity in this ex vivo circuit, KCl treatment was given for 36 hours to depolarize the cells. In this system, c-Fos levels were increased, detected by RT-qPCR (Figure 2.15D) and immunostaining (Figure 2.15C). When the BMP ligands were surveyed in the KCl treated samples, there was a reduction in BMP ligands 1, 5, 6, and 7.

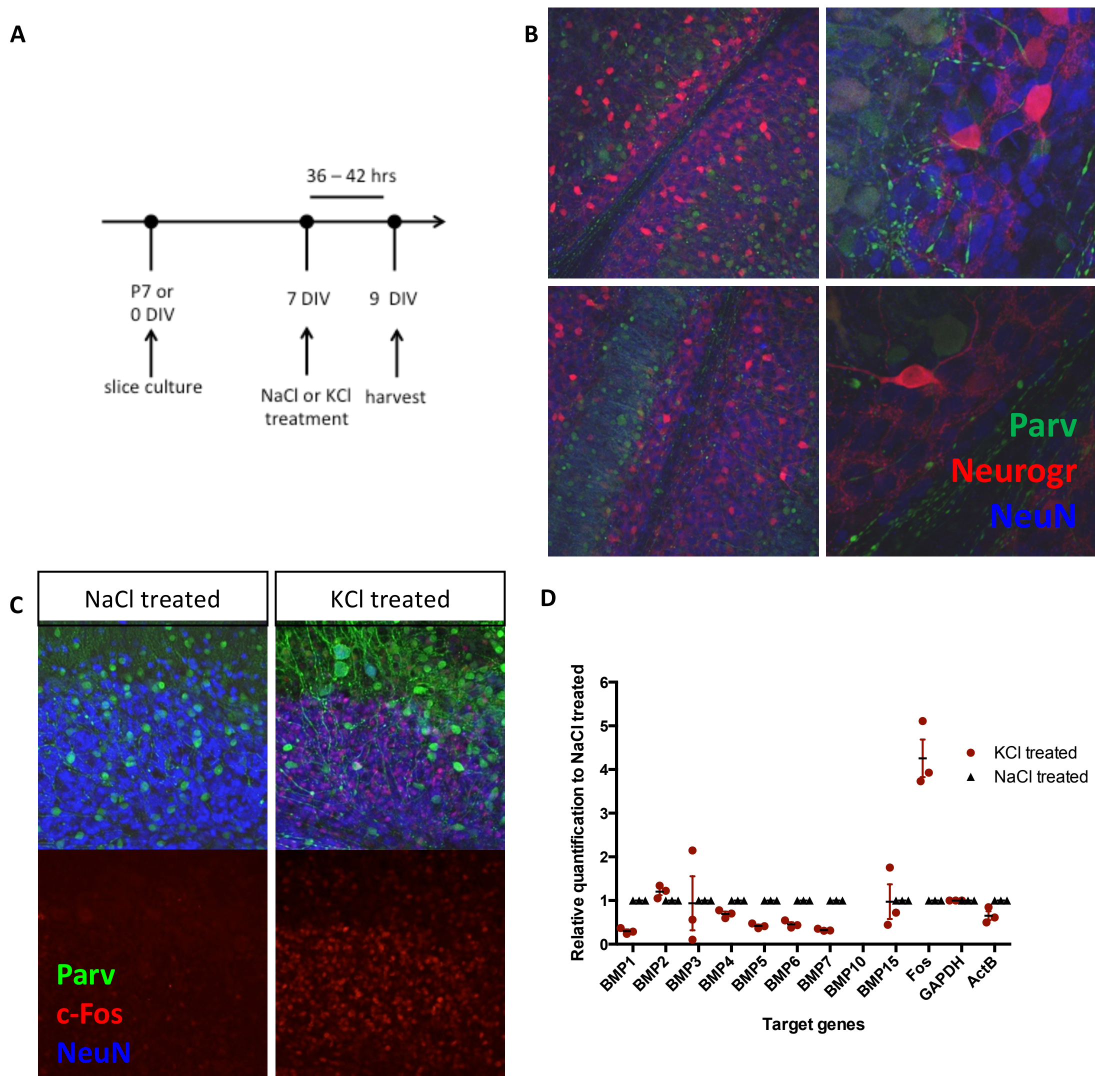

Figure 2.15 Cerebellar organotypic culture as an ex vivo circuit to manipulate activity in an intact cerebellar circuit. **A** Experimental set up. Animals were sacrificed at postnatal day 7 for slice culture, maintained for 7 days in vitro. Treated with NaCl or KCl for 36 hours before harvesting. **B** Slice cultures survive and show intact Purkinje cell layers, Golgi cells and granule cells. **C** Activation of inner granular layer by c-Fos immunostaining upon KCl treatment. **D** RT-qCPR results of KCl treated organotypic cultures show a reduction in BMP1, 5, 6, 7 mRNA levels with elevation of neuronal activity. (n = 3 animals per condition). Parv, Parvalbumin. Neurogr, Neurogranin.

To reduce neuronal activity, the ligand transcription and downstream targets of BMP signaling in the pontine gray nucleus of TeNT expressing animals were surveyed by RT-qPCR. In this case, an increase of BMP ligands 5, 6 and 7 were observed. On the other hand, in rotarod-trained animals (where there was elevated activity as seen by c-Fos levels), there was a complementary reduction of BMP ligands 5, 6, and 7 (Figure 2.16A, B).

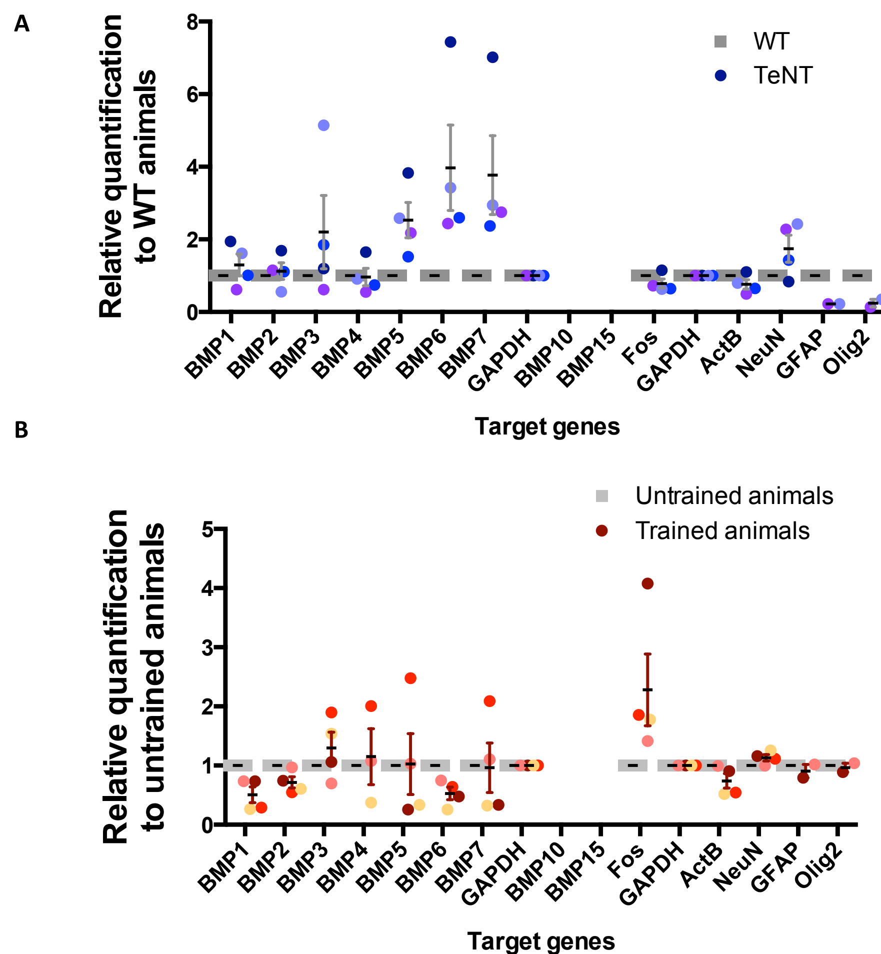

Figure 2.16 Transcription levels of BMP ligands in synaptically silenced and rotarod-trained animals. **A** TeNT and **B** rotarod trained animals. (n = 4 animals per condition).

Besides looking at the BMP ligand transcription, selected known downstream targets of BMP signaling were also examined. Of interest, there is again an increase Smad6, Id1 and Id3 transcription in the TeNT samples, while there is an opposite decrease in Smad6, Id1 and Id3 in the rotarod trained animals. In sum, neuronal activity states seem to bi-directionally regulate BMP signaling pathway (Figure 2.17A, B).

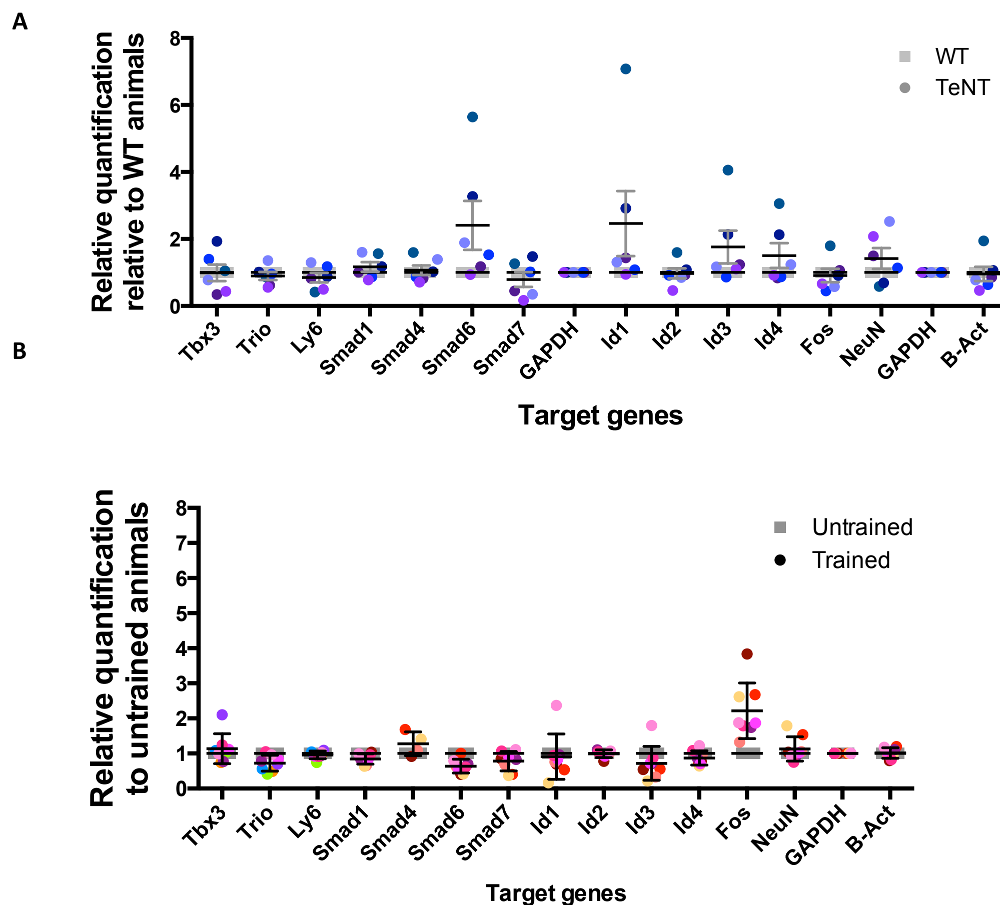

Figure 2.17 Transcription level of selected known downstream target genes of BMP signaling pathway in synaptically silenced and rotarod-trained animals. **A** TeNT. **B** Rotarod trained animals. (n = 6 animals for TeNT and n = 8 animals for rotarod trained animals).

Although these hints are interesting, there is a counter-intuitive increase of BMP pathway in activity reduced state while there is a decrease of BMP signaling in activity-enhanced states. This is because we have observed an increase in rosette complexity with increased neuronal activity (Figure 2.6A) and BMP signaling (Figure 2.1A), while a reduction in elaboration with reduced neuronal activity (Figure 2.9A) and BMP signaling pathway (Figure 2.1C). In a forward model, one would expect that an enhancement in neuronal activity by learning would increase BMP signaling, and thereby increase in rosette complexity.

As these analyses were performed in adult animals, we wondered if the same occurs in juvenile animals as well. To address this, P28 animals were trained on the rotarod and looked at the BMP ligands and downstream targets transcription as well (Figure 2.18A, B).

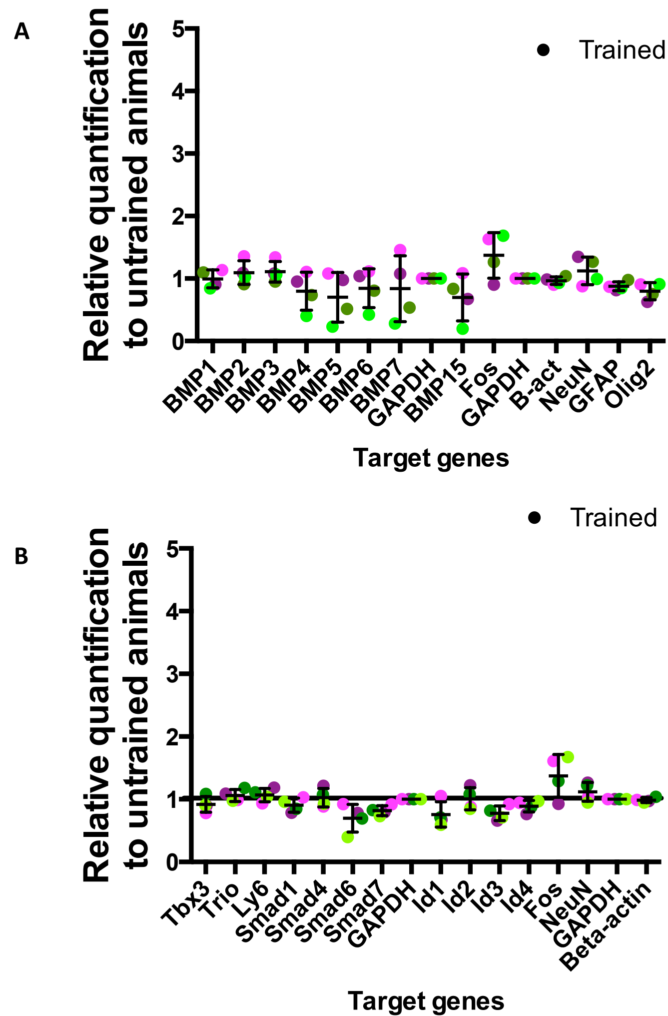

Figure 2.18 Transcription levels of BMP ligands and selected known BMP downstream target genes in juvenile (P28) animals subjected to rotarod training. **A** BMP ligands. **B** Known BMP downstream target genes. (n = 4 animals per condition)

In the juvenile animals, there is a similar trend as with the adult animals that has undergone training. There was a reduction in BMP 5, 6, and 7 ligands (Figure 2.18A) transcription while there is a reducing trend in Smad6, Id1 and Id3 transcription levels. Besides that, there is also a corresponding increase in c-Fos levels in the trained animals compared to the untrained animals (Figure 2.18B).

In sum, BMP signaling pathway may be subjected to neuronal activity-dependent regulation at the transcriptional level. However, there is a counter-intuitive increase in BMP signaling transcription in silenced neuronal and decrease in BMP signaling transcription in enhanced neuronal activity.

Discussion

DISCUSSION

The canonical BMP signaling pathway plays a role in maturation of adult cerebellar circuit

We have observed that the canonical BMP signaling has a role in the regulation of structural plasticity in the pontine mossy fibers. A gain in BMP signaling levels by BMPR2 overexpression resulted in an increase in complexity of rosette morphology (Figure 2.1A), while a loss of BMP signaling by Smad4 cKO results in a loss of rosette elaboration (Figure 2.1C). BMP-induced increase in rosette complexity resulted in putative synaptic contacts with Golgi cell, a postsynaptic partner (Figure 2.2). Furthermore, we have seen that Smad1 is not a limiting factor in the canonical BMP signaling in the regulation of rosette complexity, as overexpression of Smad1 does not significantly change rosette complexity (Figure 2.3A). In addition, we have identified Id3 a known downstream target of BMP signaling to have a role in the regulation of an aspect of mossy fiber rosette complexity (Figure 2.3C). These data strongly suggest that the canonical BMP signaling is a key pathway involved in positive regulation of structural plasticity of cerebellar mossy fiber terminals.

An emerging theme in developmental neurobiology is the recurrence of various morphogens in later stages of development. BMPs are re-used again in a few phases of neurodevelopment. Very early in embryonic development, antagonism of BMP signaling is crucial for the formation of neural tube (Liu and Niswander, 2005). Thereafter, BMP signaling specifies various cell types of the spinal cord, along with Sonic Hedgehog, to generate transcriptionally distinct progenitor domains in a gradient-dependent manner (Salie et al., 2005).

Following that, the non-canonical BMP signaling pathways have roles in axon guidance and repulsion of the commissural axons in the spinal cord (Augsburger et al., 1999, Perron and Dodd, 2011, Perron and Dodd, 2012, Phan et al., 2010). Increasing evidence from the fly neuromuscular junction (NMJ) and mouse literature that BMP signaling persists and has roles in postnatal neural circuit development. In the fly NMJ, BMP ligand secreted by postsynaptic muscle activates the presynaptic BMP receptors on the motoneuron to stimulate proper growth and function during late larval development (Aberle et al., 2002, Marques et al., 2002, McCabe et al., 2003, Rawson et al., 2003). Proper BMP receptor expression is crucial for normal innervation pattern and functional properties at the calyx of Held (Xiao et al., 2013). In early postnatal mossy fiber development (P0-P7), BMP4 secreted from Purkinje cells is pivotal in appropriate afferent targeting of developing mossy fibers to the inner granular layer (Kalinovsky et al., 2011). BMP signaling persists throughout various stages of embryonic and postnatal development, employing the canonical and non-canonical signaling pathways to mediate various roles and functions to orchestrate proper development of neural circuits.

In this study, we showed that in the mature mouse cerebellum (P21), BMP signaling pathway has a role in regulating structural maturation of mossy fiber rosettes. It is likely that the BMP signaling pathway gets activated presynaptically in the mossy fibers and travels retrograde to the cell body to initiate transcriptional changes. Evidence for this comes from cell-autonomous overexpression of BMPR2 and Smad4 cKO data, where gain and loss of canonical BMP signaling pathway results in gain and loss of rosette complexity

(Figure 2.1). As the canonical BMP signaling pathway involves changes in transcriptional profile of the cell, it is likely that activation of BMP pathway in the mossy fibers changes transcription of the cell.

One potential BMP downstream target gene in the regulation of mossy fiber complexity is Id3. Id3 overexpression results in a significant gain of function phenotype in the mossy fiber terminal complexity (Figure 2.3). It is a known downstream target gene of BMP signaling well characterized in cancer biology (Ying et al., 2003). As a dominant negative basic helix-loop-helix (bHLH) transcription factor, it inhibits a host of bHLH transcription factor. A bHLH transcription factor that is relevant for the pontocerebellar system is TCF4, which, along with Math1, is implicated in proper migration of pontine precursors to the pontine gray nucleus (Flora et al., 2007). It is possible that Id3 regulates transcription of bHLH proteins, like TCF4, in the pontine gray nucleus to regulate structural complexity of mossy fiber terminals.

Of interest, overexpression of Id3 induced a slightly different phenotype compared to BMPR2 overexpression. Both manipulations increased rosette complexity. However, a gain of Id3 expression resulted in an increase in rosette surface area (Figure 2.3E), while BMPR2 overexpression resulted in an increase in filopodia-like protrusions from rosettes (Figure 2.1B). One possibility for this phenomenon is that different signaling pathways are involved in different aspects of rosette complexity. Id3 may be one of the many downstream transcriptional targets that regulate only mossy fiber rosette surface complexity. BMPR2 gain of function may be activating a more complex downstream

transcription program that resulted in growth of filopodia-like protrusions from the rosettes.

This is reminiscent of the fly NMJ, where two downstream targets were uncovered – Trio and Lynx1 (Ball et al., 2010, Kim and Marques, 2012). Loss of these downstream targets only partially recapitulates loss of Wit (BMPR2 homologue) phenotype in the fly NMJ. Trio mainly rescued morphological growth of presynaptic terminals and to a smaller extent the functional maturation of the NMJ synapse (Ball et al., 2012). On the other hand, Lynx1 rescued synaptic function aberration of the Wit phenotype but loss of Lynx1 did not cause the morphological consequence of Wit loss of function (Kim and Marques, 2012). In this study, gain of Id3 only partially displayed BMPR2 gain of function phenotype. This suggests that there are potentially other downstream target genes regulating various aspects of mossy fiber rosette morphology. In addition, other signaling pathways, besides BMP signaling pathway, may have a role in regulating rosette morphology (i.e. Wnt signaling).

An open question in this project is the source of BMP ligand in the signaling pathway. In the cerebellar circuit, BMP4 ligands are expressed in the Purkinje cells and in the Golgi cells (Kalinovsky et al., 2011; Figure 2.0). As Golgi cell is a postsynaptic partner of mossy fibers, it is possible that the source of BMP ligand comes from the Golgi cells, similar to the fly NMJ. In fly NMJ growth, BMP ligand orthologue, Gbb, expressed in the postsynaptic muscle, is responsible for activating the BMP signaling pathway presynaptically to initiate presynaptic growth and development (McCabe et al., 2003). Given the similarity of

presynaptic activation of BMP pathway in the cerebellum to the fly NMJ, it is conceivable that a postsynaptic partner (i.e. Golgi cell) secretes the BMP ligand in the cerebellum. A direct way to test this hypothesis would be to look at the rosette morphology of mossy fibers with cKO of BMP ligand in Golgi cells.

A critical period for cerebellar mossy fiber structural plasticity

Another emerging theme from this work is that cerebellar mossy fibers are more plastic during postnatal development stage compared to adult, mature stage. Juvenile rosettes (P7 to P21) are more amenable to manipulations such as increase and decrease in BMP signaling levels (Figure 2.1) and neuronal activity manipulation (Figure 2.6). In contrast to the mature stage (P24 and beyond), BMP signaling (data not shown), neuronal activity regulation (Figure 2.6) and rotarod training in adult animals (Figure 2.13 and Figure 2.14) does not result in structural plasticity in the mossy fiber rosettes. This developmental difference might be due to the presence of a critical period where the juvenile mossy fiber terminals are in a more plastic compared to the mature stage.

Several labs have reported the occurrence of structural plasticity in the adult mouse cerebellum (Nishiyama et al., 2007; Ruediger et al., 2011; Boele et al., 2013; Carrillo et al., 2013). However, the rotarod learning experiment (Figure 2.13 and 2.14) and adult manipulation of neuronal activity by DREADDs (Figure 2.7) both suggest that the adult mossy fibers are relatively stable and do not show learning or activity-induced structural plasticity. In both cases, recent neuronal activity (examined by c-Fos expression) shows a clear recruitment and activation of pontine mossy fibers (Figures 2.4 and 2.5). This suggests that while the behavioral training and pharmacogenetic manipulation activates the pontine gray nucleus, it might not be sufficient to trigger structural plasticity in mature mossy fiber terminals.

Ruediger and colleagues (Ruediger et al., 2011) have observed structural changes in mossy fiber terminal upon rotarod learning. Observations in this study showed otherwise. The disparity between the two sets of experiment could be due to the different transgenic lines used. Ruediger et al. have used the Thy1-mGFP line, which has sparse expression of membrane-bound GFP in a small subset of neuronal populations. There is a lack of cell-type definition in this transgenic line. We have, on the other hand, used the IRX1-cre line, which has pontine-enriched expression. As such, the populations of mossy fibers examined in the two studies may represent distinct populations, with different functions and properties. Therefore, they might respond in different manners to learning. This suggests that structural plasticity in mossy fibers could be very selectively expressed in a specific sub-class of fibers only. At least, in the morphological parameters we examined, pontine mossy fiber rosettes do not show learning-induced structural plasticity.

Activity regulates BMP signaling pathway

Another aspect of my work is that BMP signaling could be subjected to activity-dependent regulation, similar to Wnt signaling. As the qPCR data suggested, BMP ligands and downstream targets of BMP signaling are differentially regulated in pontine neurons of different activity states. In activity-enhanced state (i.e. rotarod trained), there is a decrease in selected downstream targets of BMP signaling, like Smad6, Smad7, Id1 and Id3. On the other hand, there is an increase in Smad6, Smad7, Id1 and Id3 in the activity-reduced state (i.e. TeNT animals) (Figure 2.17). There was a similar change in the two different activity states when BMP5, 6 and 7 were examined as well (Figure 2.16). This data suggest that BMP signaling can be regulated during learning. However, the TeNT data needs to be interpreted with caution, as there is a strong glial activation in the use of TeNT (Figure 2.10).

In the fly NMJ, there have been reports of activity-dependent secretion and regulation of BMP signaling pathway (James et al., 2014). In addition, there are also reports of activity-dependent structural plasticity of fly NMJ requiring BMP signaling pathway (Piccioli and Littleton, 2014, Berke et al., 2013). Our finding that BMP signaling can be regulated by activity is novel because to the best of my knowledge, in the mammalian system, there have been no previous reports of activity-dependent regulation on BMP signaling pathway.

In a forward model, one would expect an increase in neuronal activity would result in an increase in BMP signaling and thus an increase in morphological complexity of mossy fiber rosettes (Figure 2.6). Yet, the result in this study

showed that increased neuronal activity by motor learning attenuates BMP signaling pathway (Figure 2.17). A possible scenario of homeostatic regulation might be at play to regulate the complexity of mossy fiber terminal to ensure stability of circuit input computation. For instance, when there is low activity level, where there is simplification of rosette elaboration, Id3 might be up regulated to promote complexity of rosette. On the other hand, when there is high activity level, Id3 might be down regulated to inhibit complexity. This mechanism could be a way the cerebellar circuit responds to differing incoming activities, but keeping it stable over time for basic afferent input computation.

Conclusions and Outlook

CONCLUSIONS AND OUTLOOK

From my work, it turned out that morphogens are re-used again in later stages of circuit function, besides their roles during early development. The canonical BMP signaling positively regulates structural plasticity of cerebellar mossy fibers in postnatal stages. I have also identified Id3 as a potential downstream target gene of BMP signaling in the context of mossy fiber structural plasticity regulation.

Besides that, I have also showed that cerebellar mossy fibers display activity-induced structural plasticity. Gain or loss of synaptic activity has an effect on complexity of mossy fiber rosettes. However, when I probed for learning-induced structural changes in the pontine mossy fiber terminals, I did not observe any morphological changes in the rosettes by parameters examined.

Lastly, selected downstream targets and ligands of BMP signaling seem to be down regulated by rotarod training. Neuronal activity could impinge on BMP signaling pathway to bring about regulation of mossy fiber rosette complexity in a homeostatic manner.

An open question and future direction for the project is to identify the source of BMP ligand that regulates structural plasticity of mossy fiber. Although the postsynaptic partner of mossy fiber, Golgi cell, expressed BMP4 ligand, direct evidence that it regulates mossy fiber complexity is missing. A possible experiment is to analyze mossy fiber rosettes with conditional ablation of BMP ligand in the Golgi cells.

Another open question is whether Id3 is a direct transcription target of BMP signaling pathway in the context of structural plasticity in mossy fibers. It is well established that Id proteins are downstream targets of BMP signaling (Ying et al., 2003) in cancer cell lines. But there is no direct evidence that Id3 is a direct target gene in the canonical BMP signaling pathway in the pontine mossy fibers. A way to test this is to survey Id3 transcription levels by RT-qPCR in pontine BMPR2 overexpression animals.

Another direction to follow up on is to find out if activity-induced structural plasticity of mossy fibers depends on BMP signaling. We have observed that BMP signaling and activity manipulation results in similar gain and loss of complexity of the terminals. As such, a logical ramification is to ask if these two pathways interact with each other, or not. A way to address this is to examine rosettes that are stimulated by hM3Dq with a Smad4 cKO background.

Materials and Methods

MATERIALS AND METHODS

Immunostaining

Postnatal animals, deeply anaesthesized, were subjected to trans-cardial perfusion using 4% paraformaldehyde in phosphate buffer (4% PFA in PB). Brain samples were sectioned using the Vibratome (50 um) and processed for immunofluorescence staining. Samples were stained overnight at room temperature in primary antibodies. Samples were washed three times in 1x PBS 0.05% Triton-X and incubated in their respective secondary antibodies for 2 hours in the dark (Jackson Immuno, or Life Technologies). Finally, samples were mounted to dry and kept in Fluomount-G (Southern Biotech) after a wash in 1x PBS. Primary antibodies used were as follows: GFP (Life Technologies, 1:1000), RFP (homemade, 1:1000), cFos (Calbiochem, 1:2000), Calbindin (Covance, 1:2000), Smad4 (Milipore, 1:100), pSMAD1/5/8 (Cell Signaling, 1:200).

RT-qPCR and data analysis

Rotarod trained or virus injected animals were dissected into 100 ul Trizol for RNA preservation and tissue lysis. Samples were grinded up using eppendorf compatible pestles and passed through sequentially in 19G and 26G needles in 1 ml of Trizol. 200ul of formaldehyde was added to the sample. After centrifugation, the transparent aqueous interface was extracted and purified for RNA using Qiagen RNeasy Mini Kit (74104) using product manual.

Purified RNA was subjected to reverse transcription using Promega Improm-II reverse transcriptase kit (A3800).

cDNA from previous step was diluted 1:10 for quantitative PCR reactions using Taqman gene expression assay kit. Comparative delta CT values were calculated from raw CT values of qPCR reactions and relative quantification (RQ) values were obtained for comparison between experimental conditions.

In utero electroporation

Timed-pregnant NMRI mice were anaesthetized with Ketamine/Xylazine mix when embryos were E14.5 old. After exposing uterus, expression plasmid of interest (e.g. pENTRE-syn-GFP at 1 ug/ul) was injected into the fourth ventricle of embryo using a micropipette. Forcep-shaped electrodes were positioned to cover bilateral ends of the fourth ventricle. 5 square pulses of 50 mV per 50 ms were delivered by the electroporator to the embryo (Dipietrantonio and Dymecki, 2009, Kalinovsky et al., 2011, Kawauchi et al., 2006). Uterine horns were re-positioned into abdomen; peritoneal wall and skin were sutured separately. Pups were brought to term and positively electroporated animals were analyzed at postnatal stages (P18, P21, P28).

Stereotaxic injection

P11 (adolescents) or P23 (adults) animals were head-fixed on stereotaxic injection setup. Bregma and lambda points of the skull were adjusted to the same plane. An error rate of ±0.05 was accepted. For targeting pontine gray nucleus at P11-P13, the coordinates X: 0.2; Y: -1.1 from lambda; Z: -6.3 to -6.5 were used. As for P23-24 animals, the coordinates X: 0.2, Y: -1.15 from lambda;

Z: -5.5 to -6.0 were used. AAV viruses were suspended in 1x PBS. Animals were analyzed 10 to 14 days post-surgery.

Rotarod

Postnatal animals (4 weeks or 8 weeks) were trained for either 5 or 8 days on an accelerating Rotarod protocol (after Ruediger et al. 2011 with slight modifications). Each subject underwent 4 trials per day on an accelerating protocol running from 5-50 rpm in 5 minutes unless otherwise stated. No pre-trial was done for the animals, as their learning curves were to be assessed by taking their latency to fall. The surface of the running rod was taped with 3M scotch tape make task more difficult and engaging. To make task even more challenging for younger animals (as they are more agile), a sequential increment of speeds were made over training days shown in the table below.

Day of training	Speed (rpm)	Duration (mins)	Acceleration
1	5-50	5	9
2	5-50	5	9
3	6-60	5	10.8
4	7-70	5	12.6
5	8-80	5	14.4

After 5 or 8 days days of training, animals were sacrificed half an hour after the last trial. They are either dissected into Trizol for RNA extraction, or perfused in 4% PFA in PB for immunostaining.

CNO administration

Animals expressing DREADD receptors were treated with 5 consecutive days of Clozapine-N-Oxide dissolved in saline (1 mg/kg) or saline only.

Image analysis

Z-stack images were acquired on LSM500 and analyzed using Imaris software. Lobules 6/7 and 9 hemispheres of the cerebellum were imaged, as these are the main projection area of pontine mossy fibers. Surfaces of complete rosettes, with clear axonal projections, were reconstructed three dimensionally by surface rendering tool in Imaris. Filopodia-like structures were quantified by tracing through the confocal stacks of regions of interest. Rosette sphericity (a calculation of how spherical a structure is) and filopodia number are used to quantify rosette terminal complexity.

Cerebellar organotypic cultures

P7 animals were sacrificed by acute beheading. Dissected cerebellum, with meninges removed, was quickly put into cold Gey's wash solution (Gey's solution and 0.5% glucose). The cerebellum was sliced into 200 um para-sagital sections using tissue chopper. Sections were then transferred on to 0.4 um organotypic culture insert (Milipore) soaked with BME/25% horse serum (100 ml Basal Medium Eagle, Gibco 41010-026; 50 ml 1x Hank's Balanced Salt Solution, Gibco 14060-032; Horse Serum, Gibco 16050-122; 1 mM L-glutamine; 2 ml Glucose; 2 ml Penicillin Streptomycin) at 37 ^oC. Cultures were kept for a week at 37 ^oC and 5% CO_2 before KCl or NaCl treatment (25mM) for

depolarization or control, respectively, for 36 hours. Sections were either fixed in 4% PFA in PB or extracted for RNA using Qiagen RNeasy Mini Kit (74104).

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List of Abbreviations

LIST OF ABBREVIATIONS

AAV	Adeno-Associated Virus
bHLH	basic helix-loop-helix
BMP	Bone Morphogenetic Protein
BMPR1	Type I BMP receptor
BMPR2	Type II BMP receptor
BMPR2-fl	Type II BMP receptor full length
BMPR2-sh	Type II BMP receptor short
CA3	Cornus Ammonis 3
cAMP	cyclic Adenosine Monophosphate
cKO	conditional knockout
CNO	Clozapine-N-oxide
CREB	cAMP Response Element Binding
CreERT2	Cre recombinase fused to a modified Estrogen Receptor
DREADD	Designer Receptor Exclusively Activated by Designer Drug
Dkk	Dickkopf
Dsh	Disheveled
EE	Enriched Environment
FFI	Feedforward inhibition
Gbb	Glass bottom boat
GEF	Guanine nucleotide Exchange Factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescence Protein
GIRK	G protein-couple Inward Rectifying Potassium channel
GSK	Glycogen Synthase Kinase

Id protein	Inhibitor of DNA-binding protein
KCl	Potassium Chloride
LIMK1	LIM domain Kinase 1
LMT	Large Mossy fiber Terminals
LRP	Lipoprotein-related Receptor Protein
LTD	Long-term Depression
LTP	Long-term Potentiation
MAPK	Mitogen-activated protein kinase
NaCl	Sodium Chloride
NMJ	Neuromuscular Junction
TeNT	Tetanus Toxin
RT-qPCR	Reverse Transcriptase-quantitative Polymerase Chain Reaction
TGF-β	Transforming Growth Factor- β
PI3K	Phosphoinositide-3 Kinase
TCF4	Transcription factor 4
vGlut1	vesicular Glutamate transporter 1
VAMP2	Vesicle-associated membrane protein 2
WCST	Wisconsin Card Sorting Test
Wg	Wingless
WT	Wildtype

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PERSONAL INFORMATION

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RESEARCH EXPERIENCE

UNIVERISITY OF BASEL, BIOZENTRUM
Basel, CH
2011 – 2015
Supervisor:
Prof. P. Scheiffele

GRADUATE STUDENT

- My work revolved around understanding how Bone Morphogenetic Protein regulates structural plasticity in cerebellar mossy fibers.
- We uncovered that the canonical BMP signaling pathway and neuronal activity can bi-directionally influence structural complexity of mossy fiber rosettes.
- Trained in *in vivo* techniques such as in utero electroporation and stereotypic virus injection in mice.
- Familiar with confocal microscopy and image analysis by Imaris.

DUKE-NUS GRADUATE MEDICAL SCHOOL
Singapore, SG
2009 – 2010

Supervisor:
A/Prof. H. Wang

RESEARCH ASSISTANT

- My work was focused on finding molecular machinery that regulates asymmetric cell division in *Drosophila* neuroblasts.
- In particular, we uncovered that the transcription factor Zif regulates aPKC levels and localization to regulate neuroblast polarity and self-renewal.
- Trained in molecular techniques such as Western Blotting, RT-PCR and cloning.
- Familiar with confocal microscopy and fly genetics.
- I also handled administrative jobs, such as purchase order, health and safety protocols and basic lab jobs.

UNIVERISITY OF LEEDS
Leeds, UK
2009 – 2009
Supervisor:
Prof. N. Gamper

FYP STUDENT

- My research interest was focused on dissecting the molecular mechanisms that regulated KCNQ K^+ channels.
- I found that modifying tonic PIP_2 levels can change the interaction between KCNQ2 channels and Ca^{2+}/Calmodulin complex.
- I learnt molecular techniques such as transient cell transfection, Western Blotting and co-immunoprecipitation.

EDUCATION

UNIVERISITY OF BASEL, BIOZENTRUM
Basel, CH
2011 – 2015

PhD NEUROBIOLOGY (2011 – 2015)
MSc MOLECULAR BIOLOGY (2011 – 2011)

- See "Work Experience" for an overview of project undertaken and key skills acquired.

UNIVERISITY OF LEEDS
Leeds, UK
2006 – 2009

BSc NEUROSCIENCE

- Key modules include: *In vivo* techniques, neuroanatomy, topics in toxicology.
- Top student for neuroscience cohort (class of 2009) for 2 years in a row.

AWARDS & PUBLICATION

AWARDS

Basel International PhD, Fellowship for Excellence recipient (Aug 2011 – Dec 2014)
A poster prize for Gordon Research Conference, Hong Kong (Molecular and Cellular Neurobiology, July 2014)

PUBLICATION

Chang et al. (2010) Interplay between transcription factor Zif and aPKC regulates neuroblast polarity and self-renewal. *Dev Cell*. 19(5). pp. 778-85.

VOLUNTEER WORK

CAREGIVERS ALLIANCE LTD
Singapore, SG
2013 - 2013

- I was working at Caregivers Alliance Ltd (CAL) as a volunteer consultant in Neurobiology for their Caregivers-2-caregivers (C2C) course; I helped to improve topics pertaining to general knowledge about the brain and psychiatric mediations.
- I also helped to expand resources for their mandarin C2C course by translating and subtitling some videos that they use in their English C2C course.
- In addition, I also helped with generating flyers and information brochures for events and courses available at CAL.

OTHER SKILLS

- Handicraft making
- Volleyball