A Comparative Approach to Cerebellar Circuit Function

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ABSTRACT

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The approaches available for unlocking a neural circuit – deciphering its algorithm's means and ends – are restricted by the biological characteristics of both the circuit in question and the organism in which it is studied. The cerebellum has long appealed to circuits neuroscientists in this regard because of its simple yet evocative structure and physiology. Decades of efforts to validate theories inspired by its distinctive characteristics have yielded intriguing but highly equivocal results. In particular, the general spirit of David Marr and James Albus's models of cerebellar involvement in associative learning, now almost 50 years old, continues to shape much research, and yet the resulting data indicates that the Marr-Albus theories cannot, in their original incarnations, be the whole story.

In efforts to resolve these mysteries of the cerebellum, researchers have pushed the advantages of its simple circuit even further by studying it in model organisms with complimentary methodological advantages. Much early work for example was conducted in monkeys and humans taking advantage of the mechanically simple and precise oculomotor behaviors at which these foveates excel. Then, as genetic tools entered the scene and became increasingly powerful, neuroscientists began porting what had been learned into mouse, a model system in which these tools can be deployed with great sophistication. This was effective in part because cerebellum is highly conserved across vertebrates so complimentary insights can be made across different model systems.

Today genetic prowess has been further augmented by rapid advances in optical methods for visualizing and manipulating genetically targeted components. The promise of these new capabilities provides grounds for exploring additional model organisms with characteristics particularly suited to harnessing the power of modern methodology.

In the following chapters I explore the promise and challenges of adding a new organism to the current pantheon of most commonly studied cerebellar model organisms. In chapter 1, I introduce the cerebellar circuit and a sampling of the historically equivocal outcomes met by efforts to test Marr-Albus theories in the context of a classical cerebellar learning paradigm: vestibulo-ocular reflex adaptation.

In chapter 2, I detail my efforts to establish a method for population calcium imaging in cerebellar granule cells (GCs) of the weakly electric mormyrid fish, *gnathonemus petersii*. The unusual anatomical placement of GCs in this organism, directly on the surface of the brain, is ideal for optical methods, which require the ability to illuminate structures of interest. Furthermore, in the mormyrid, GCs play analogous role in two circuits -- the cerebellum and a purely sensory structure, the electrosensory lobe, which has a cerebellum-like structure. This latter circuit is unusually well-characterized and appears to employ a Marr-Albus style associative learning algorithm. This could provide a helpful context for interpreting the purpose of GC processing, shared by this circuit and the cerebellum proper. However, taking advantage of these qualities will require overcoming methodological hurdles presented by imaging in this as-yet not genetically tractable organism. While I was able to load and image evoked transients in these cells, and twice observed spontaneous transient, I did not find a loading method that allowed routine observation of spontaneous levels of activity.

In chapter 3, I introduce the larval zebrafish, *danio rerio*, an organism in which optical and genetic methods are already quite established. The zebrafish is genetically tractable and orders of magnitudes smaller than other vertebrate model systems, making it extremely accessible to optical monitoring and manipulation of neural activity. However, in contrast to the mormyrid, very little is known about the physiology of the cerebellar circuit components in this organism or the behaviors to which they contribute.

In chapter 4 I detail my efforts to contribute to this modest foundational knowledge by characterizing the electrophysiological activity of Purkinje cells of larval zebrafish during the optomotor response (OMR)—a behavior with similarities to cerebellar-dependent visual stabilization behaviors that have been studied extensively in mammals. I observe a diversity of structured motor and visual activity that suggests that Purkinje cells could contribute to adjusting swim speed during the OMR and other behaviors.

In chapter 5, I outline some of the upfront work that remains before cerebellar researchers are likely to fully harness the power of optical and genetic methods in the zebrafish as well as the types of experiments that may become possible if we do.

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CHAPTER 1
INTRODUCTION TO THE CEREBELLAR CIRCUIT

CHAPTER 1: INTRODUCTION TO THE CEREBELLAR CIRCUIT

Historical context and motivation

The simple architecture of the cerebellum has tantalized neuroscientists for decades. Its small number of cell types are connected in a highly regular structure whose very form seems to suggest function. Based on the distinctive architecture of the two major inputs to cerebellum, described in detail below, David Marr and James Albus conjectured that the circuit might serve as a flexible module for supervised learning and adapting of desired responses to particular sensorimotor contexts (Albus 1971; Ito and Kano 1982; Marr 1969). It has been theorized that such a capacity to learn and continually adjust input-output rules might offer organisms a way to keep sensorimotor transformations properly calibrated in the face of such physical changes as growing bodies or tiring muscles.

However, while these theories make concrete, testable predictions of circuit activity, it has proven challenging to conclusively evaluate them. This is in part due to methodological constraints. Fortunately, different model organisms have different inherent methodological strengths and weaknesses. As the cerebellum is largely conserved across vertebrates, methodological limitations could be minimized by choosing different model organisms with features best suited to address different question. In this dissertation I explore methodological opportunities and challenges in two particular species—the mormyrid electric fish and the zebrafish—that could make it easier to monitor and precisely manipulate cerebellar circuitry at the population level during behavior. Such methods could allow acquisition of cerebellar data that can otherwise be prohibitively taxing to collect.

The cerebellar circuit

The cerebellum has a strikingly simple anatomy that is largely uniform across subregions that are engaged by different sensorimotor behaviors. This regularity, despite input sources and output targets that vary, has led neuroscientists to suppose that the circuit carries out a single canonical function in the variety of tasks in which it is involved. The unique connectivity of the less than ten cells that make up this circuit (Fig. 1) has spurred hypotheses about what that cerebellar algorithm might be.

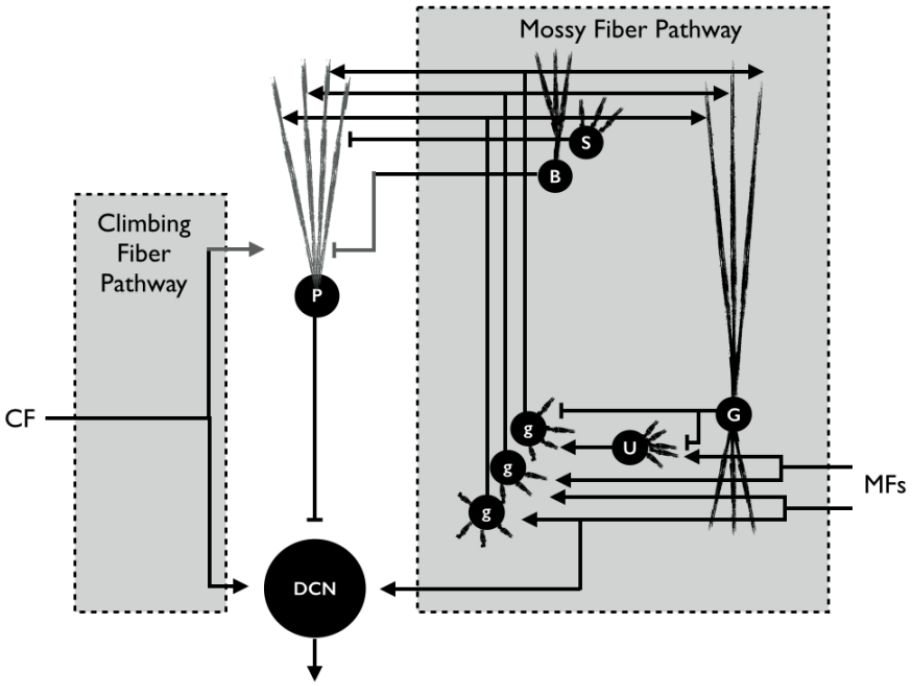

Figure 1.1 The cerebellar circuit.
The cerebellum receives two inputs with quite different characteristics. A single climbing fiber from the inferior olive innervates each Purkinje cell (PC) directly while many mossy fibers contact PCs indirectly via a massive network of excitatory granule cells and other less numerous interneurons, mostly inhibitory. PCs inhibit the output cells of the cerebellum, found in the deep cerebellar nucleus in mammals, which also receive direct input from mossy fibers and climbing fibers. **CF**: climbing fiber, **MFs**: mossy fibers, **G**: Golgi cell, **U**: unipolar brush cell, **g**: granule cell, **S**: stellate cell, **B**: basket cell, **P**: Purkinje cell, **DCN**: deep cerebellar nucleus. (Lugaro cells, a less studied interneuron that inhibits Golgi, basket, and stellate cells and receives inputs from granule cells and Purkinje cells, has not been included in this schematic for simplicity of visualization).

Marr/Albus theories of cerebellar learning hinge on the contrasting features of the cerebellum's two inputs: mossy fibers and climbing fibers. These pathways eventually converge (after substantial interneuronal processing in the case of mossy fibers) on a population of cerebellar interneurons called Purkinje cells. Strikingly, just one climbing fiber (CF), as compared to many thousands of mossy fibers, drives each Purkinje cell. This and other physiological and anatomical differences, discussed below, inspired the theory that during learning a teaching signal conveyed by the CF sculpts PC responses to sensorimotor state information provided by the mossy fiber pathway.

The mossy fiber pathway into the cerebellum provides PCs with a broad range of sensory and motor information from a variety of brain regions and from the spinal cord. Before this information reaches PCs, it undergoes a good deal of pre-processing by an enormous population of excitatory interneurons, called granule cells, along with a handful of other less numerous interneurons (most inhibitory—called Golgi, basket, stellate and Lugaro cells—and one excitatory—called the unipolar brush cell). The axons of granule cells, called parallel fibers, course perpendicularly through fan like dendritic arbors of PCs, which are stacked like pancakes. Parallel fibers likely contact nearly every PC they pass (Brand et al. 1976). The mossy fiber pathway thus does not seem to be highly discriminating in what information it provides to individual PCs, but rather offers each PC a multimodal inundation of processed information about an organism's sensory and motor state.

The climbing-fiber pathway on the other hand stems exclusively from the inferior olive. Every PC receives a single, direct CF input. This CF makes such extensive contacts onto a PC that its firing drives an unusual second action potential type, called a "complex spike" in mammals or, more generally, a "CF response".

A PC's regular action potentials—driven by the combined effect of excitatory drive from parallel fibers, intrinsic PC activity and feed-forward inhibition from stellate and basket cells—fire at high baseline levels and can reach frequencies of more than 200 Hz when responding to stimuli. Numerous studies have indicated that sensory and motor information can be read out from the firing rates of these action potentials, called simple spikes. The CF response, on the other hand, is driven at quite low frequencies, on the order of 1 Hz. These frequencies do not seem compatible with having a strong influence on a rate code in PCs. This observation helped inspire the Marr/Albus theory of CF activity as a teaching signal.

PCs directly inhibit the output cells of the cerebellum. In mammals these output cells are separated into the deep cerebellar nuclei, while in teleosts they are found in the same region as the PCs that innervate them. In either case, the outputs of the cerebellum contact a wide range of brain regions, including strong connections to spinal cord, via relays in the brainstem, as well as connections to the forebrain. (Of note, there is one region of cerebellum, the vestibulocerebellum, in which PCs do not project to the deep cerebellar nuclei, but have an analogous connection to the vestibular nuclei, which, in addition to connections to spinal cord and thalamus, directly innervate motoneurons in the oculomotor nuclei that control eye movements). The activity of these output cells mediates the cerebellum's influence on downstream structures, most notably on motor behaviors.

A cerebellar algorithm

Motor deficits in patients with cerebellar damage or hereditary cerebellar degenerative diseases offer clues about the algorithm executed by the cerebellum. Cerebellar patients exhibit difficulty with movements normally carried out automatically, including impairment of gait

(ataxia), lack of posture stability (tremors) and disordered eye movements (Diener et al. 1993; Dietrichs 2008; Fine et al. 2002; Tilikete and Pelisson 2008). In the words of one cerebellar patient, “The movements of my left arm are done subconsciously, but I have to think out each movement of the right (affected) arm” (Holmes 1939). Based on these types of deficits and the observed differences between mossy fiber and CF pathways, Marr and Albus postulated theories that the unique organization of cerebellar circuitry learns to automate smooth motor responses to learned sensory contexts. Specifically, they proposed that a CF teaching signal adjusts the weights of a basis of contextual information, provided by parallel fiber inputs, to sculpt desired motor responses to particular sensorimotor contexts.

The crux of this theory of the cerebellum has been likened to a theoretical pattern associator called an associative net (Tyrrell and Willshaw 1992). At its most basic, an associative net consists of a set of input lines carrying distinct pieces of sensory information and a set of output lines controlling individual components of motor responses (Fig. 2). During a training period, analogous to conscious learning, a teaching signal activates the appropriate output lines to generate a desired motor response. Any input lines that happen to be simultaneously activated by the current sensory context will have their connections to the activated output lines strengthened. As a result, the sensory context that was present during training will now be sufficient to drive the desired combination of motor outputs without supervision by the teaching signal. In short, each output line receives a full basis of contextual information from which appropriate input combinations can be chosen to drive automatic activation of that output line in the future. In terms of motor-learning, this framework could allow supervised-automation of motor responses to sensory contexts.

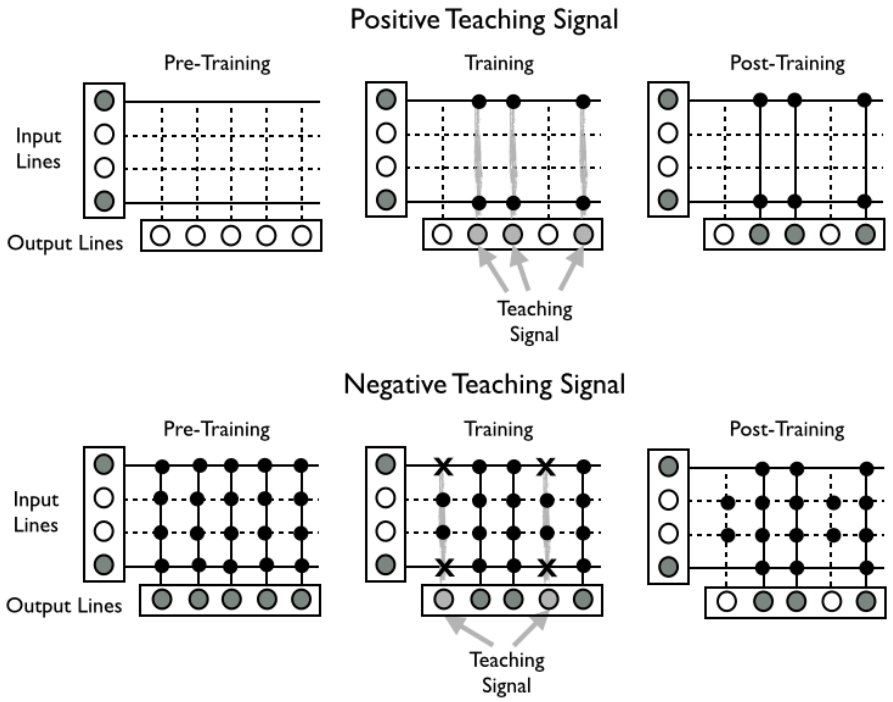

Figure 1.1 An associative net model of learning.
Marr's original proposal of the learning algorithm of carried out in the cerebellum was similar to a theoretical pattern associator called an associative net that learns to generate specific patterns of output in response to specific input pattern contexts. This can theoretically be accomplished by a positive or negative reinforcement teaching signal. In the case of the former (*top row*), the teaching signal acts as an example, as was theorized by Marr. Before training, the basis of input signals has no ability to drive the outputs, which are driven instead directly by the teaching signal. During training, inputs that are coactive with the output lines driven by the teaching signal have their connections to those outputs activated. After training the learned input pattern contexts can drive the appropriate output automatically without need for the direct teaching signal activation. A complimentary series of events can accomplish learning via a negative teaching signal (*bottom row*). A network in which input lines initially drive all output lines indiscriminately receives an error signal that indicates whenever this results in an inappropriate activation of an output line. Any input lines that are active at the time of erroneous output line activations have their connections to those lines removed. After training this results in input patterns driving only the desired output patterns. Albus hypothesized that the teaching signal was of the latter, error-indicating variety, though his model differed from the simple associative net model in a number of other ways, including weighted synapses and proportional rather than binary plasticity.

Validating theories of cerebellum

Because Marr and Albus's theories were grounded in the actual biology of the cerebellum, they made remarkably concrete predictions about how the various components of the

cerebellum should behave. This began a history in cerebellar research of testing biologically grounded theories and modifying them to account for new data.

For example, Marr initially predicted that the proposed teaching signal should serve as an "example signal" in the manner described above (i.e. any incoming fibers simultaneously active with the teaching signal should be strengthened). Conversely, Albus predicted the signal would be an error signal that signifies when an output line should NOT have been active and would therefore weaken inputs proportionate to their activity at the time of the error (Albus 1971). In this manner an appropriate PC output could be carved out from a basis of contextual information provided on the parallel fibers. (For simplicity, both the "example signal" and "error signal" trained circuits shown in figure 2 use binary synapses that are either active or inactive as in Marr's original model, although Albus's non-binary synaptic weights are more biologically accurate). Marr and Albus's theories thus differed in their predictions of whether incoming contextual fibers simultaneously active with the CF should be strengthened or weakened. A seminal experiment by Ito (Ito et al. 1982) demonstrated that stimulation of the CF drove long term depression (LTD) in co-active parallel fibers, supporting a role for CFs as an error signal rather than as an example signal,

Today, parallel fiber LTD-mediated supervised learning of input-output associations is often referred to as the Marr/Albus/Ito model of the cerebellum's function and remains an underlying pillar of many modern theories. However the concretely testable postulates of this classic model have continued to support data-driven questioning and modification of its tenants. Despite the proven existence of parallel fiber LTD, for example, there is evidence that the nature, locations, and interplay of all of the plasticity driving cerebellar learning is most likely much more complex than this single factor.

The VOR as a case example

One behavioral paradigm in which cerebellum has been extensively studied is gain adaptation of the vestibulo-ocular reflex (VOR). The VOR is a visual stabilization reflex that counters head movements with rapid and proportionate eye movements in the opposite direction to keep visual targets fixed on the retina (Boyden et al. 2004; Broussard and Kassardjian 2004; du Lac et al. 1995; Ito 2013). Successful stabilization depends on properly transforming head movements to compensatory eye movements. The scalar for this transformation, defined as the ratio between eye and head velocities, is called the VOR gain. Too low of a gain results in insufficient eye movement to fully counteract the effects of head movement and a residual slip of the image across the retina in the opposite direction of head movement. Too high of a gain over-corrects, causing slip of the image across the retina in the same direction as head movement.

The appropriate gain setting can change over both long timescales, as an organism grows, and over short timescales, due to fatigue, injury, or, in the case of humans, a new glasses prescription that changes the magnification of the field of view. VOR-gain adaptation therefore allows organisms to adjust their gain setting when visual feedback indicates that it has become too low or too high. Adaptation can be triggered artificially by moving the visual scene at the same time as the head. If the scene is moved in the opposite direction from the head, the appropriate amount of compensatory eye movement increases, and the image initially slips somewhat on the retina until the higher gain is learned. Conversely, if the image is moved in the same direction as the head, the amount of compensatory eye movement that is necessary decreases, causing an initial overshoot slip until the gain decreases accordingly.

At its most basic then, the task of VOR gain machinery is to learn to drive the appropriate adjustment of oculomotor output in response to information about head movement. Such an undertaking aligns remarkably well with the theorized capabilities of the classic Marr/Albus/Ito model. Indeed, in 1972 Ito proposed an explicit mapping of the components of the Marr/Albus/Ito model onto the circuitry of the cerebellar floccular complex, known to be involved in VOR adaptation (Ito 1982; 1972).

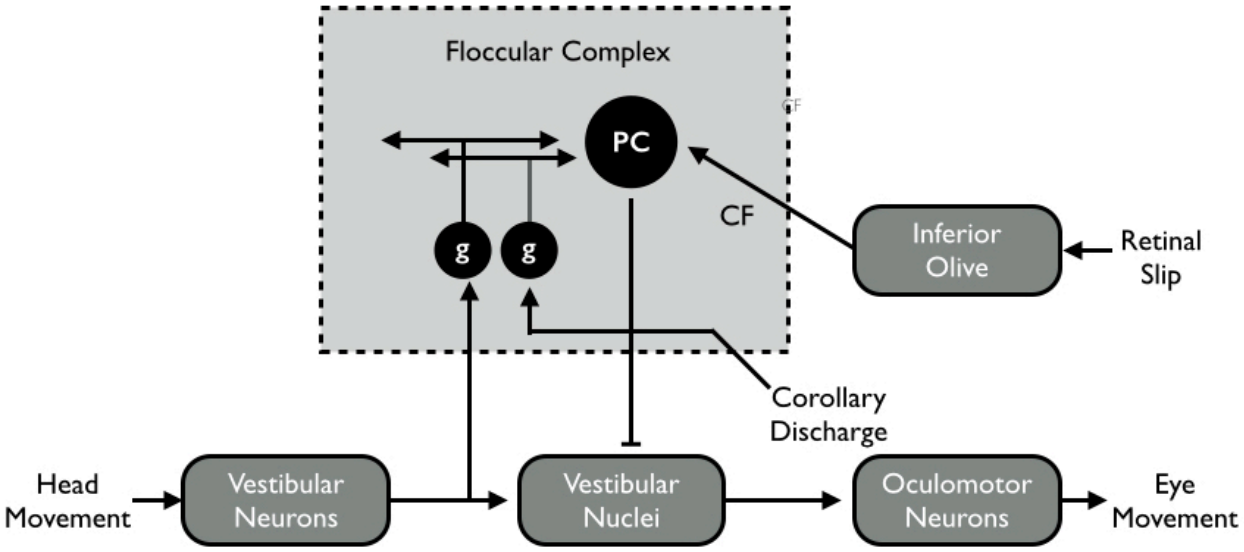

Figure 1.3 Vestibulo-ocular reflex adaptation circuitry.
Head movement velocity drives vestibular inputs, which influence eye movements via a direct route through the brainstem and an indirect side-loop through the cerebellum. In addition to vestibular information, Purkinje cells in the floccular complex of the cerebellum receive oculomotor corollary discharge information as well as retinal slip information via climbing fiber inputs from the inferior olive. Vestibulo-ocular reflex adaptation was originally thought to be mediated by LTD at the synapses between vestibular parallel fibers and Purkinje cells, driven by an error signal conveyed by the climbing fiber. This hypothesis has since been complicated by identification of various sites of plasticity with different apparent contributions to vestibule-ocular reflex adaptation. PC: Purkinje cell, CF: climbing fiber, g: granule cell.

The floccular complex is situated as a side loop relative to a direct pathway responsible for carrying out the VOR. The direct pathway consists of a simple three synapse arc. The vestibular nerve sends information about head movement to brainstem vestibular nuclei. These

neurons from the vestibular nuclei contact downstream oculomotor neurons responsible for eye movements. The vestibular nerve also contacts the vestibular nuclei indirectly via the cerebellar side loop. The same vestibular information is provided via mossy fibers to the floccular complex, which sends inhibitory output, via PCs, to the neurons of the vestibular nucleus, called floccular target neurons (FTNs).

Ito proposed that PC inhibition appropriately modulates FTNs during VOR gain by increasing inhibition in gain-down adaptation and decreasing it during gain-up (Ito 1982; 1972). This was theorized to occur via a classic Marr-Albus-Ito mechanism in which image retinal slip provides an error signal that drives CF activity, which in turn drives LTD in coincidentally active parallel fibers carrying vestibular information. This decreases the strength with which PCs were driven by that context in the future.

Observed changes in PC activity after VOR gain training aligned with the idea that learned changes in PC firing modify FTN activity appropriately to adjust the gain of downstream eye movements. After VOR gain-down training, for example, PCs fired in phase with vestibular neurons, thereby providing counteracting inhibitory drive to the FTNs driving oculomotor neurons (du Lac et al. 1995; Watanabe 1984; 1985)

However, other data called a contribution of parallel fiber LTD to VOR adaptation into question (Miles et al. 1980; Miles and Lisberger 1981). In 1980, Miles et al. attempted to isolate the effect of head movement inputs to PCs, Ito's presumed site of motor learning, on PCs with the clever use of a behavioral paradigm known as VOR cancellation. In this paradigm monkeys' heads were rotated with the same dynamics that would usually induce the VOR, but their trained visual fixation point was moved coincidentally such that to stay fixated monkeys had to keep their eyes directed forward in their heads, overriding or cancelling the VOR. After monkeys had

been trained using a VOR gain up or gain down protocol, Miles et al. recorded from PCs during cancellation of the VOR to probe for changes in the drive of head movement neurons to PCs. They found activity modulations in response to head movements, but in the wrong direction to cause the observed changes in the VOR. That is, after gain-down training, PC responses to isolated head velocity during VOR cancellation was actually modulated OUT of phase with the vestibular inputs, suggesting that vestibular-driven PC inhibition of FTNs not only don't mitigate vestibular drive directly onto FTNs after gain-down training, but exacerbate it compared to pre-training.

Miles et al. therefore concluded that LTD at vestibular parallel fibers onto PCs cannot be the primary mechanism underlying VOR gain change. They suggested that changes in PC activity during the adapted-VOR that appear appropriate to drive the gain change in this behavior can be explained by changing drive from a second mossy fiber input that had been recently identified, which carries oculomotor corollary discharge (Lisberger and Fuchs 1978). They further suggested that the change in drive from this input isn't due to plasticity of its weights, or indeed to plasticity in the cerebellar cortex at all. Rather, they posited that it is a side-effect of plasticity occurring outside of the cerebellum in the direct pathway, from vestibular inputs onto FTNs. The resulting changes in eye movements would feed back onto PCs via oculomotor corollary discharge inputs; they would drive corresponding changes in the PC's activity rather than vice versa -- a classic chicken-and-the-egg mix-up.

Since then understanding sites of plasticity in the cerebellar cortex and vestibular nuclei has been the subject of much research. A comprehensive picture has yet to emerge, but a variety of plasticities has been definitively demonstrated at multiple synapses in the cerebellar cortex and vestibular nuclei. Both LTD (Ito and Kano 1982) and LTP (Lev-Ram et al. 2002; Salin et al.

1996) can occur at parallel fiber to PC synapses as well at other locations in cerebellar cortex, including from inhibitory molecular layer interneurons to PCs (Kano et al. 1996; Kawaguchi and Hirano 2002) and even from mossy fibers to granule cells (D'Angelo et al. 2005). In the vestibular nuclei, various forms of plasticity have also been demonstrated including at the mossy fiber to FTN neurons as was first surmised by Miles and Lisberger (Gittis and du 2006; McElvain et al. 2010).

There is little consensus about the contribution of, or necessity for, any of these sites in VOR adaptation, including the classic CF-mediated LTD of parallel fibers. For example, a cleverly designed behavioral paradigm, using opposing target and background motion to null out a visual scene's net retinal slip, can drive VOR learning without engaging the CF (Ke et al. 2009), suggesting that the CF is not necessary for learning at all. Yet, mice with multiple CF inputs to cerebellar PCs exhibit impaired VOR adaptation, indicating that the CF can influence learning regardless of whether it is necessary for it (Kimpo and Raymond 2007). Similarly, it has been demonstrated that optogenetic activation of the CF paired with vestibular input can induce VOR gain increases. Yet it was not found possible to drive VOR gain in the decreased direction, signifying that different sites of plasticity may be engaged in different aspects of VOR learning (Nguyen-Vu et al. 2013).

Current theories tend to surmise that multiple sites of plasticity work synergistically and perhaps in some instances redundantly to achieve VOR adaptation. An experiment using mice with impaired parallel fiber to PC LTD found that these animals did not demonstrate short-term VOR adaptation, but could learn some amount of adaptation over an extended 8 day detraining period (van Alphen and De Zeeuw 2002), raising the possibility that parallel fiber to PC LTD may be required for rapid VOR adaptation while other independent plasticity mechanisms work

on a longer timescale. Although cerebellar mice with impaired and likely more specific mossy fiber to PC LTD have shown no deficits in VOR adaptation at all (Schonewille et al. 2011), there remains considerable interest in the theory that initial learning of a VOR gain change is carried out in the cerebellar cortex, but then transferred to the vestibular nuclei over a longer time course of training (Anzai et al. 2010; Galiana 1986; Kassardjian et al. 2005; Nagao and Kitazawa 2003; Peterson et al. 1991; Titley et al. 2007).

A time varying role of the dependence of learning on cerebellar cortex would help explain discrepant findings regarding the effect of cerebellar inactivation on VOR adaptation as there has been considerable variability in training time across experiments (Luebke and Robinson 1994; McElligott et al. 1998; Nagao and Kitazawa 2003; Partsalis et al. 1995; Pastor et al. 1994; Robinson 1976). A number of studies have addressed this time course explicitly. In cats, pharmacological inactivation of excitatory transmission in the cerebellum, by injecting the glutamate antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) into the flocculus, reversed gain adaptation if applied after 60 minutes of gain-decrease VOR training, but not when it was applied after 3 days of learning (Kassardjian et al. 2005). Interestingly, it has been shown that after 60 minutes of training, VOR adaptation could also be undone by continued VOR performance in the dark with no visual feedback, but an hour of no VOR movement in the interim prevented VOR adaptation loss by performance in the dark (Titley et al. 2007). More recently, in monkeys it was demonstrated that injection of lidocaine, a blocker of neuronal activity, into the flocculus after 2 hours of gain-decrease VOR training caused the VOR to revert to baseline but had little effect when administered after 3 days of VOR training (Anzai et al. 2010).

Methodological challenges in studying cerebellar learning

While there is good general evidence of differential engagement of multiple sites of plasticity in the cerebellum and its target nuclei, it has been challenging to arrive at a comprehensive understanding of how specific sites and mechanisms of plasticity are engaged and interact to exert their effect on VOR adaptation. For example, if indeed plasticity in the cerebellum is necessary for short term memory, while plasticity in the vestibular nucleus is necessary for long term consolidation, a question will be whether this latter plasticity depends on the former. If so, this would be in agreement with a trigger-and-storage model of cerebellar motor learning (Medina et al. 2002). Alternatively, the two could proceed independently, as suggested by evidence that VOR adaptation can occur without instructive CF input (Ke et al. 2009) and by the fact that mice with impaired mossy fiber to PC LTD do not demonstrate short-term VOR adaptation, but can learn some amount of adaptation over extended 8 days training (van Alphen and De Zeeuw 2002).

Part of the challenge in teasing apart the various plasticity mechanisms implicated in VOR adaptation arises from methodological limitations in monitoring and precisely manipulating the circuit. For example, though learning is an extended process that unfolds over time, historically using electrophysiological techniques to monitor large numbers of cells and their interaction throughout this time course has been prohibitively difficult. Instead, researchers have tended to compare states of the circuit before and after learning to deduce the possible ways by which such changes might have occurred. Such an approach makes it difficult to tightly constrain explanations for observed changes or conversely to resolve apparently paradoxical outcomes from insights that could be gleaned from direct observation of dynamics.

A second methodological challenge has concerned how to selectively monitor and manipulate one site of plasticity without affecting other processes in vivo. It is difficult to restrict the effects of pharmacological manipulation to plasticity at specific sites, without affecting other cellular processes or sites of plasticity. Genetic manipulations offer greater control, but even when manipulations have been made specifically to LTD (Schonewille et al. 2011), removal of one type of plasticity could push another source of plasticity into a compensatory role, obfuscating the one that the abolished plasticity would normally play. Therefore, the power to monitor site-specific plasticity during regular VOR adaptation, not just observe the effects when it is removed, could provide critical traction in untangling the various contributions of different sites of plasticity.

I have focused on sites of plasticity in cerebellar learning as one example of the ambiguity that has arisen from efforts to validate the original theories of the Marr/Albus/Ito model. But similar ambiguity pervades understanding of the rest of the cerebellar circuit and resolving it faces similar methodological challenges. Another example, which will be discussed in depth in chapter 2, is the massive recoding in granule cells of mossy fiber information en route to PCs. Briefly, Marr and Albus proposed concrete theories regarding the purpose of this expansion (Albus 1971; Marr 1969), but electrophysiological recordings from these cells have been equivocal in validating or refuting their hypotheses. A complete answer to such a population based question may require the ability to sample activity in a larger fraction of the enormous granule cell population than can practically be done by traditional electrophysiological means.

In efforts to resolve questions like the purpose of multiple sites of plasticity or granule cell expansion, researchers have pushed the numbers of cells that can be recorded from

electrophysiologically to impressive limits and have paired them with clever manipulations of behavioral paradigms in combination with lesions and pharmacological manipulations as well as in vitro examination of detailed physiological mechanisms. They have succeeded in both supporting aspects of the theorized Marr-Albus-Ito circuit, while convincingly demonstrating that it is not the whole story in cerebellar learning. But the challenge remains to fully elucidate the individual contributions of circuit elements and sites of plasticity and to determine how they do interact to achieve the cerebellum's algorithm.

Methodological advantages of alternative cerebellar model systems

A historically profitable biologist trick of the trade has been to make use of model systems with unique features that are especially conducive to particular methods of inquiry. A push to study VOR in mice for example was motivated in part by the promise of genetic tools not available in monkeys (Koekkoek et al. 1997). Today genetic control combined with modern optical tools for monitoring and manipulating cellular activity offer a powerful means of contending with some of the issues noted above, including precisely and reversibly activating and inactivating identified circuit components, selectively interfering with plasticity within these components, monitoring their activity at the population level over long periods of time, and even monitoring subcellular plasticity at the molecular level in real time. While some of these tools can be employed in mice, some less commonly studied cerebellar model systems have features that are particularly suited to these methods. In the remainder of this thesis I will discuss two such model systems with uniquely accessible anatomies as well as a number of other methodological advantages for studying the cerebellum.

In particular, in Chapter 2, I will introduce the mormyrid *Gnathonemus petersii*, a teleost fish. The layered structure of the cerebellum is inverted in mormyrids, placing granule cell bodies, which are usually located below PCs, on the surface of the brain where they may be amenable to population imaging. In addition, this granule cell population projects not only to PCs of the cerebellum, but also to Purkinje-like cells of a nearby cerebellum-like structure, the electrosensory lobe. A Marr/Albus/Ito style coding strategy that uses contextual sensory motor information to learn to cancel out self-generated electrosensory afference has been largely substantiated in this structure. This unusually well characterized circuit could provide a convenient framework for addressing the use of the processing of mossy fiber inputs carried out by granule cells for Marr/Albus/Ito systems (Bell et al. 2008; Kennedy et al. 2014; Requarth and Sawtell 2011; Sawtell 2010). I will discuss the mixed results of my efforts to establish a method for population imaging of these granule cells.

In Chapter 3, I will introduce a second teleost, the larval zebrafish, on which I've focused the majority of my research efforts. The zebrafish is broadly recognized as a methodologically tractable system, well-suited to approaching questions that require population imaging and optogenetic control. In Chapter 4, I will detail my work probing the sensory and motor properties of PC activity during optomotor behavior in this fish. And, in Chapter 5, I will discuss the immediate challenges of continuing this work in zebrafish cerebellum as well as the exciting future prospects in this system if such challenges can be surmounted.

CHAPTER 2
CALCIUM IMAGING IN CEREBELLAR GRANULE CELLS IN WEAKLY ELECTRIC MORMYRID FISH

CHAPTER 2: CALCIUM IMAGING IN CEREBELLAR GRANULE CELLS IN WEAKLY ELECTRIC MORMYRID FISH

Introduction

Marr and Albus proposed that mossy fiber inputs to the cerebellum provide contextual sensory and motor state information that can be used by Purkinje cells (PCs) to sculpt appropriate motor responses (Albus 1971; Marr 1969). However en route to the PCs, information carried in on mossy fibers undergoes expansive recoding by cerebellar granule cells (GCs), an enormous population of cells that outnumbers their mossy fiber inputs 200 to 1. The expanded GC representation then reconverges on the much smaller population of PCs. A number of intriguing theories have been postulated about the purpose of the GC's massive expansion (Albus 1971; Fujita 1982; Marr 1969; Medina and Mauk 2000). But, the striking abundance of GCs, which represent more than half of all the cells in the entire brain, and their associated small size and dense-packing makes them difficult to study by *in vivo* electrophysiological methods. Furthermore, while a few labs have published data using such challenging and time-consuming approaches (Chadderton et al. 2004; Jorntell and Ekerot 2006; Kennedy et al. 2014; Sawtell 2010), sampling one cell at a time is not ideal for evaluating hypotheses of GC function which tend to be population-focused.

Two-photon calcium imaging would be an ideal alternative method for studying GCs and indeed there have been two reports of calcium transients recorded in vivo from these cells in mammals: one in the granule cell axons (parallel fibers) of anesthetized mice (Wilms and Hausser 2015), which in mammals course through the uppermost layer of the cerebellum, and one in the putative GC bodies of awake, locomoting mice (Ozden et al. 2012). However, in mammalian model systems, GCs lie deep in the cerebellum below highly light scattering tissue,

which may account for the relative paucity of calcium imaging studies of GC bodies in mammals *in vivo*. I therefore sought, with mixed results, to establish methods of GC population imaging in the mormyrid electric fish, a model system in which GCs are located right at the surface of the brain, making high resolution calcium imaging potentially more straight-forward.

Of note, since the time that I began this work, there has also been an initial report of GC imaging in zebrafish during optomotor behavior (Sylvester et al. 2011). As the entire brain is quite accessible in zebrafish, this represents an exciting development in the direction of establishing GC imaging with optimal optical accessibility. This is especially true given the genetic tractability of this organism, which opens up additional tools for studying GC function. Indeed, my work in subsequent chapters is directed towards building foundational electrophysiological knowledge of cerebellar activity in zebrafish to support the further use of complementary optical methods. However, GC imaging in mormyrid has unique advantages as well. Most notably, the characterization of cerebellum and cerebellum-like circuits in which mormyrid GCs participate is quite advanced, especially compared to our current knowledge in zebrafish. This could provide a helpful framework for interpreting imaged GC activity, as will be described in more detail below.

Theories of GC expansion

The early models of Marr and Albus suggested that GCs might transform incoming contextual information into a higher dimensional space that could be advantageous for subsequent processing by PCs (Albus 1971; Marr 1969). Each GC has on average 3-4 short dendrites (Eccles et al. 1967; Kennedy et al. 2014)—with claw-like endings—and most likely receives only a few mossy fiber excitatory inputs. Based on this morphology, GCs were surmised

to combine small numbers of mossy fiber inputs carrying different contextual information. This would allow PCs to learn responses to particular multimodal contexts that could be different than a linear sum of responses to the separate comprising inputs. This would allow the significance of the activity of a given mossy fiber input to vary depending on the greater multimodal sensory context.

In the years since GC combinatorial encoding was first proposed, a different type of higher dimensionality stimulus recoding has been hypothesized based on the notion that a response to a particular stimulus may be desired at some delay relative to that stimulus. For example, rabbits exposed to a training paradigm in which a tone is followed by a puff of air (a stimulus that causes them to blink in the manner of an unconditioned stimulus) can learn to blink at variable delays after the tone in a good prediction of when the air puff will occur. Research has shown that this learning depends on the cerebellum (McCormick and Thompson 1984) and it has been hypothesized that in order for PCs to respond at a delay, GCs must copy the incoming stimulus into many different time frames, providing the PC with a temporal basis from which to sculpt a response (Medina and Mauk 2000). In this case, rather than aiding PCs to respond differently to different stimulus patterns, this type of recoding would allow different responses to the same original stimulus by recoding it into a series of different timeframes.

The mormyrid as a model system for studying GCs

The mormyrid has a number of biological features that make it particularly amenable to probing theories of GC expansion. The connectivity of its cerebellar components appears quite similar to that of mammals, other than the observation that its Golgi cells may not send dendrites into the parallel fibers and an apparent lack of basket cells. However the physical layout of these

components differs from that of mammals. The output cells of the cerebellum are located in the same layer as PCs, rather than in the deep cerebellar nuclei. And, most notably for the purposes of this chapter, the GC layer is located right on the surface of the brain, rather than below the PC bodies as in mammals. This superficial location could make population imaging of GCs more straight-forward than in mice, where cell bodies lie near to the limits of optical accessibility, or in even larger animals where they cannot be imaged at all.

In addition to its purely anatomical appeal, the mormyrid has a unique, cerebellum-like sensory structure, the electrosensory lobe (ELL). An evidently Marr-Albus based information processing task carried out in ELL is much better understood than in cerebellum. Furthermore, ELL receives projections from the same population of GCs as the cerebellum proper, thereby providing a well-studied framework for exploring the purpose of GC processing. These GCs encode diverse sensory and motor corollary discharge information that is used by Purkinje-like cells to predict and cancel out electrosensory effects driven by the fish's own behavior (Bell et al. 1997; Kennedy et al. 2014; Sawtell 2010).

In particular, it has been shown that Purkinje-like cells in ELL, called medium ganglion (MG) cells, can learn to predict electrosensory stimuli if delivered yoked to a fish's own electric organ corollary discharge (EOCD), which normally drives generation of an electric field used for active electrolocation and communication (Bell 1981; Bell et al. 1993; Sawtell and Williams 2008). The experimentally controlled paired electrosensory stimulation drives responses in these MG cells initially, which fade over time. Removal of the electrosensory stimulation at this point reveals a learned response to the EOCD that is a "negative image" of the original electrosensory response. There is good evidence that this process occurs in a Marr/Albus manner in which LTD driven by a teaching signal sculpts the negative image from a basis of granule cell inputs about

the current sensorimotor state (Requarth and Sawtell 2011). The major difference between this structure and the fish's cerebellum proper is that the putative teaching signal in this process takes the form of an electrosensory input from the periphery rather than a climbing fiber from the inferior olive. Understanding the nature of the types of negative images that can be formed in ELL can provide a helpful context for understanding the types of processing that is likely to occur in granule cells (Kennedy et al. 2014; Requarth et al. 2014; Sawtell 2010).

Combinatorial expansion in mormyrids

Electrophysiological recordings from GCs have provided mixed evidence regarding the existence of combinatorial encoding. In decerebrate cat, in-vivo whole cell recordings have demonstrated that mossy fibers of a given somatosensory or joint-related type innervate separate GCs (Bengtsson and Jorntell 2009; Jorntell and Ekerot 2006). In contrast, a study in mormyrids provided the first clear demonstration of multimodal integration in GCs, showing that many GCs receive both proprioceptive and EOCD inputs (Sawtell 2010). However the full extent of multimodal integration in mormyrids has not been probed, as this study focused exclusively on these two inputs. Furthermore, a number of second order questions about this multimodal integration remain, such as whether activation of multiple distinct mossy fiber inputs is required for GCs to fire action potentials.

Temporal expansion in mormyrids

The EOCD in mormyrids drives short pulses of electric organ discharge (EODs) used for active electrolocation. This discharge can interfere with the electroreceptors of a separate, passive electrosensory system, causing a ringing pattern that lasts much longer than the initial

EOD (Bell and Russell 1978). Negative images of the ringing can be learned with temporal specificity in order to mitigate these interference effects (Bell 1981). A recent study in mormyrid demonstrated convincingly that GC activity in response to the brief motor commands that drive the EOD provide MG cells with a temporally expanded basis from which to sculpt negative images of the ringing interference that can extend long after the initial corollary discharge signals have ended (Kennedy et al. 2014). In that study a heroic number of GCs was recorded from intracellularly. Population imaging could greatly facilitate follow-up experiments, addressing such issues as the distribution of electric organ corollary discharge response timings amongst the population of GCs as well as temporal expansion in other modalities and their interaction with any combinatorial coding discussed above.

Calcium imaging of GCs in mormyrids

Combinatorial encoding and temporal expansion hypotheses both propose that GCs transform raw contextual material into higher dimensional inputs better suited to driving the desired downstream activity. These hypotheses are by no means mutually exclusive and neither has been proven. Because these hypotheses are highly population based, monitoring activity in large number of GCs simultaneous could greatly facilitate progress in their evaluation.

Currently, spiking activity in populations of neurons is most effectively imaged by the proxy signal of changes in calcium concentration in active cells because direct indicators of voltage have, amongst other issues, relatively poor signal to noise ratios. Calcium changes can be read out using fluorescent indicators composed of calcium-selective chelators hybridized to fluorescent chromophores.

Though population-based imaging of mormyrid GCs would offer a powerful means of addressing the hypotheses explored above, a protocol for loading and imaging these cells has yet to be reported. I therefore sought to establish such a method, with mixed results. I succeeded in bulk loading GCs with calcium indicator and was able to see evoked transients. I also observed spontaneous fluorescence modulation in response to a sensory stimulus in two cells. However, I never saw wide spread spontaneous activity in loaded GCs. I will discuss the possible methodological and physiological explanations for this as well as possible avenues for evaluating and addressing them.

Results

Loading granule cells

A number of dye-loading methods have been successful in various brain regions and model systems. These include different types of dyes as well as different methods of administering the dyes (Adams 2010; Eilers and Konnerth 2009; Garaschuk and Konnerth 2010; Helmchen and Nevian 2007). Acetoxymethyl ester (AM) dyes are particularly suited to loading large numbers of cells locally, as they are engineered to allow simple passive transport of normally hydrophobic indicator across the cell membrane (Kimura 2007; Tsien 1981). Conjugating an acetoxymethyl ester group to the dye confers a sufficient degree of hydrophobicity on the molecule, which upon entry to the cell is reversed by intracellular esterases that cleave the AM group, trapping the dye inside. I found that it was possible to label large populations of GCs with AM-dye via multicell bolus loading, a well-established method for loading cells in vivo (Fig. 2.1) (Stosiek et al. 2003).

In particular for bolus loading I used a modified version of Ohki and Reid's bolus loading protocol (Ohki and Reid 2014). To make the dye, I added 6 μL of freshly prepared 20% pluronic-F127 solution in DMSO, to a 50 μg tube of Oregon Green 488 BAPTA-1 AM dye. After vortexing for 1 min, I added 72 μL of calcium-free ACSF and 2 μL of 2 mM sulfrhodamine 101. Just before use, the entire solution was sonicated for 5 minutes and filtered through 0.45 μm Millex filter (#SLHVR04NL). I found sonication and filtration was a critical step for preventing pipette clogs during injection and needed to be done in close proximity to when the dye was to be used. Dye was loaded into a glass patch pipette with tip broken to 5-10 μm.

The injection itself was made under visualized control on a 2-photon microscope. I followed Ohki and Reid's protocol for navigating to 100 μm below the surface of the GC body region (EGp). Low continuous pressure (usually 7 psi applied for 7 min) resulted in the best labeling, although short higher pressure pulses could work as well (5 psi applied in 25-50 pulses of 200-300 ms each). Importantly, sulfrhodamine-101 allowed visualization of the injection in real time as OGB does not become highly visible until it is taken up and hydrolyzed by cells.

Approximately 90 minutes after injection, this resulted in labeling of densely packed GCs (Fig 2.1). Cell bodies were individually distinguishable in the first 1-2 layers of cells. Below that, labeling appeared a more diffuse green, which might be due to the highly light scattering properties of GC tissue.

For surface loading of GCs, I used the same recipe as above and applied it directly to the surface of EGp. Again, 90 minutes after labeling densely packed GCs were apparent, though this resulted in a broader but shallower labeled region of GCs as compared to bolus loading.

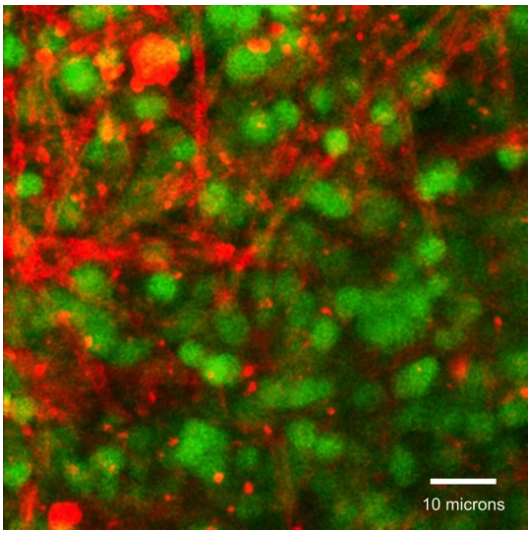

Figure 2.1 Granule cells loaded with calcium indicator.
A dense field of granule cells loaded with OGB-1 (green). Red counter-labeling represents sulfrhodamine-101, which allows visualization of the dye during loading and preferentially labels astrocytes.

Positive control for proper function of dye and imaging apparatus

As a positive control to verify that our imaging apparatus and dye were capable of capturing known action potential activity, we recorded intracellularly from a Purkinje cell in the caudal lobe of mormyrid cerebellum. These cells are known to fire calcium-mediated dendritic spikes at approximately 1 Hz in response to all-or-none activation of CF synapses. After filling a patched PC dendrite with OGB 1, we observed large fluorescence transients synchronous with the recorded calcium spike (Fig. 2.2).

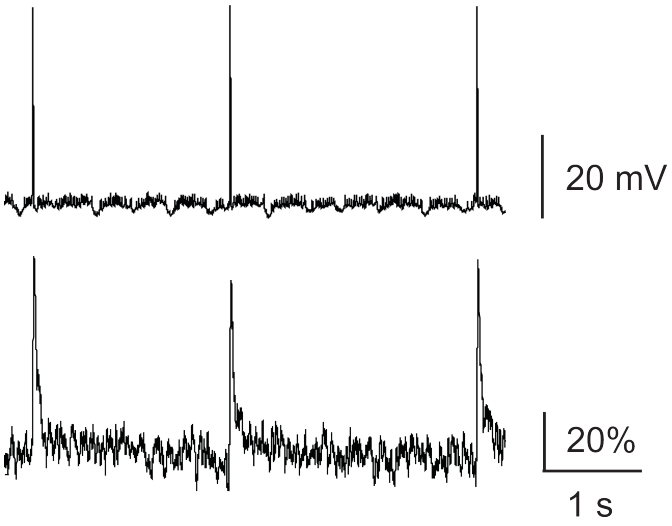

Figure 2.2 Purkinje cell transients.
Whole cell recording (top row) was conducted simultaneously with imaging of a filled Purkinje cell. Spontaneous climbing fiber activity corresponded to large transients in imaged fluorescence.

Evoked transients in GCs

Using stimulation protocols similar to those that evoke GC synaptic activity and spiking in vitro in cerebellar slices[26], I was able to reliably evoke large transients in single trials in nearby GCs (Fig. 2.3). A glass electrode with the tip broken to 4-5 μm was placed approximately 50 μm away from the imaging window. 10-50 μA current pulse trains drove strong and reliable transients in many GCs. The stimulating electrode was grounded against muscle tissue in the rostral trunk of the mormyrid. ROIs were imaged at a frame rate of 40 Hz using 920 nm illumination throughout. Transients in neighboring cells could be of quite different amplitudes, suggesting that firing was driven synaptically via activation of mossy fiber inputs rather than by direct electrical activation.

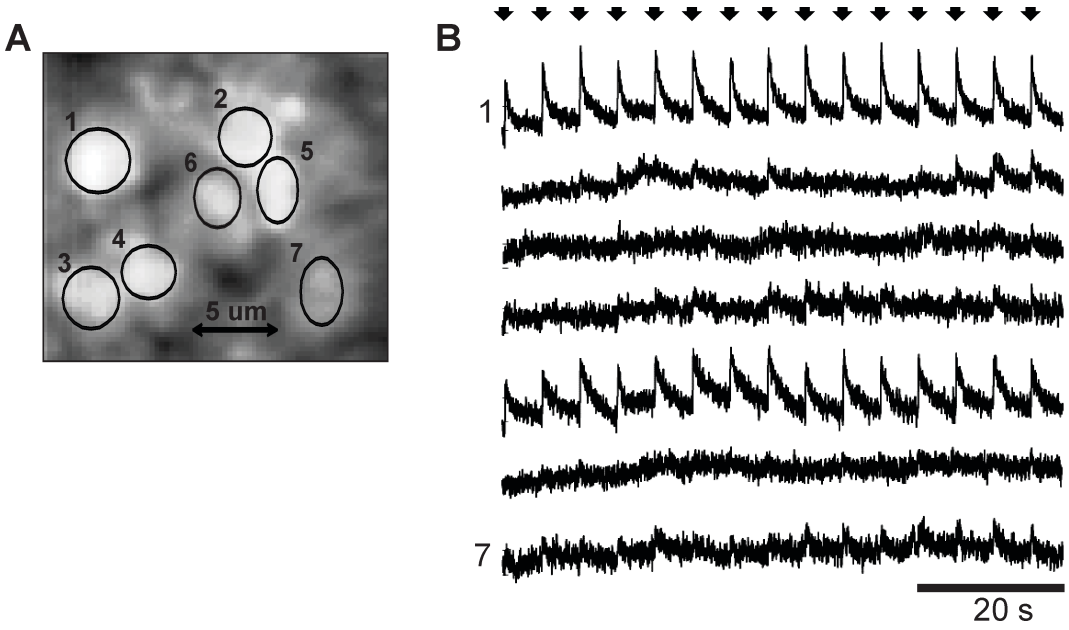

Figure 2.3 Evoked calcium transients in granule cells.
Electrical stimulation evoked calcium transients in granule cells up to 50 μm away. Amplitudes of transients varied suggesting activation occurred synaptically via activation of mossy fiber inputs rather than by direct electrical activation.

Spontaneous transients

In my initial search for more naturalistic activity in GCs, I initially monitored the fish's EOCD during simultaneous imaging. I did not observe fluorescence modulations locked to the EOCD, despite knowledge from published whole cell recordings that many GCs receive inputs from mossy fibers carrying this information and that in at least some GCs, though not the majority, this input is sufficient to drive action potentials (Kennedy et al. 2014; Sawtell 2010). Specifically in one quantitative study, approximately 15% of recorded GCs fired action potentials time-locked to the EOCD (Sawtell 2010).

I next tried to drive GC responses using a low frequency electrosensory stimulus of the variety known to engage passive (ampullary) electrosensory mossy fibers. In comparison to the brief, pulsatile input from EOCD mossy fibers, low frequency electrosensory stimuli might drive more sustained depolarization and spiking in GCs, which could allow summation of calcium

from multiple action potentials, resulting in a stronger fluorescence signal. Two cells in one fish exhibited fluorescence modulations of a frequency that graded with the frequency of a 5 μA sinusoidal stimulus, ranging from 0.125 to 0.5 Hz, grounded against an electrode in the mormyrid's stomach (Fig. 2.4). However, no further cells with such response properties were observed across hundreds of cells.

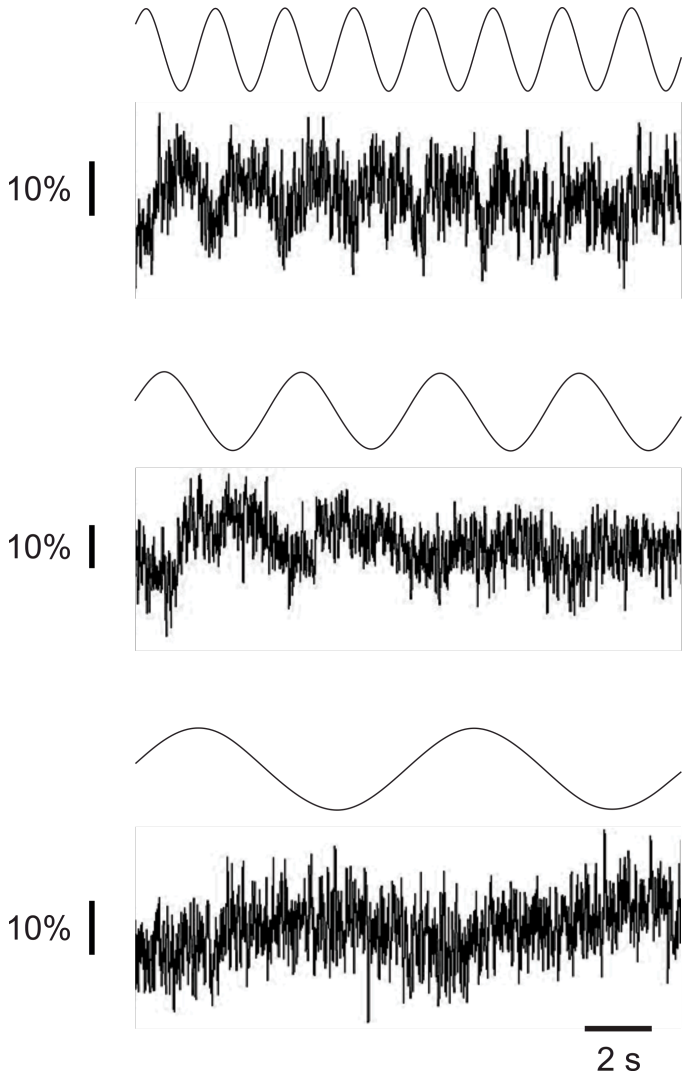

Figure 2.4 Sensory-evoked calcium transients in granule cells.
Example traces from a GC with apparent responses to electrosensory stimulation. Low frequency stimulation, known to activate ampullary electroreceptors, corresponded to fluorescence modulation of the same frequency. Amplitude of sinusoidal stimulation was 5uA amp applied to an electrode in the tank, grounded against an electrode in the fish's stomach.

Discussion

It is possible that the observed paucity of fluorescence modulation associated with the EOCD or ampullary electrosensory stimulation was a true reflection of extremely sparse activity in GCs. However, it is more likely that a significant fraction of the GCs observed were indeed firing action potentials, as suggested by whole cell recordings (Sawtell 2010), but that this activity could not be detected in the fluorescence signal.

Such a situation could arise for a number of reasons. Fluorescence changes are the result of circuit intrinsic dynamics that determine how many action potentials fire and cell intrinsic dynamics that determine how much free calcium becomes available when they do, combined with properties of the loaded indicator, which govern the size of the fluorescence signal that an action potential-mediated calcium fluctuation generates. Strategies to increase stimulus-driven activity or indicator responsiveness are both potentially viable approaches for achieving visualization of spontaneous activity in mormyrid GCs.

Driving increased activity in GCs

My ability to reliably evoke transients in GCs with a stimulating electrode suggests that some level of activity can be monitored using my stated loading parameters. However mossy fiber inputs must not be adequate to drive GCs this strongly under the experimental conditions described. Engaging more sensory modalities could increase levels of activity in GCs. For example, proprioceptive inputs activated by tail movement are known to drive mossy fibers (Bell et al. 2008; Requarth et al. 2014). Perhaps GCs that receive EOCD, proprioceptive and electrosensory inputs on different dendrites would fire more strongly to a combination of these

inputs. A caveat to this approach is that some theories hold that inhibitory interneurons work to maintain GC firing rates in a fixed regime, independent of variation in the numbers of active innervating mossy fibers (Albus 1971; Marr 1969). Furthermore, if engagement of multiple modalities is necessary to see activity, this will limit the types of experiments that can be conducted.

Optimizing loading concentration

Increasing the sensitivity of the loaded indicator so as not to require extremely high levels of activity for detection would be a preferable approach. The major determinants of a loaded indicator's signal size stem from inherent properties of the selected indicator and the concentration at which it is loaded. The effect of loading concentration is not monotonic. Initially, increasing indicator increases the resulting signal to noise ratio (SNR). Ultimately though, the change in fluorescence to change in calcium ratio follows a saturation function because as all calcium becomes bound, continuing to increase indicator cannot continue to increase signal. On the other hand, baseline noise, which is a direct property of dye concentration, can continue to increase. Once the growing noise outpaces the slowing fluorescence to calcium ratio, SNR peaks and begins to descend (Gobel and Helmchen 2007).

At optimal SNR, exogenous calcium buffering capacity from the loaded dye is approximately equal to that of endogenous calcium buffers. Without knowing the calcium buffering properties of mormyrid GCs though, this can't be calculated precisely. But, electrophysiological recordings from GCs combined with simultaneous imaging of cells loaded directly via patch pipette could be used to empirically evaluate signal responses to evoked simple spikes. This could be done in vivo, or in slice. The latter would allow for convenient visualized

targeting of whole cell recordings, but comes with the added challenge of generating slices, which can be tricky in mormyrids, and wouldn't guarantee the same loading concentrations as in vivo.

Unfortunately, the concentration of AM-dyes achieved via bolus loading is not easy to finely control. Cellular concentration of indicator is generally much higher than, and not directly determined by, extracellular loading concentration. A recent paper however suggests that loading concentration can be affected at least bluntly by varying the concentration of pluronic F-127 and DMSO (Hamad et al. 2015).

Optimizing indicator selection

Different calcium indicators have characteristics that render them better suited for visualizing signals with different requirements. Two important characteristics are an indicator's calcium binding affinity and its dynamic range. Binding affinity is a measure of the attraction between the indicator and the desired ligand, in this case calcium. A quantitative metric for this, the indicator's calcium dissociation constant (K_d), is defined as the concentration of intracellular calcium necessary for 50% of indicator molecules to bind a Ca^{2+} ion at equilibrium. Fluorescence changes in response to changing Ca^{2+} concentrations, [Ca^{2+}], are most robust when an indicator has a K_d within one order of magnitude of resting [free Ca^{2+}]. This balances a high enough affinity to capture small calcium influxes with sufficient signal to noise (STN) with a low enough affinity to avoid saturation of indicator in response to large calcium influx (Yasuda et al. 2004).

In the case of GCs, where spiking activity may be low enough that detection of single action potentials is critical, biasing this balance toward higher-affinity indicators might be

justified, despite the converse possibility of saturation in the face of trains of APs. The resting [Ca^{2+}] concentration of mormyrid GCs is not known, which makes precise calculation of the appropriate K_d impossible. However, at rest, most neurons have an intracellular calcium concentration in the range of 50–100 nM that can rise transiently during electrical activity to levels that are ten to 100 times higher (Berridge et al. 2000). In good accordance, [Ca^{2+}] measured in GCs in slices from 5 day old mice have been estimated in the 40-50 nM range (Harkins et al. 2000; Womack et al. 1998). Conservatively, an indicator with a K_d of 5-500 nM would be appropriate. The K_d of OGB-1 , a particularly high-affinity calcium indicator, is around 200 nM (the precise value depends on the particulars of parameters like pH, and temperature), which is well within these limits (Russell 2011; Yasuda et al. 2004). Indeed OGB-1 has proven a popular choice for measuring activity in vivo in many organisms and cell types (Dombeck et al. 2010; Ohki et al. 2005; Sullivan et al. 2005; Sumbre et al. 2008; Wachowiak et al. 2004).

Importantly, indicator binding affinity has other effects that should not be overlooked. An indicator, like OGB-1, with higher binding affinity, can have effects on a cell's calcium dynamics by operating as an exogenous calcium buffer. High binding affinity can also adversely affect the linearity of the relationship between [Ca^{2+}] and fluorescence, preventing quantitative read out of spiking activity from fluorescence. However these are perhaps secondary considerations to be dealt with after finding appropriate parameters for visualizing GC spiking at all.

A second important factor to consider is the dynamic range of an indicator, determined by its basal fluorescence and its change in fluorescence upon binding calcium. This affects its signal to noise. Fluo4, which has very low baseline fluorescence, has a better dynamic range in

this sense than OGB (Paredes et al. 2008) and may be a fruitful dye to use. I did in fact load GCs with Fluo4 via bolus and surface loading in a number of experiments, but didn't have noticeably improved results.

An alternative approach

An alternative strategy to pursue population imaging of GCs would be to make use of an even more methodologically tractable model system. While the mormyrid fish has an enticing population of GCs right on the surface of the brain, it is not currently a genetically tractable system, limiting some approaches to labeling. For example, the genetically encoded calcium indicator, GCaMP6, was recently shown to read out action potentials with higher fidelity than OGB-1 (Chen et al. 2013). GCaMP6 could possibly be introduced into mormyrid GCs, but would have to be done virally, an approach that has not been established in this organism. It is also not clear how deep we will ultimately be able to see into the highly light-scattering tissue where granule cells are located, as the loading achieved thus far suggests discriminating cell bodies more than a couple of cell layers beneath the surface may be challenging.

In another model organism, the larval zebrafish, which is small and clear, it has been shown possible to genetically label and image throughout the entire brain using pan-neuronal expression of GCaMP (Ahrens et al. 2012). Furthermore, as noted above, a first account of calcium imaging specifically in the granule cells of this organism has already been reported (Sylvester et al. 2011).

In the remainder of this thesis I explore the potential for zebrafish as a model system for studying the cerebellum. However, there are compelling reasons to continue pursuing imaging of GCs in mormyrids in parallel. The inputs to mormyrid GCs are already well characterized in this

system as are many of their downstream targets. In particular, the mormyrid's cerebellum-like-structure, the ELL, receives inputs from cerebellar GCs and a theory of Marr-Albus based processing in it is largely substantiated. Studying GCs in the context of this well characterized ELL circuit would provide a helpful framework for interpreting the purpose of the massive coding expansion in GCs.

CHAPTER 3
THE ZEBRAFISH AS A CEREBELLAR MODEL

CHAPTER 3: THE ZEBRAFISH AS A CEREBELLAR MODEL

The tools considered requisite for reverse-engineering a circuit include a well-described wiring diagram and the ability to monitor and separately manipulate the activity of identified components within it. Neuroscientists generally have had to make do with a severely restricted version of this toolkit, but advances in imaging and genetic techniques are changing that. The small size and genetic tractability of the zebrafish, *Danio rerio*, make it particularly compatible with these techniques and have increased its prominence as a model organism for studying neural circuits (Baier and Scott 2009; Del and Wyart 2012; Friedrich et al. 2010; Portugues et al. 2013; Sumbre and de Polavieja 2014). In this chapter I introduce the cerebellar circuit in zebrafish and discuss the methodological capabilities available for studying it as well as some of the challenges that will need to be overcome to use the zebrafish as a standard model organism for cerebellar studies.

Comparison of zebrafish versus mammalian cerebellum

The structure of the cerebellar circuit is largely conserved between mammals and teleosts, like the zebrafish, though there are a number of differences (Hibi and Shimizu 2012; Meek 1992). The teleost cerebellum is divided into three lobes: the valvula, the vestibulolateral lobe, and the corpus cerebella. The valvula is a unique teleost specialization and the vestibulolateral lobe is a region thought to be homologous to the vestibulocerebellum of mammals. The corpus represents the remaining, and in zebrafish the largest, part of the cerebellum. The corpus is a three-layered structure. GCs occupy the deepest layer, Purkinje cells the middle, and the molecular layer, where parallel fibers and stellate cells contact Purkinje cells,

occupies the top. As in mammals, mossy fiber and climbing fiber pathways converge on Purkinje cells, with the former contacting Purkinje cells indirectly via GC and stellate cell axons, after being processed in the GC layer by a network of GCs, Golgi cells, and unipolar brush cells (Hibi and Shimizu 2012).

Some differences from the mammalian circuit include a lack of inhibitory basket cells projecting to Purkinje cell somas, a lack of Golgi cell dendrites in the molecular layer, and a different anatomical location of the cerebellum's output cells. Similar to the deep cerebellar nucleus neurons in mammals, the glutamatergic efferent cells of the zebrafish cerebellum, called eurydendroid cells, are the targets of Purkinje cell inhibition; however, these cells are located in the vicinity of Purkinje cells rather than in a separate nucleus (Alonso et al. 1992; Meek et al. 1992). They also receive parallel fiber input rather than direct mossy fiber input as is the case for deep cerebellar nuclear cells in mammals.

The time course over which the cerebellum matures in larval zebrafish has not been entirely characterized, but it is known that cerebellar cell types have begun differentiating by 3 days post fertilization (dpf) and the layered structure of the cerebellum forms by 5 dpf (Bae et al. 2009). Also by 5 dpf, Purkinje cells fire two types of action potentials, simple spikes and climbing fiber responses, which can be modulated by sensory stimulation in the form of luminance changes, indicating the existence of functional sensory inputs to the cerebellum (Hsieh et al. 2014). Climbing fiber rates increase until 5 dpf and then decrease to a steady state over the next couple of days, suggesting that multiple climbing fiber innervations of Purkinje cells are pruned over this period (Hsieh et al. 2014). The sources of mossy fiber afferents to the zebrafish cerebellum are not all known, but inputs from the dorsal tegmental nuclei (Bae et al. 2009) and pretectal nuclei have been reported (Volkmann et al. 2010).

Comparison of mammalian vestibulo-ocular reflex response and zebrafish optomotor response

In chapter 1, I described a mammalian cerebellar-dependent behavioral paradigm, the vestibulo-ocular reflex (VOR), in which an image is kept steady on the retina, despite head movements, by compensatory eye movements driven by vestibular input from semicircular canals. Zebrafish also exhibit the VOR (Beck et al. 2004; Moorman et al. 1999), though the role of the cerebellum in it has not been well-studied. A different visual stabilization behavior in zebrafish for which the cerebellum has received some attention is called the optomotor response (OMR) (Ahrens et al. 2012).

The OMR shares some algorithmic requirements with the VOR. In this behavior, zebrafish swim in the direction of whole field motion, stabilizing themselves relative to their visual surroundings (Portugues and Engert 2009). Forward visual motion drives forward swimming, while other directions first cause turning to orient along the axis of motion, followed by forward swimming (Orger et al. 2008; Orger et al. 2000). Such a behavior could be useful for these aquatic creatures to maintain their position in the face of water currents. In both the VOR and the OMR, the brain is faced with the need to translate sensory information about a displacement (vestibular information about displacement of the eyes by head movement in the case of the VOR and visual information about displacement of the body by an external current in the case of the OMR) into compensatory stabilizing movements.

The appropriate motor response to sensory destabilization information can change over time as an organism's size and strength change. Just as the gain of the VOR can change in response to errors indicating a need for sensorimotor recalibration, it has recently been shown that the gain of the OMR can be adjusted by manipulating the relationship between sensory

information about drift velocity and the appropriate magnitude of compensatory response to achieve stabilization (Ahrens et al. 2012; Portugues and Engert 2011). This has been demonstrated in virtual OMR paradigms in which an immobilized larva can control a visual stimulus projected onto a screen beneath it. In one study, fish were held in place by partial embedding in agar, but their tails were left free to move (Portugues and Engert 2011). The amount of tail movement was then quantified and fed into a computer program to allow the fish to control movement of the visual stimulus. In a subsequent study, fish were paralyzed and recordings from their motor nerves were used to drive entirely fictive swimming (Ahrens et al. 2012).

When the relationship between motor output in these studies and its effect on the velocity of the visual stimulus was increased or decreased, fish initially overshot or undershot their previous level of stabilization, but subsequently adjusted their motor output in the direction that would reestablish the previous level of stability. It is important to note that "increased gain" in OMR terminology is used to connote the opposite of its meaning in VOR terminology. Specifically, an increased gain in the VOR means larger eye movements in response to the same vestibular input. In OMR terminology, an increased gain means an increased effect of motor output on drift velocity, which actually necessitates *smaller* swim movements in response to the same visual drift input.

One study has suggested that the cerebellum may play an analogous role in this behavior to its role in VOR gain-adaptation (Ahrens et al. 2012). Calcium imaging in this study revealed strong engagement of the cerebellum during the OMR, including some cells that were specifically active during gain changes. Consistent with this hypothesis, when the climbing fiber input to the cerebellum was inactivated by lesioning of the inferior olive, OMR gain changes

were prevented, while sparing the OMR itself (Ahrens et al. 2012). Lesions are not a highly specific means of inactivating a region as processes passing through the region will also be inactivated, so the effects of lesioning on OMR gain could have been due to damage of neurons other than the inferior olive. However, if OMR gain change is cerebellum-dependent, it could provide a useful framework for studying cerebellar learning in the zebrafish.

A hypothetical mechanism for OMR gain change

While similar to the VOR in some respects, the OMR also has some clear differences. To begin with, the likely purpose of the OMR requires considerably less precision than the VOR. In the VOR, preventing blurred vision requires very tight moment-to-moment coupling of compensatory eye movements to destabilizing head movements. The OMR is a much less temporally precise behavior. Fish swim in the direction of whole field motion on average, but not in a tightly locked manner. This makes some ethological sense as displacement due to current drift can be prevented with swimming that is only counteractive on average.

The looseness of coupling between sensory motion and motor response is further compounded in larval zebrafish by the structure of swimming at this immature stage. Larval zebrafish swim in discrete units, called bouts, each of which consists of a series of generally less than 10 tail beats carried out over brief periods, generally less than 200 ms, interspersed with pauses in movement of similar or slightly longer duration (Severi et al. 2014). The average speed of a sequence of bouts can be adjusted by changing tail beat frequency, bout duration, or bout frequency (Severi et al. 2014). This allows larval zebrafish to adjust average swim speed in response to OMR whole-field motion stimuli of varying drift velocities. However, even with average swimming perfectly tuned to cancel out drift over long time periods, at any given

moment during the OMR the fish either overshoots the instantaneous rate at which the visual stimulus moves (during bouts) or undershoots it (between bouts).

The equation relating instantaneous sensory input to motor output is thus quite different during performance of the OMR compared to the VOR. Theoretically, the desired eye velocity in the VOR is simply equal to the sensory information about head velocity scaled by some gain factor, g, and inverted such that:

$$V_{eye} = -g * V_{head}$$

During the OMR, however, calculating the appropriate swim velocity of a bout from visual drift velocity information requires integrating the total displacement that has been incurred since the previous bout terminated to produce a counteracting displacement over the duration of the current bout. Assuming for simplicity a bout with a square pulse of uniform velocity V_{bout} , then for the nth bout with duration d_n and start time t_n, the desired velocity would be:

$$V_{bout}(n) = -\frac{\int_{t_{n-1}}^{t_n} V_{drift}(t)\partial t}{d_n}$$

The gain of such a calculation could be scaled before integration, which could be interpreted as adjusting the transformation between perceived drift and actual drift.

$$V_{bout}(n) = -\frac{\int_{t_{n-1}}^{t_n} g \times V_{drift}(t)\partial t}{d_n}$$

Or it could be scaled at the end, which could be interpreted as adjusting the transformation between intended velocity and achieved velocity. (Though both formulations are mathematically equivalent, they could suggest different biological locations of implementation).

$$V_{bout}(n) = -g \times \frac{\int_{t_{n-1}}^{t_n} V_{drift}(t) \partial t}{d_n}$$

In the simplest Marr/Albus/Ito model, adjusting VOR gain was proposed to occur by simply adjusting the strength of vestibular drive onto Purkinje cells in the opposite direction from the desired gain. Since Purkinje cells inhibit the vestibular nuclei, which drive the VOR, modulating their output in one direction was theorized to drive the strength of the VOR in the other. Such a simple process requires that the input to Purkinje cells be directly proportional to the desired output. As can be seen in the equation above, the integration step and the division by the bout duration, d_n, which varies over bouts, breaks this requirement for V_{bout}'s relationship to V_{drift}.

However, one can imagine small modifications that would resolve this issue, if stabilization did not need to be precise on a bout to bout timescale. Indeed, as discussed above, the OMR is not executed with bout to bout precision. V_{drift} could be integrated upstream of parallel fibers onto Purkinje cells. A structure that carries out integration of eye velocity to generate an eye position signal, called the oculomotor integrator, demonstrates that this type of mathematical integration can be carried out by neural circuitry and mechanisms for how have been proposed (Aksay et al. 2001; Aksay et al. 2007; Arnold and Robinson 1997; Joshua and Lisberger 2015; Robinson 1989). The quantity specified by the integral above is not simply a

general position signal however, but the specific change in position, or displacement, incurred since the termination of the previous bout. Using the oculomotor integrator as an example, the zero point from which it measures displacement is determined by its time constant, which interestingly has been shown to depend on cerebellum (Chelazzi et al. 1990; Robinson 1974). Unless an integration time constant could vary substantially on a bout to bout timescale it would not allow precise integration over varying interbout intervals. However, because the OMR need not be precise on this timescale, the time constant could be set to some constant value that would overestimate the drift displacement in some bout instances and underestimate it in others, but the sum of these estimates would approach the correct accumulated displacement with time.

The issue of division by a bout duration that varies with time can be resolved similarly by accepting division by an average bout duration, which would result in overshooting of compensatory displacement by longer-than-average bouts and undershooting by shorter ones, but again should achieve accuracy on longer timescales. Bout duration then becomes a simple constant and the new equation for bout velocity becomes:

$$V_{bout}(n) = -g * \frac{P_n}{d}$$

This equation could now be carried out as simply as the basic VOR model described above. Change in position during approximately the interval between the current and previous bouts, P_n, calculated by a visual drift velocity integrator, would arrive via parallel fibers. The strength with which these parallel fibers drive Purkinje cells would be determined by the combined value of a fixed "bout duration" scalar, d, and an adjustable "gain" scalar, g. In a low gain paradigm (which for the OMR means a decreased effect of motor output on drift velocity),

decreasing the weight of integrator inputs to Purkinje cells would decrease Purkinje cell inhibition of downstream motor control apparatus, increasing motor output appropriately to regain stability under the new "decreased strength" conditions of the lower gain paradigm.

There are some additional issues that would need to be addressed for this simple model to successfully learn to adjust VOR gain. Purkinje cells should only affect motor apparatus during swim bouts for example. This could be addressed by a gating mechanism that only allows this drive to reach motor apparatus during bouts, presuming bouts are initiated outside of the cerebellum as is the case for head movements in the VOR. Similarly, the change in position to be stabilized should only be evaluated during interbout intervals, but should be acted upon during bouts. An expanded temporal representation of the interbout displacement, perhaps in the granule cells, could allow use of this signal at a variety of delays.

Another issue would be defining an appropriate teaching signal. In the case of the VOR there is controversy about what the teaching signal actually encodes, but at least theoretically a simple slip signal would be sufficient to indicate to a Purkinje cell that an error in stabilization had occurred. In the OMR, even perfect execution would still be associated with slip due to the discrete bout nature larval of swimming. An error then should be evaluated over the timescale of at least one bout. A possible error signal would be the integration of velocity over the preceding interbout interval through the termination of the current bout. If this were 0 it would indicate perfect stabilization, whereas a negative value would indicate too low of bout strength and a positive value too high of one. To be effective, a teaching signal occurring at the end of a bout would have to affect the weights of inputs that drove the bout itself over a window of approximately 150 – 200 ms prior to the teaching signal. Notably, in mammals, it has been demonstrated that parallel fiber to Purkinje cell LTD driven by activation of parallel fibers and

climbing fibers is greatest when climbing fiber activation followed parallel fiber activation by 50 – 200 ms (Wang et al. 2000). This timing also aligns wells with observations that timing of gain adaptation during the VOR can be predicted from the correlation between complex spike firing and the simple spike firing rate 100 ms before (Raymond and Lisberger 1998).

As addressed above, it has not been definitively proven that the cerebellum is involved in OMR gain change, but if it is, the algorithmic requirements to drive OMR gain change behavior, suchas those outlined above, could provide a rich territory for evaluating theories of cerebellar processing. Furthermore, whether this behavior or another turns out to be cerebellum-dependent, there is strong motivation to pursue understanding of cerebellar processing in zebrafish behaviors because of the methodological advantages in this system that could provide powerful approaches for experimental evaluation of these sorts of theories.

Methodological advantages of zebrafish

A small organism

The advantages proffered by the larval zebrafish stem from its minute size as compared to other vertebrate model organisms. The brain of a larval zebrafish has less than 100,000 neurons at 7 dpf (Hill et al. 2003) as compared to 70,000,000 in mouse (Herculano-Houzel et al. 2006). The motor control apparatus of the zebrafish is similarly relatively simple compared to higher vertebrates. The reticulospinal neurons, which channel information from the brain to the spinal cord, comprise only about 300 neurons, including many that can be individually identified across animals (Kimmel et al. 1982). Such minimal circuits may be easier to dissect than those of more complex model systems. For example, high speed swimming during the optomotor response was recently demonstrated to depend on a particular subset of about 20 reticulospinal

neurons, located in the nucleus of the medial longitudinal fasciculus. Furthermore, activity in two distinct sets of two nMLF neurons corresponded to specific speed-related parameters: tail beat frequency and bout duration (Fig. 2.1). In addition to such opportunities for simple direct observation of activity, small numbers of neurons present the potential for modeling of interconnected populations to understand network dynamics using biologically accurate numbers of neurons. This is a significant consideration as the importance of population level dynamics to circuit function often cannot be addressed at full scale in models.

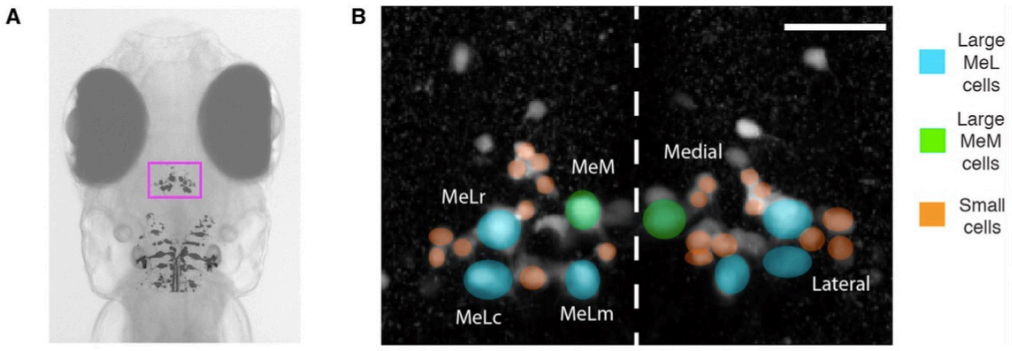

Figure 3.1 Individually identifiable neurons of the nMLF.
A: labeled reticulospinal neurons including those of the nMLF (magenta box) superimposed in their location over an image of a zebrafish larva. B: The neurons of the nMLF (approximately 20 in total), including 4 pairs of 4 individually identifiable cells (MeLr, MeLm, MeM, MeLc), some of which appear to have unique roles in controlling tail beat frequency or bout duration of high speed swim responses to optomotor response-driving visual stimulation. Reproduced from (**Severi et al. 2014**).

Imaging Activity

Beyond the intrinsic value of simpler circuits, small size is a physical trait of great value in the context of modern neuroscience's potent optical tools for monitoring and manipulating neural activity. Advances in noninvasive optics-based techniques have made it possible to image activity in large populations of genetically identifiable cells and to activate and inactivate them

using directed light. These techniques are powerful, but depend on illumination of the neurons in question.

Under two-photon microscopy, imaging at depths up to 600 μm is fairly standard and can be pushed up to 1 mm with appropriate methods in some brain regions (Helmchen and Denk 2005; Kleinfeld et al. 1998; Svoboda and Yasuda 2006). In other vertebrate model systems this range severely limits the regions of the brain that can be non-invasively imaged and optically stimulated. For example, recent studies involving chronic imaging of hippocampal cells in mice, which are more than 1 mm below the brain surface in mice, required aspiration of the cortex for visualization (Dombeck et al. 2010; Kaifosh et al. 2013). The accessibility of cerebellar circuitry in mouse varies, with neurons of the deep cerebellar nuclei at one extreme, on the order of 2 mm below the brain surface (Lu et al. 2013).

In contrast, the brain of the small, transparent zebrafish is only 0.5 mm thick at 5 dpf, rendering all brain regions accessible to functional imaging at single cell or even subcellular resolution. For example, a recently published method for monitoring internalization of GFP-labeled AMPA receptors in vivo (Zhang et al. 2015) could allow direct observation of plasticity in sub-cellular structures. This would offer a particularly elegant way to evaluate hypotheses of multiple sites of plasticity in cerebellum that, as described in Chapter 1, have been historically difficult to test.

While optical imaging allows monitoring of activity in whole populations of cells, the rate of scanning limits the temporal resolution with which these data can be acquired. Much progress has been made in recent years in increasing the speed at which populations of neurons can be imaged. In 2014, light-sheet imaging made it possible to image more than 80% of the larval zebrafish brain with single cell resolution at a rate of 0.8 Hz (Ahrens et al. 2013). In 2014

light-field imaging was used to image a similar proportion of the zebrafish brain at 20 Hz, though with slightly lower resolution and substantial computational time required for the reconstruction techniques necessary to process the data (Prevedel et al. 2014). Focusing imaging on just the cerebellum, rather than whole brain, should allow an order of magnitude increase in this rate and the rapid improvements in these methods in recent years bode well for further increase as well.

Manipulating Activity

The genetic tractability and optical accessibility of zebrafish also make it possible to use optogenetic actuators to manipulate the activity of cells. A variety of light-gated ion channels can be expressed in genetically targeted cells, resulting in luminance-controlled electrical excitability of neuronal subtypes. When cells expressing channelrhodopsin-2 (Nagel et al. 2003) are exposed to blue light, the light-gated ion channel permits influx of non-specific cations. The neuronal firing that results from this luminance activation can be controlled with temporal precision (Boyden et al. 2005; Li et al. 2005). Conversely, halorhodopsin, a light-driven chloride pump, causes hyperpolarization when activated by light in cells in which it is expressed (Gradinaru et al. 2008; Zhang et al. 2007).

Critically, the ability to transiently turn on and off populations of identified cells makes it possible to test theories of causality involving the necessity and sufficiency of circuits and circuit components for controlling behaviors. These methods have been successfully applied to show causal roles for neurons in zebrafish behavior. For example, a region at the boundary between the spinal cord and hindbrain has been demonstrated to drive swimming when activated via channelrhodposin-2 (Arrenberg et al. 2009). Interestingly, discovery of this region's involvement

began with an observation of swimming after a release from inhibition of the entire brain driven by pan-neuronal expression of halorhodopsin. The authors were thus initially agnostic about what regions of the brain might be causing this response. Using a fiber optic to focus light on 30 μm ROIs, they were able to zero in on the responsible region, the commissura infima Halleri, which drives swimming when released from inhibition or activated directly by channelrhodopsin-2. Activating spatially segregated subsets of cells in this manner is an important capability for circuit dissection and a number of approaches exist for realizing it. For example, digital mirror devices (Wyart et al. 2009) and holographic pattern illumination (Vaziri and Emiliani 2012) have been employed in addition to fiber optic control.

Genetic Targeting

Calcium indicators and optogenetic actuators can be introduced to cells via the injection loading techniques described in chapter 2 (Daie et al. 2015), but the genetic tractability of zebrafish makes genetic introduction of calcium sensors, and light-gated ion channels possible as well. This facilitates targeting of indicator to specific, identified cell types in addition to obviating the need to manually label individual organisms once a line is created with a desired expression pattern.

Currently there are a number of methods for introducing genetic material into identified cell types. Transgenesis is commonly achieved in zebrafish using the Tol2 transposon system (Daie et al. 2015; Kawakami et al. 2004). Briefly, mRNA for the transposase, Tol2, is injected into zebrafish embryos along with plasmid DNA containing the protein to be expressed, for example GFP. Tol2 inserts in the zebrafish genome in a stochastic manner. If it is inserted in

such a way that it can hijack or "trap" an enhancer from an existing gene, the result is a trap line with unique expression patterns depending on the enhancer that was trapped.

Tol2 transgenesis has been used powerfully in combination with the combinatorial Gal4-UAS system, which allows for mixing and matching of lines in which the location for transgene expression has been defined and lines that define which protein to express (Asakawa et al. 2008; Ogura et al. 2009; Scott et al. 2007). This is accomplished by separating Gal4, a yeast transcription activator protein from the enhancer that it binds to, Upstream Activation Sequence (UAS), which itself drives transcription of a desired gene. Trap lines with unique Gal4 expression lines can be made as detailed above. Separate "reporter lines" contain the UAS region adjacent to a desired gene expressed pan-neuronally in all cells. When a Gal4 trap line is crossed with a reporter line for a protein of interest, the resulting progeny express the desired protein only in the subset of cells specified by the Gal4 trap line.

Large numbers of Gal4 lines can be made using Tol2 as described above and then screened for desired expression patterns by crossing with a UAS:GFP reporter line to visualize the distribution of affected cells. The combinatorial nature of the Gal4-UAS systems can confer Gal4 expression patterns on any desired protein for which a UAS reporter line exists, thereby maximizing the utility garnered from each trap line. Extensive collections of Gal4 lines have been created in this manner (Asakawa et al. 2008; Ogura et al. 2009; Otsuna et al. 2015; Scott et al. 2007).

The stochastic nature of Tol2 transgenesis can make the development of a line with a given particular expression pattern arduous. More recent methodological developments, such as TALENs (Sander et al. 2011) and CRISPR (Hwang et al. 2013) allow targeted DNA sequence mutations. CRISPR has recently been used to begin converting established zebrafish lines

expressing GFP to express Gal4 instead, opening these expression lines to the combinatorial power of the Gal4-UAS system (Auer et al. 2014). CRISPR can also be used to introduce transgenes directly into the genetic material of naïve zebrafish (Hisano et al. 2015; Kimura et al. 2014).

Additional Genetic Tools

Calcium activity monitoring and optogentically-controlled transient gain of function and loss of function experiments are two of the most heralded methods that can make use of the zebrafish's small size and genetic tractability. However, additional tools for more specialized tasks exist as well.

Long term suppression of synaptic output can be achieved by expression of tetanus toxin light chain (TeTxLC), a permanent blocker of synaptic vesicle release. UAS-Gal4 driven expression of TeTxLC in different brain regions has successfully demonstrated effects on mechanosensory and olfactory behaviors (Asakawa et al. 2008; Koide et al. 2009).

Monitoring intracellular calcium levels without requiring external illumination can be realized by expressing the Calcium-sensitive photoprotein (GFP)-Aequorin (Baubet et al. 2000) in defined cell populations. Aequorin emits photons in the presence of increased calcium. The emitted light signal does not provide spatial information about the location of active cells, but can provide information about overall levels of activity in the labeled cells. This has been used to monitor activity levels in free swimming fish during behaviors like the startle response or spontaneous swimming (Naumann et al. 2010).

Tagging cells at distinct points in development is possible in chx10:Kaede transgenic animals (Ando et al. 2002). Kaede is a fluorescent protein that can be photo-converted from

green to red, thereby marking any cells that were present at the time of the conversion event red, while leaving cells that develop subsequently green. This approach has been used to study developing glutamatergic hind brain cells that likely drive motor output. Researchers found that older neurons were located more ventrally and were involved in different types of swimming than those that were more dorsal (Kinkhabwala et al. 2011; Koyama et al. 2011). The ability of kaede to label cells of different ages could be a particularly important tool given that studying zebrafish larva means studying an animal that is not fully developed. I discuss this challenge further below, but the existence of tools like kaede should be helpful in determining the timeframe of maturation of the cerebellum in zebrafish.

Challenges in zebrafish

Technological challenges

Despite the impressive array of tools available for use in research conducted on zebrafish, such work comes with a number of challenges. One class of hurdles to be surmounted derives from the new tools themselves. The power of optical imaging and stimulation is remarkable, but it is not immediately clear how the signals measured and driven relate to the spiking activity of targeted cells. Electrophysiological recording from cells in tandem with optical stimulation or calcium imaging should be helpful in calibrating this relationship.

A related problem is that we don't know what signals will be possible to image using calcium indicators. While calcium sensitive dyes allow monitoring of individual cells, their slow dynamics do not provide as sensitive a read out of neuronal activity as traditional electrophysiological voltage recordings. Furthermore, the amount of calcium fluctuation caused by spiking varies by cell type. In mammalian Purkinje cells, the calcium influx caused by simple

spikes has not appeared to be substantial enough to read out in fluorescence traces so only monitoring of climbing fiber responses via calcium imaging has been reported in these cells (Kitamura and Hausser 2011; Najafi et al. 2014; Schultz et al. 2009; Sullivan et al. 2005). However, the sensitivity of genetically encoded calcium indicators (GECIs) has improved continuously in recent years (Akerboom et al. 2012; Zhao et al. 2011). The latest GECIs, while still not capable of capturing simple spikes in Purkinje cells, are nearing reliable, single spike sensitivity in other neurons, though slow kinetics restrict them to detecting action potentials that are temporally sparse (Akerboom et al. 2012; Chen et al. 2013).

Genetically encoded voltage indicators (GEVIs) represent a more distant but potentially promising avenue for directly monitoring membrane potential voltage some day. This would side-step the issue of insufficient calcium influx for some action potential types and would also allow observation of subthreshold activity. Practical use of GEVIs still appears to be quite a ways off. Achieving sufficient signal to noise, for example, is a challenge in voltage sensors, which tend to be membrane bound and so cannot provide the larger cytosolic signal available with calcium sensors. But when and if this method does overcome the various hurdles currently before it, the zebrafish would be an ideal organism in which to put its power to use.

Behavioral Challenges

A second class of challenges in the larval zebrafish has to do with the developmental state of the animal. The larval zebrafish is generally studied within two weeks of fertilization. It is not clear how sophisticated of a behavioral repertoire such an immature animal can be expected to have. The cerebellum tends to wield a subtle role in behavior, contributing to the gain settings of a motion initiated elsewhere, for example, rather than exerting absolute control

of the movement. It's conceivable that at this early stage in development, zebrafish larvae may not yet engage in sufficiently complex behaviors to require the cerebellum. On the other hand, even at the larval stage, zebrafish face stringent demands on their motor skills as pressures to hunt and avoid predation begin within the first week of life.

Two larval zebrafish behaviors have so far been described with evidence of cerebellar contribution. OMR gain adaptation and the evidence for a cerebellar role in it were described above. Another potentially cerebellar behavior involves an associative conditioning task in which a conditioned visual stimulus drives an enhanced motor response after pairing with an unconditioned touch stimulus (Aizenberg and Schuman 2011). In parallel with this behavioral change the authors observed facilitation of luminance responses in cerebellar neurons after conditioning. Interestingly, laser ablation of the cerebellum arrested acquisition and extinction (dependent on when during training the lesion was made) but not retention of the learned response. The activity in this experiment was visualized using bolus loading of synthetic dye, so it is not known which cerebellar cell types were involved in the observed responses. Furthermore, as noted above, laser ablations are not cell type specific, allowing for the possibility that non-cerebellar processes in the vicinity could have been affected. Nevertheless, this paradigm is a promising one given its similarity to mammalian cerebellar-dependent classical eyelid conditioning paradigms (Thompson and Steinmetz 2009).

A sizable number of other behaviors have been described in zebrafish, perhaps reflecting the motor demands larvae face from the earliest stages. These behaviors, in which a role for cerebellum has not been probed, include a variety of locomotor maneuvers such as slow forward swims, routine turns, and escape responses (Budick and O'Malley 2000; Fetcho and O'Malley 1995; Gahtan et al. 2002). An assortment of visually driven behaviors have been described

including the dorsal-light response, in which the dorsal surface is tilted towards a light source (Nicolson et al. 1998), the optokinetic response, in which eyes track rotational field motion (Daie et al. 2015; Easter and Nicola 1996; Mueller and Neuhauss 2010; Rinner et al. 2005) and prey capture (Bianco et al. 2011; Borla et al. 2002; Gahtan et al. 2005; McElligott and O'Malley 2005) in which larvae enact a stereotyped sequence of maneuvers in response to a paramecium-like visual stimulus or in the presence of live paramecium.

Beyond visuomotor behaviors, larvae have been shown to exhibit other sensory behaviors including rheotaxis (Olszewski et al. 2012) olfactory (Mathuru et al. 2012) and taste (Boyer et al. 2013) induced behaviors. Finally they have demonstrated learning and memory capabilities (Roberts et al. 2013; Valente et al. 2012), and also exhibit circadian rhythms and sleep (Chiu and Prober 2013; Elbaz et al. 2013; Naumann et al. 2010), Thus a wide assortment of behaviors are enacted by zebrafish even at the larval stage, though it remains to be seen if any are definitively cerebellar dependent.

Developmental Challenges

The immaturity of the larval zebrafish also raises the issue of whether circuit function can be properly studied during development, when rapid changes in immature circuits might not provide enough stability to study a circuit's steady state function effectively. As detailed above, basic circuitry of the cerebellum is wired up and active by 6 dpf. Nevertheless additional cells continue to be incorporated into the circuit well beyond this age. Indeed, at one extreme, GC production continues at quite substantial levels through adulthood (Kaslin et al. 2013). Kaede studies should be helpful in determining the time course of cerebellar development so that an

optimal age for studying this circuit can be selected that balances the retention of optical accessibility with a sufficiently mature cerebellum.

Foundational knowledge challenges

A final class of challenge has to do with the simple lack of foundational knowledge about the cerebellum in larval zebrafish as compared to established cerebellar model systems. Beyond anatomical information about the major circuit components, our knowledge is limited. In particular, there have been only two published papers with electrophysiological recordings in zebrafish cerebellum at the time of writing (Hsieh et al. 2014; Sengupta and Thirumalai 2015) and only a handful of imaging studies that have focused on it (Ahrens et al. 2012; Aizenberg and Schuman 2011; Matsui et al. 2014; Sylvester et al. 2011) so we know very little about the spiking properties of cerebellar components or how spiking is engaged by sensory and motor stimuli. We know similarly little about the nature of the information carried by the inputs into the cerebellum or by its outputs to downstream targets or about how activity in the inputs, outputs, or cerebellum proper relates to behavior.

My work aims to build some of this foundational knowledge to help provide a context from which the full power of tools available in zebrafish can be brought to bear on dissection of the cerebellar circuit in this animal. In the following chapter, I use electrophysiological recordings to evaluate the activity of larval zebrafish Purkinje cells in the context of the optomotor response.

CHAPTER 4
ELECTROPHYSIOLOGAL CHARACTERIZATION OF PURKINJE CELL ACTIVITY DURING OPTOMOTOR BEHAVIOR

CHAPTER 4: ELECTROPHYSIOLOGAL CHARACTERIZATION OF PURKINJE CELL ACTIVITY DURING OPTOMOTOR BEHAVIOR

Introduction

Despite decades of intensive investigation, links between the highly-ordered and relatively simple circuitry of the cerebellum and its function remain elusive. Though the majority of work on the cerebellum has focused on mammals, core features of cerebellar circuitry are conserved across vertebrate phylogeny (Finger 1983; Hibi and Shimizu 2012; Larsell 1967; Meek 1992; Nieuwenhuys 1967). Hence studies of simpler vertebrates may provide a useful perspective on cerebellar function. The larval zebrafish is particularly promising as a model organism for studying the cerebellum. First, the number of cerebellar neurons is far smaller than in other systems in which the cerebellum has traditionally been studied. There are roughly 300 Purkinje cells in the 7 days post fertilization (dpf) larval zebrafish cerebellum (Hamling et al. 2015) compared to 1 to 2 million in adult cat (Mwamengele et al. 1993; Palkovits et al. 1971) and roughly 100,000 in adult mice (Herrup and Trenkner 1987). These small numbers together with the optical transparency of the larval zebrafish offer the potential to monitor the activity of all of the neurons in the cerebellum (together with activity in other brain regions) simultaneously during behavior (Ahrens et al. 2012; Ahrens et al. 2013). This unique potential for large-scale activity monitoring along with rapidly emerging technologies for mapping circuits and manipulating genetically identified cell types make the larval zebrafish a uniquely attractive model organism for cerebellar studies (Okamoto 2014).

Core features of cerebellar circuitry are shared between mammals and larval zebrafish, including the presence in both of mossy fibers, granule cells, parallel fibers, Purkinje cells,

climbing fibers, molecular layer interneurons and Golgi cells (Aizenberg and Schuman 2011; Bae et al. 2009; Takeuchi et al. 2015). A difference between the cerebellum in mammals and teleost fish, including zebrafish, is the location of the glutamatergic neurons that receive input from Purkinje cells and project to brain regions outside the cerebellum. Whereas in mammals such neurons are located in separate deep cerebellar or vestibular nuclei, in fish the large majority are located adjacent to Purkinje cells (Bae et al. 2009; Finger 1978; Heap et al. 2013). This proximity could, in fact, be a major advantage for understanding how Purkinje cells shape cerebellar output—a question that has been extremely difficult to address in mammals.

Several lines of evidence suggest that the cerebellum is functional at larval stages. Developmental studies have shown that by 5 dpf Purkinje cell and granule cell layers have formed and that the two major input pathways to Purkinje cells—the mossy fiber-granule cell-parallel fiber pathway and the olivocerebellar climbing fiber pathway--are in place (Bae et al. 2009; Takeuchi et al. 2015). An electrophysiological study has shown that larval zebrafish Purkinje cells exhibit both simple spikes and climbing fiber responses (CFRs) with firing patterns that change little after 6 dpf (Hsieh et al. 2014). Optogenetic activation or silencing of larval Purkinje cells alters swimming movements during the OMR (Matsui et al. 2014). Finally, lesioning of the olivocerebellar pathway prevents motor adaptation in a closed-loop, fictive OMR paradigm (Ahrens et al. 2012; Sengupta and Thirumalai 2015).

Though calcium imaging studies have revealed that cerebellar neurons are active during the OMR (Ahrens et al. 2012; Matsui et al. 2014), the nature of the signals they convey remains unclear. For example, the limited temporal resolution of calcium imaging has not allowed for a detailed analysis of how cerebellar activity relates to the structure of swimming behavior in larval zebrafish, which consists of rapid tail beats organized into discrete bouts. Moreover,

calcium responses in Purkinje cells could be due to simple spikes, CFRs, or some combination of both.

To address these issues, we used whole-cell and cell-attached recordings to characterize activity in genetically identified Purkinje cells during the fictive OMR. Though subthreshold membrane potential and simple spikes were modulated by an OMR-inducing visual stimulus, such responses generally did not exhibit the direction or velocity sensitivity that would be appropriate to drive the OMR. On the other hand, most Purkinje cells exhibited prominent subthreshold membrane potential and simple spikes modulations during fictive swim bouts. Temporal characteristics of these responses suggest that they could play a role in encoding and/or controlling aspects of swimming behavior, such as swim speed. CFRs were most common during fictive swim bouts and could also be driven by visual stimuli but did not appear to encode a visual error signal during bouts that would be appropriate for adjusting the gain of the OMR. Finally, individual Purkinje cells exhibited diverse relationships between simple spikes and CFRs, highlighting the importance of distinguishing between these responses in calcium imaging experiments.

Methods

All experiments performed in this study were approved by the Columbia University Institutional Animal Care and Use Committee. Most experiments were conducted in transgenic aldoca:gap43-Venus fish to allow visualization of Purkinje cells (Takeuchi et al. 2015; Tanabe et al. 2010) or in Nacre or Casper strains to facilitate visualized recordings and imaging.

Experimental preparation

6-10 days post fertilization (dpf) larva were embedded in a small block of low gelling temperature agarose (Sigma-Aldrich #A0701), which was then glued to the glass-bottom of a slice recording chamber. Agar was removed from above the head and adjacent to the right side of the trunk, from muscle segments 7 to 24, to allow placement of the neural and motor recording electrodes. Fish were paralyzed using 1 mg/mL alpha-bungarotoxin (Tocris) applied for one minute locally to the exposed portion of the trunk, where a small nick in the skin around muscle segment 23 facilitated paralysis. The skin over the cerebellum was gently removed using a bent tungsten dissecting needle (Roboz Surgical Instrument #RS-6063). During the experiment, the recording chamber was continuously perfused with aerated Evans solution containing, in mM: NaCl (134);KCl (2.9); $CaCl_2$ (2.1); $MgCl_2$ (1.2); HEPES (10); (pH 7.8, 280-290 mOsm).

Visual Stimuli

Visual stimuli were presented on a screen beneath the fish (approximately 1 cm). In luminance experiments (Fig. 4.5 and 4.10), movies showed alternating 3-6 second presentations of all- black and all-white screens. In the OMR open-loop experiments (Figs. 4, 8, 9 and 10), movies showed a square wave grating with spatial period 20 mm that moved alternately in 10-25 second periods of "OMR-inducing drift" (tail-to-head motion) and "OMR-suppressing drift" (head-to-tail motion), with a 5 second period of no drift between each drift period. Each trial consisted of four rounds of this alternating drift with increasing speed 0.4 cm/s to 1.2 cm/s. There were 2-6 trials per cell.

Closed loop/Playback experiments

In the OMR closed loop/playback experiments (Figs. 4.3 and 4.8), we based our methods on a published fictive swimming paradigm (Ahrens et al. 2012). Fish were again presented with trials containing four rounds of OMR-inducing and OMR-suppressing drift, however this time the baseline drift was combined with a "virtual swimming" drift component, driven by the fish's recorded motor nerve output.

The magnitude of the underlying drift in these experiments was kept constant at 1 cm/s and alternated directions. The virtual swimming drift component was always in the head-to tail direction (i.e. the direction of visual drift that results from forward swimming), defined here as positive, and was added linearly to the underlying drift. The magnitude of the virtual swimming drift component was calculated based on the recorded motor nerve signal.

Fish swim in discrete units of swimming called bouts, which are apparent in the recorded motor signal as transient increases in variance. The motor signal was processed by first taking the standard deviation of the raw motor trace over a sliding window of 10 ms. During a bout (detected automatically when the processed motor signal crossed a baseline threshold, set manually during periods of no swimming) the swim-related component of the grating's velocity, v_S, was calculated as the average of the processed motor signal since the last update, m_{av}, minus the baseline threshold, multiplied by a constant of proportionality, k, such that $V_s = k \cdot (m_{av} - b)$. The constant of proportionality was set experimentally for each fish such that the fish was able to roughly stabilize the grating during OMR-inducing periods. After each bout, the swim related component decayed back to zero at a rate of -15 cm/sec^2.

The total grating velocity experienced by the fish was equal to this swim velocity plus the baseline drift (±1 cm/s or 0 cm/s). This total grating velocity was updated on average at greater than 200 Hz and was smoothed at each update so that (effective drift velocity) = $\alpha \cdot$ (calculated

velocity) + (1 − α) · (previous effective drift velocity), with α = 0.3. The change in grating position at each update was equal to the total grating velocity multiplied by the time since the last update.

Each of these closed loop trials generated a unique visual stimulus movie resulting from the combination of the preset underlying drift and the fish-controlled virtual swim component. After a closed loop trial, a playback period was initiated in which this same visual stimulus movie was played again, now entirely unyoked from the fish's motor output, in order to be able to dissect the visual and motor components of any activity modulations seen in closed loop.

Electrophysiology

Motor nerve recordings were made based on published methods (Ahrens et al. 2012; Masino and Fetcho 2005). Briefly, a glass microelectrode filled with Evan's solution and beveled to lay flat against the fish's side was placed, with light suction, on a myotomal cleft between muscle segments 11 and 16. Purkinje cells were targeted for cell-attached or whole-cell recordings using Dodt contrast microscopy. Recordings were made using glass microelectrodes (8-17 MΩ) filled with internal solution for whole-cell recordings (see below) or Evan's solution for cell-attached recordings. Pipettes were wrapped in parafilm to reduce capacitance. Internal solution contained, in mM: K-gluconate (122); KCl (7); HEPES (10); Na2GTP (0.4); MgATP (4); EGTA (0.5); and alexa-594 (0.05) (pH 7.2, 280-290 mOsm). The calculated liquid junction potential was 15.9 mV and was not corrected for in the figures. Motor nerve and brain recordings were digitized at 40 kHz and 20 kHz respectively (CED Micro1401-3 hardware and Spike2 software; Cambridge Electronics Design, Cambridge, UK).

In a subset of cells, which were labeled by alexa-594 in the recording pipette, morphology and location within the cerebellum were visualized on a 2-photon microscope (Chameleon Ultra II, Coherent laser at 850 and 920 nm wavelength and for data collection PrairieView software, Prairie Technologies). In these experiments, Purkinje cell identity was verified by visualization of Venus fluorescence around the soma of the recorded cell. In experiments in wild-type fish, Purkinje cell identity was established electrophysiologically by the presence of two distinct spike types (see Results).

Data analysis and statistics

Data were analyzed off-line using Spike2, Matlab (MathWorks, Natick, MA), and SPSS (IBM Corp., Armonk, NY). Only cells recorded in fish that swam in response to OMR-inducing stimuli were analyzed.

A cell was determined to have a simple spike (extracellular) or membrane potential (intracellular recordings) motor bout response if the extreme in a response window 0-150 ms after bout onset was more than three standard deviations larger than the extreme in the same direction in a baseline window 75 to 225 ms before the bout onset (Fig. 4.6-7). This criterion was used for assessing closed-loop and playback (motor and visual) responses as well (Fig. 4.3). Student's t- tests were used to evaluate statistical significance (alpha=0.01). Spike train data used in this analysis was first transformed into a smoothed waveform by convolving with a triangular kernel of width 50 ms.

When evaluating correlation with speed of OMR-inducing drift, measurements of simple spike rates and membrane potential were taken from a 0.1 s period before each swim bout executed during OMR-inducing drift (Fig. 4.4). This ensured that measurements were taken

during the same conditions across all drift speeds and were relatively uncontaminated by motor responses.

Assessments of simple spike rate or membrane potential during drift as compared to no drift were based on an ANOVA for each cell comparing the simple spike rate or membrane potential (average over a 0.1 s window) measured at 2 second intervals during drift periods (OMR-inducing or OMR-suppressing) to the same measurements during pauses in grating drift (Fig. 4.4). To contend with the possibility that significant differences during OMR-inducing drift as compared to pause periods could be due to differences in motor activity, a significant increase in activity during both OMR-inducing and OMR-suppressing drift was required for a cell to be considered to have a drift response.

Cells were evaluated for CFR motor bout responses by evaluating whether the extreme in the response window 0 - 150 ms after bout onset was more than 3 standard deviations larger than the extreme in the same direction in the baseline window 75 to 225 ms before the bout onset (Fig. 4.8). This criterion was used for assessing closed-loop and playback (motor and visual) responses as well (Fig. 4.8). Student's t- tests were used to evaluate statistical significance (alpha=0.01). Spike train data used in this analysis was first transformed into a smoothed waveform by convolving with a triangular kernel of width 150 ms.

The effect of OMR-inducing drift speed on identified CFR bout responses was evaluated by counting the number of bouts at each drift speed in which a CFR did or did not occur (Fig. 4.8). Binary logistic regression was then used to assess whether the likelihood of a CFR occurring during a bout was affected by drift speed.

A cell was determined to have a transient CFR drift response based on an ANOVA comparing the peak response in a 1 second window after drift (OMR-inducing or OMR-

suppressing) to the peak response in a 1 window second after pause onset. Cells with responses that were significantly different ($p < .01$) for one or both drift directions, as compared to the pause periods, were considered to have CFR responses (Fig. 4.9).

Results

We obtained visualized recordings (n= 28 whole-cell; n=10 cell-attached) from Purkinje cells in the corpus cerebelli of 6-10 dpf zebrafish. Consistent with previous studies of Purkinje cells in a wide variety of species, including larval zebrafish (Hsieh et al. 2014), Purkinje cell recordings exhibited two types of action potentials that differed in their waveforms, rate of occurrence, and dependence on the underlying membrane potential (Fig. 4.1*A*-*D*). The smaller, more frequent events likely correspond to simple spikes and the larger, infrequent events to olivary climbing fiber responses, referred to here as CFRs (for cell-attached recordings: 10.4 ± 7.7 Hz for simple spikes versus 0.55 ± 0.50 Hz for CFRs; n=10) (Fig. 4.1*C*). In whole-cell recordings we observed that simple spike firing could be abolished by injection of small hyperpolarizing currents, with no apparent effect on CFR rates (Fig. 4.1*A*), consistent with the separate origins of simple spikes and CFRs. Finally, the amplitude of CFRs was always larger than the amplitude of simple spikes. Though different from the complex spikes evoked by climbing fiber activation in mammalian Purkinje cells, most notably in their lack of multiple spikelets, the appearance of CFRs in our *in vivo* recordings is consistent with that reported previously for fish, including larval zebrafish (Hsieh et al. 2014; Sengupta and Thirumalai 2015) and mormyrid fish (Alvina and Sawtell 2014; de Ruiter et al. 2006; Han and Bell 2003; Zhang and Han 2007).

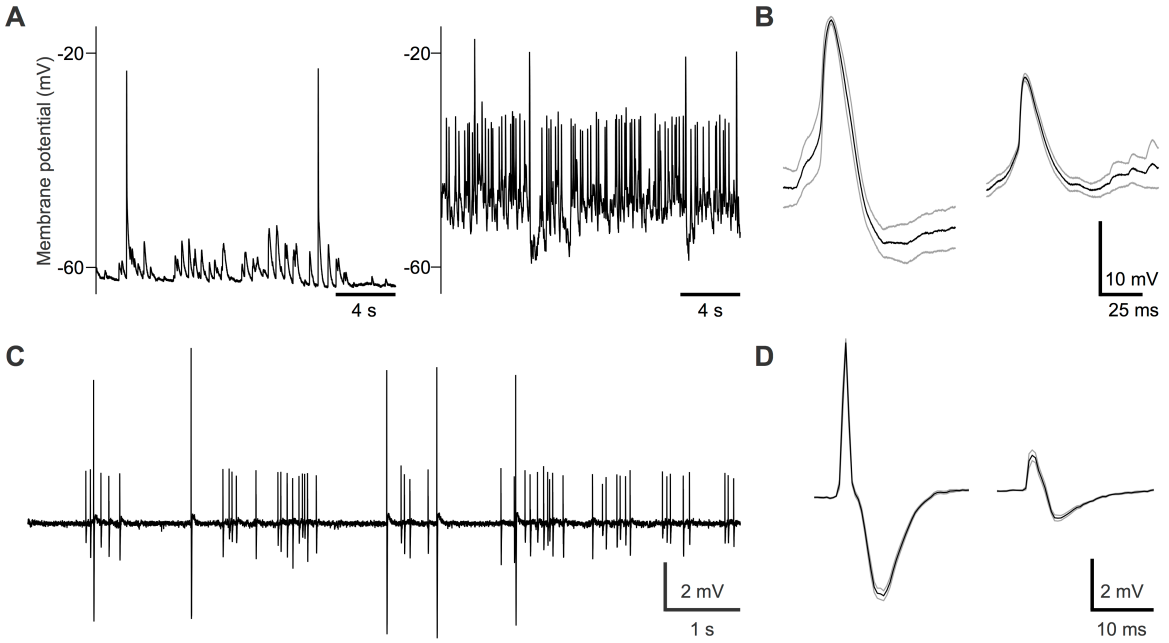

Figure 4.1 Electrophysiological properties of zebrafish Purkinje cells.
A: Intracellular trace from a Purkinje cell held at two different resting membrane potentials (left: -9 pA, right: -7 pA). Simple spike (SS) rate is strongly modulated by resting membrane potential while climbing fiber response (CFR) rate is relatively independent and consistently low. *B:* Average CFR (*left*) and SS (*right*) waveforms from recording in *A* (10 events each, grey traces indicate 1 SEM) *C:* Extracellular cell-attached recording from a Purkinje cell exhibiting spontaneous SSs and CFs. *D:* Average CF (*left*) and SS (*right*) waveforms from recording in *C* (10 events each, grey traces indicate 1 SEM).

In a subset of recordings (n=20) performed in transgenic fish (aldoca:gap43-Venus) in which the fluorescent protein Venus was selectively expressed in Purkinje cells (Tanabe et al. 2010), we were able to confirm Purkinje cell identity based on a halo of Venus fluorescence around the cell body (inset, Fig. 4.2*A*). An additional 7 cells exhibited only a single type of spike and were not Venus-positive (data not shown). During recording, we filled cells with a fluorescent dye to visualize their morphology and position within the cerebellum. Purkinje cells had extensive dendritic arbors, which appeared to be densely studded with spines (Fig. 4.2*B*). In some cases a thinner beaded process was also visible, likely the Purkinje cells axon. To map the relative location of Purkinje cells within the cerebellum across fish, we used the Venus expression in the Purkinje cell population to make a standard image of the shape and extent of

the Purkinje cell region and then used a point transformation to map individual recorded cells onto it (Fig. 4.2*C*). For cells whose locations could not be visualized using fluorescence (e.g. cells recorded extracellularly or not in transgenic aldoca:gap43-Venus fish), the location of the recording pipette tip was noted manually under Dodt visualization when possible (Fig. 4.2*C*, dotted outlines). A majority of Purkinje cells were recorded from the corpus cerebelli, though one or two cells may have been recorded at the edge of the valvula (Fig. 4.2*C*).

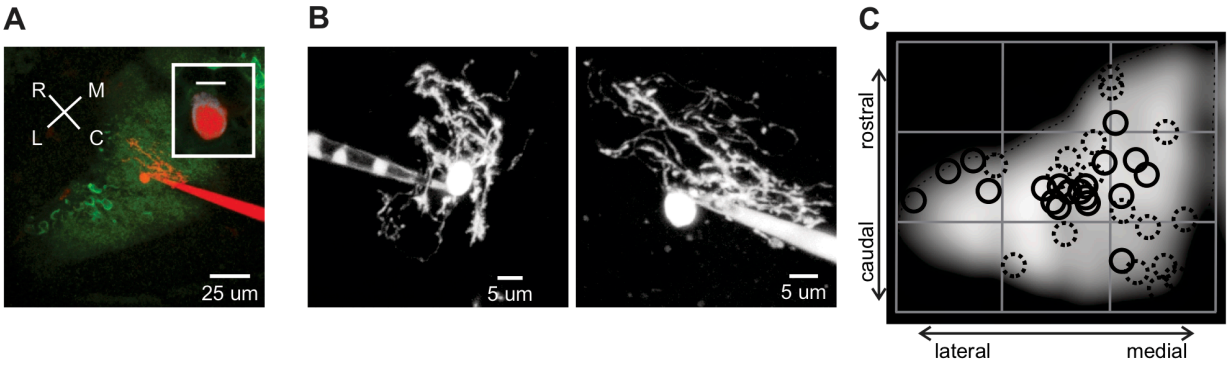

Figure 4.2 Morphological properties of zebrafish Purkinje cells
A: Purkinje cell visualized against Venus-labeled Purkinje cell population. Inset of z-stack through center of nucleus shows Venus localized to cell membrane (scale bar: 5 microns). *B:* Purkinje cells filled with Alexa-594 *C:* Recording locations of recorded cells mapped onto a standardized hemisphere of Venus-labeled cerebellum. Solid outlined cells were mapped using a point transformation of each filled cell relative to Venus background. Dotted-outlined cells could not be fluorescence-visualized for point transformation and were instead plotted based on manually noted coordinates of pipette tip under Dodt visualization.

Subthreshold and simple spike responses during closed loop optomotor behavior

A previous study using whole-brain calcium imaging revealed responses in the cerebellum during a closed loop OMR paradigm in which motor commands related to swimming (monitored in paralyzed fish by recordings from trunk motor nerves) are used to control the motion of a visual display (Ahrens et al. 2012). Under these conditions, fish can transiently stabilize the position of a tail-to-head drifting grating by emitting swim commands. However, this study could not distinguish which cell types were responsible for the calcium responses or whether calcium responses were due to simple spikes, CFRs, or both. Furthermore, the temporal

resolution of whole-brain calcium imaging in this study was not sufficient to relate activity to the detailed structure of larval zebrafish motor behavior, which is composed of rapid tail beats (~30 Hz) organized into discrete bouts of swimming lasting on the order of a few hundred milliseconds. We took a complementary approach by recording subthreshold, simple spike, and CFRs from individual Purkinje cells in the context of a similar closed loop fictive OMR paradigm.

Purkinje cell activity during the OMR could relate to the visual stimulus, the fish's swim commands, or to both. To differentiate between these possibilities we compared Purkinje cell activity in closed loop conditions in which fictive swim bouts controlled the position of a grating stimulus (*Closed loop*, Fig. 4.3*A*) to activity recorded when the same visual stimulus was played back independent of the fish's motor commands (*Playback*, Fig. 4.3*B*). We focus initially on subthreshold and simple spike responses. CFR responses will be discussed in a later section. Under closed loop conditions, most Purkinje cells exhibited strong subthreshold (6 of 7) and simple spike (3 of 4) modulations with onsets similar to individual fictive swim bouts (Fig. 4.3*A*). Responses to bouts under playback conditions were similar to those observed under closed loop conditions (Fig. 4.3*B*). On the other hand, not a single cell exhibited clear visual responses (i.e. membrane potential responses triggered on the onset of visual motion) during playback (*Visual-triggered*, Fig. 4.3*C*). These observations strongly suggest that the bout responses observed under closed loop conditions were largely motor (as opposed to visual) responses.

In some cases, small differences in bout responses were observed between closed-loop and playback (Fig. 4.3*C*, *Cell 1*). Such differences could reflect interactions between visual and motor signals, e.g. a component of Purkinje cell responses related to a mismatch between actual and expected visual input related to motor bouts. However given our limited read-out (i.e. a

motor nerve recording at a single site), we cannot rule out the possibility that differences in neural responses are due to differences in the fish's fictive swim behavior under closed loop versus playback conditions.

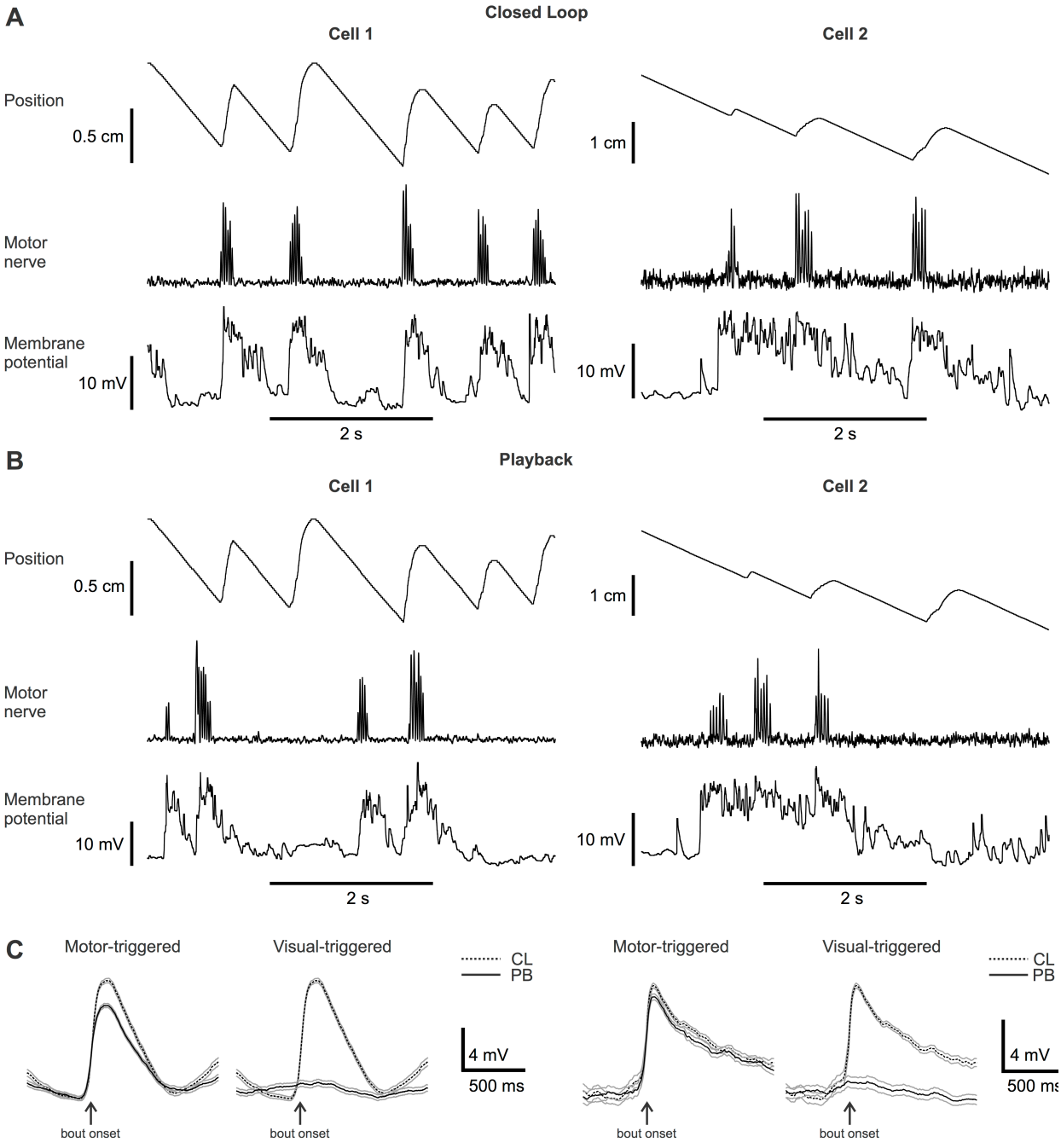

Figure 4.3 Visual and motor activity during closed loop OMR behavior.

A: Example cells with responses to motor bouts during Closed loop (CL) stimulus. In CL trials, fictive backwards displacement (simulated by a grating drifting in the tail to head direction) can be transiently stabilized by the fish's motor nerve output. Under these conditions membrane potential responses during bouts (row 3), could be related to either motor nerve activity (row 2) or its visual consequences (row 1). B: Responses during Playback (PB). In PB trials the visual stimulus that was generated by motor nerve activity during CL is played back independent of ongoing motor nerve activity. Therefore, motor bouts executed in PB (row 2) are not yoked to the played back visual consequences of bouts executed in CL (row 1), allowing us to separately probe the motor and visual-related components of neural activity (row 3). C: Average membrane potential (+/- 1 SEM) during bouts in CL, motor bouts in PB, and the visual consequences of bouts replayed during PB trials. Both cells have modulations during bouts in CL (dotted line) and in PB show similar modulation to bouts (solid line, left), but not to the bout-driven visual consequences replayed from CL (solid line, right). The motor bout driven responses were similar in shape between CL and PB, but could have somewhat different amplitudes.

Subthreshold and simple spike responses to sustained visual motion

OMR behavior allows larval zebrafish to maintain their position relative to a visual stimulus, however such stabilization is not instantaneous but rather is achieved on a timescale substantially longer than that of individual swim bouts. Hence the presence of sustained visual motion might be a relevant signal for controlling the OMR. To test whether such signals were present, we examined 29 Purkinje cells in the context of a simple open loop visual stimulus in which drift velocity was held constant at different values. Specifically, we presented fish with alternate periods of tail-to-head (OMR-inducing) and head-to-tail (OMR-suppressing) drift. Drift periods were interposed by periods in which the grating was stationary (Fig. 4.4*A*). There were 4 rounds of forward-backward drift pairs per trial, with drift speed increasing successively across rounds (0.4cm/s, 0.8 cm/s, 1.2 cm/, 1.4 cm/s), and 2-6 trials presented per cell. Consistent with previous observations, fictive swim bouts were most frequent at the onset of OMR-inducing drift and were quite rare during pauses in drift or OMR-suppressing drift (data not shown).

Surprisingly, we did not observe marked sensitivity to the velocity or direction of grating drift in Purkinje cells. Only 4/29 cells exhibited even a modest grading of membrane potential or simple spike responses to drift velocity (3/24 intracellular, 1/ 5 extracellular, $R>.3$, $p<.05$) (Fig. 4.4*A*,*B*). In some cells however, we noted a dramatic shift in the membrane potential at

transitions between periods of drifting versus stationary gratings (Fig. 4.4*A,C*). When we evaluated this effect quantitatively, we found 14/29 Purkinje cells exhibited sustained membrane potential depolarization and simple spike rate increases in response to drift in both directions as compared to a stable grating velocity (13/24 intracellular, 1/5 extracellular, ANOVA $p<0.05$) (Fig. 4.4*C*). These responses were present in the OMR-inhibiting as well as inducing direction, indicating they were not a simple result of the motor responses in the OMR-inducing direction. In some of the cells with particularly strong drift responses we noted oscillations in the membrane potential that increased in frequency with increasing speed of visual motion (Fig. 4.4*D*). Given that the frequency of the oscillations were similar to the spatial frequency of the grating stimulus, such responses could simply be due to changes in luminance. Indeed responses to luminance change have been reported previously in larval zebrafish Purkinje cells (Hsieh et al. 2014).

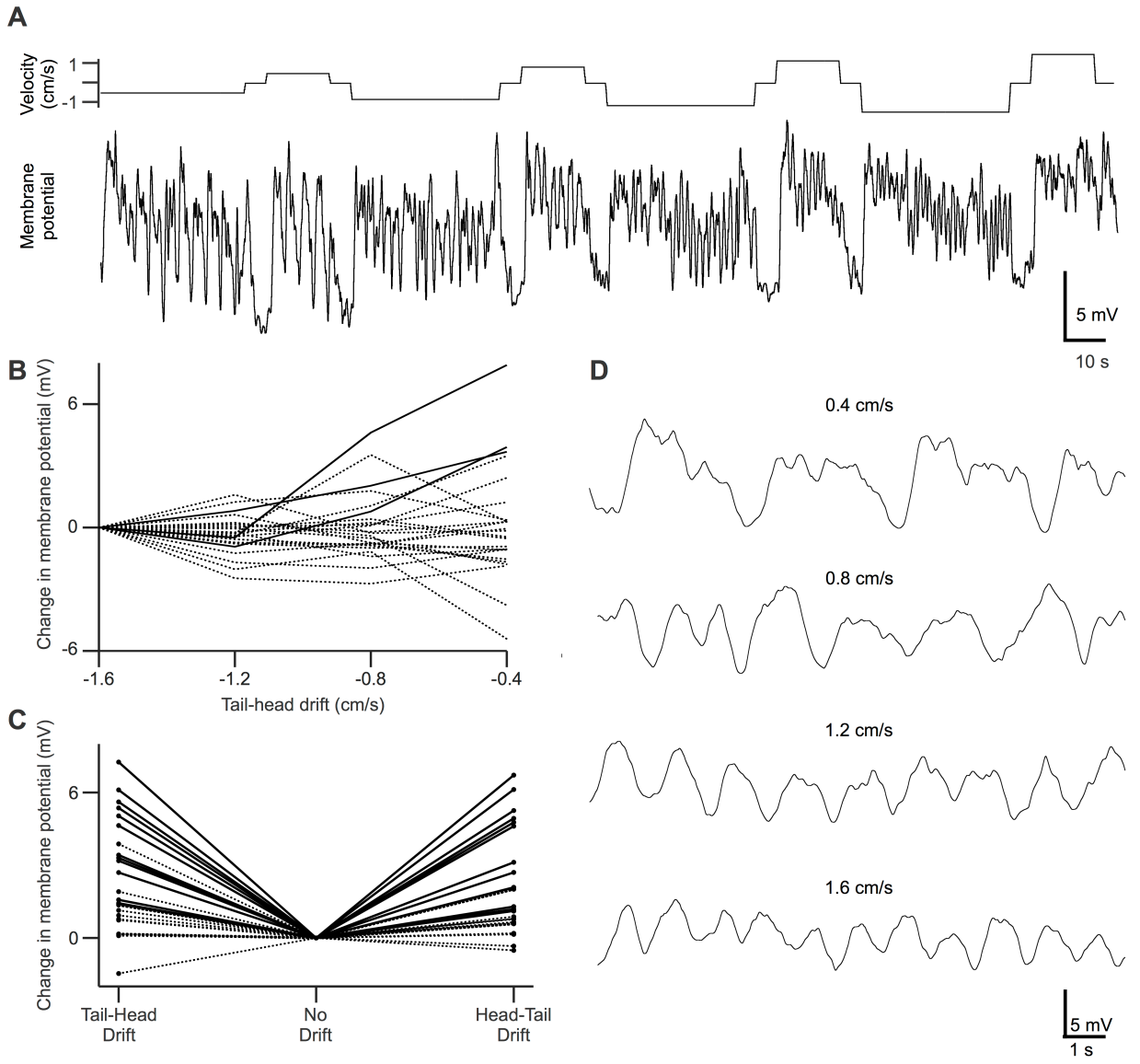

Figure 4.4 Visual modulation of membrane potential.
A: Example of long timescale membrane potential data at various speeds during three drift states: OMR-inducing, no drift, and OMR-suppressing. Drifting in either direction and at all speeds strongly drives activity as compared to no drift periods. *B:* Summary of membrane potential responses to graded OMR-inducing drift velocities. Only four cells (*solid lines*) exhibited even a moderate grading in response (3/24 intracellular, 1/ 5 extracellular, $R>.3$, $p<.05$) *C:* Summary of membrane potential responses to OMR-inducing and suppressing drift (irrespective of speed) as compared to no drift. Many cells were significantly modulated by drift in both directions (*solid lines*) (13/24 intracellular, 1/5 extracellular, ANOVA $p<0.05$), suggesting the modulation is not an artifact caused by responses to the motor activity during OMR-inducing drift. *D:* Oscillations in membrane potential (smoothed) of cell from A at different OMR-inducing drift speeds. Frequency of oscillation scales with magnitude of drift velocity. This would be consistent with activity driven by the luminance changes caused by the alternating black and white bars of the OMR-inducing stimulus.

Luminance responses were tested directly using a full-field visual stimulus that alternated from black to white with a period of 3-5 seconds (Fig. 4.5A-C). 23/31 Purkinje cells showed clear modulations of simple spikes or membrane potential related to luminance changes. Responses varied across cells both in terms of their polarity (whether cells were excited by light onset and/or offset) and their temporal profiles (whether they were relatively sustained or quite transient). In addition to explaining the observed frequency-dependent membrane potential oscillations, these luminance responses likely account for some portion of the drift responses we observed due to increases in activity as the grating's darker or lighter regions moved past. In theory, a large enough luminance response with an appropriate decay constant could result in speed modulation as well as a general sensitivity to motion, but any such effect did not appear to be strong in our data. Taken together these results suggest that although individual Purkinje cells receive strong visual input, they do not encode drift direction or velocity signals that would be appropriate for controlling the OMR.

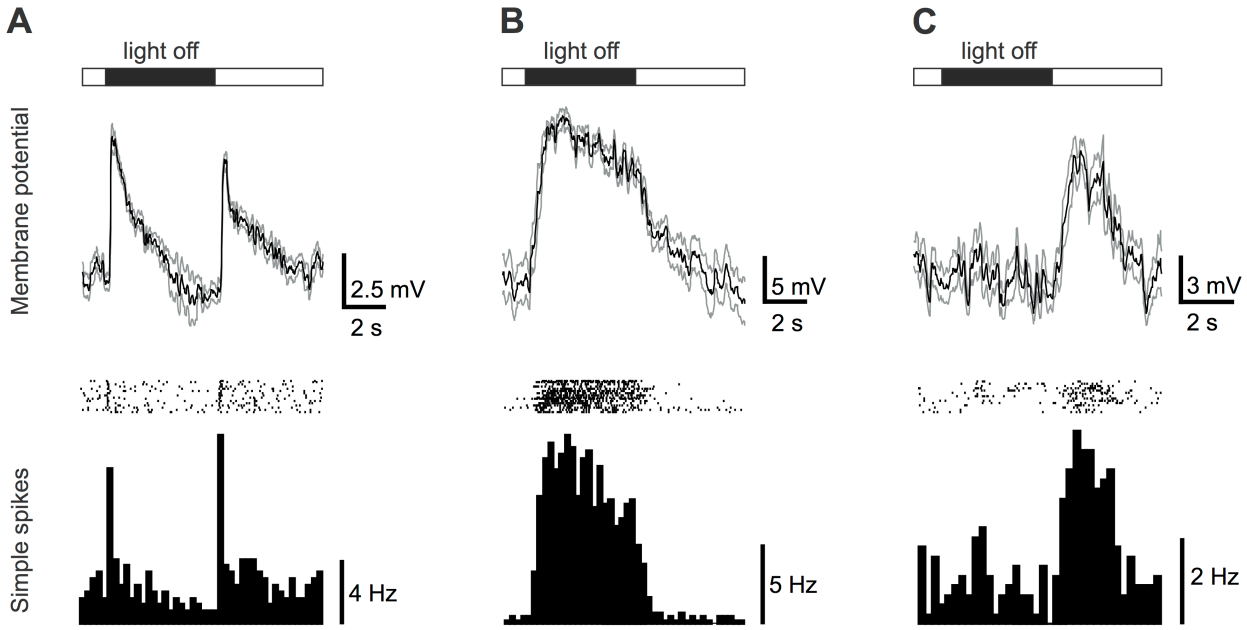

Figure 4.5 Luminance modulation of membrane potential.
A: Luminance response of cell with graded oscillation frequency response shown in Fig 4 D. Cell exhibits transient response to changes in luminance (both onset and offset). *B:* Example cell with membrane potential depolarization and increased simple spikes in response to dark. *C:* Example cell with membrane potential depolarization and increased simple spikes in response to light. (Gray traces indicates 1 SEM).

Subthreshold and simple spike responses to fictive swim bouts

Larval zebrafish control swim speed, including stabilization in the context of the OMR, by adjusting a small number of swim bout parameters, namely: bout frequency, bout duration, and tail beat frequency (Budick and O'Malley 2000; Buss and Drapeau 2001; Masino and Fetcho 2005; Severi et al. 2014). The high temporal resolution afforded by electrophysiological recording allowed us to look in more detail at how Purkinje cell membrane potential and simple spiking responses related to parameters of swim bouts. We examined the bout responses of 28 Purkinje cells (including the 11 cells initially examined in the closed loop OMR paradigm) and found that a majority of Purkinje cells had significant modulations of membrane potential (16 of 19 whole-cell recordings) or simple spikes (7 of 9 cell-attached recordings) (Student's T-test, $p<0.01$). 18 of the 23 bout responding cells had membrane potential depolarizations or increases in simple spike firing responses during bouts (Fig. 4.6*A*). The onsets of these responses were closely tied to the onset of the bout itself, recorded at the motor nerve (Fig. 4.6*B*). The average response onset preceded the first recorded motor burst by 39.8 ± 4.27 ms, consistent with the possibility that Purkinje cell activity participates in initiating and or shaping swim bouts. Bout responses had a variety of time courses (Fig. 4.6*A,C*). In some instances responses did not peak until after bout termination (Fig. 6*C*, *left panel*). Even in instances where the peak occurred early after bout onset, responses tended not to return all the way to baseline until after bout termination (Fig. 4.6*C*, *right panel*). Finally, three Purkinje cells exhibited bout-related simple spike firing that was clearly time-locked to the simultaneously recorded motor nerve bursts, the frequency of

which corresponds to the tail beat frequency in freely swimming fish (Fig. 4.6*D*). Hence, these Purkinje cells could participate in signaling or controlling tail-beat frequency, which relates directly to swim power.

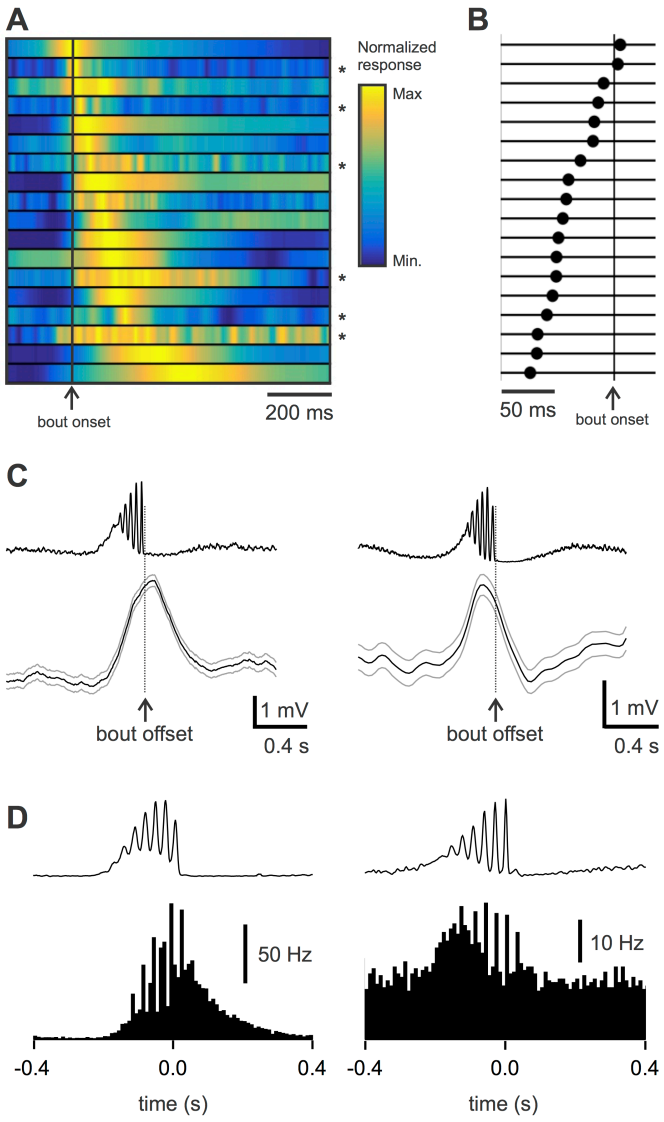

Figure 4.6 Bout-related increases in membrane potential and simple spike activity.
A: Heat map of normalized bout-triggered responses shows time course of increased membrane potential and simple spike responses (sorted by time of peak response). Black line shows bout onset recorded at motor nerve. Asterisks indicate extracellular recordings. *B:* Response onset relative to bout onset recorded at motor nerve (t=0). Response onset preceded recorded motor onset in most cells. *C:* Examples of bout response activity triggered off of termination of motor nerve bout activity. *Top*: Average motor nerve traces processed with a 5 ms RMS sliding window. *Bottom*: average membrane potential. Many bout responses extended well beyond the termination of motor nerve bout activity. *D:* Two examples of tail-beat frequency substructure in bout responses. *Top*: Average motor nerve traces processed with a 5 ms RMS sliding window, which is necessary for resolving bursts in multi-bout

averages. Bursts correspond to individual tail beats. *Bottom*: simple spike histogram triggered on final burst in a bout. Simple spike frequency exhibits modulation at same frequency as motor bursts.

A smaller subset (n=5) of the bout-responsive cells exhibited membrane potential hyperpolarization or simple spike rate decreases during motor bouts (Fig. 4.7). Unlike the varied timing of the peaks of the bout responses with opposite polarity, the troughs of these cells were generally near bout termination (Fig. 4.7*A*). In two cells we recorded enough bouts to subdivide Purkinje cell subthreshold responses according to the number of tail beats per bout, which is directly related to bout duration. When we compared response timing across these groups we found a strong correlation between the number of tail beats per bout and the time of the response trough (Fig. 7*B*; R=0.95 and R=0.96, $p<0.005$). Hence these cells could participate in signaling or controlling the timing of bout termination.

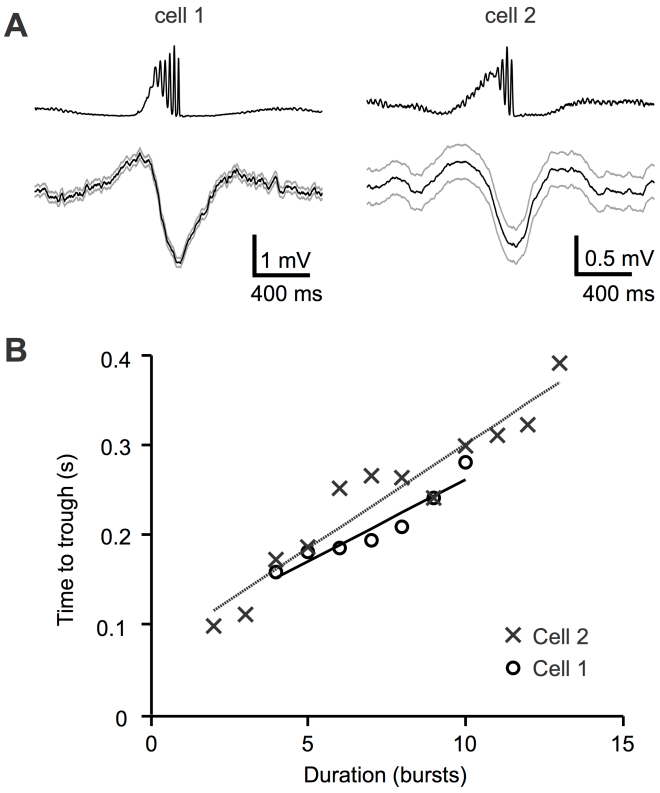

Figure 4.7 Bout-related decreases in membrane potential and simple spike activity.
A: Processed motor nerve recording (*top*) and membrane potential (*bottom*) triggered on final burst in a bout in two example cells. Membrane potential recordings (shown as average +/- 1 SEM) exhibit troughs coincident with bout terminations. *B:* Motor bout duration (binned by number of bursts per bout) vs. time of membrane potential trough in the example cells from A. As bout duration increases, time of trough increases linearly in both cells.

Climbing Fiber Responses during OMR behavior

Theories of cerebellar function posit that climbing fiber input to Purkinje cells conveys a teaching or error signal that serves to sculpt appropriate patterns of simple spike firing via plasticity at parallel fiber synapses (Albus 1971; Ito 1972; Marr 1969). If motor command signals related to swim bouts are subject to this type of error-driven correction, CFRs should preferentially occur during or following swim bouts. Indeed, we found that most Purkinje cells (17/28) had CFRs that were modulated during fictive swim bouts (Student's T-test, $p<.01$) (Fig. 4.8*A*).

A potentially relevant teaching signal for adjusting swimming in the context of the OMR would be one that grades based on the stabilization success of a swim bout. If this were the case, the probability of a CFR occurring during a bout would be expected to grade with the magnitude of tail-to-head drift (lack of stabilization) that occurred over its duration. However, when we examined the probability of a CFR during bouts in the context of open loop graded drift velocities, we saw little evidence of corresponding CFR grading. A binary logistic regression was performed to ascertain the effect of velocity on the likelihood that a CFR would occur during a bout. In only 2 of 11 cells with CFR bouts responses was the logistic regression model statistically significant ($p < 0.05$) and even in those cases the model explained only 9 and 13 % (Nagelkerke R^2) of the variance in CFR firing across bouts (Fig. 4.8*B*).

Consistent with this apparent lack of effect of drift velocity on CFR bout responses, examination of CFRs of cells recorded under closed loop/playback conditions showed that all

cells with increased CFR activity during motor bouts in closed loop (7 of 11) had similar responses to motor bouts in playback (*Motor-triggered*, Fig. 4.8*C*). Furthermore, no cells had CFR responses to the closed loop visual stimulus played back independent of the fish's motor commands (*Visual-triggered*, Fig. 4.8C).

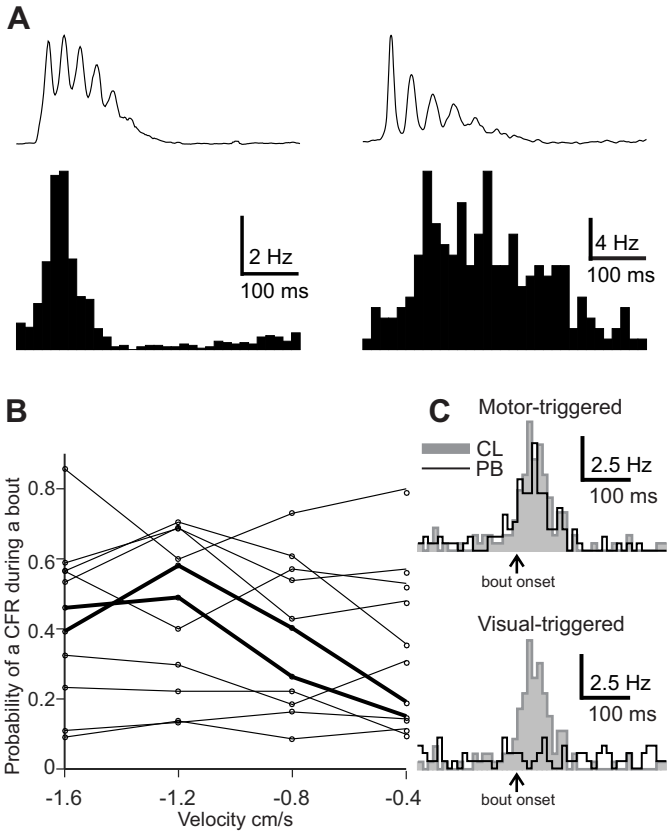

Figure 4.8 Bout-related modulations of climbing fiber response rate.
A: Examples of bout-related modulations of climbing fiber response (CFR) rate. Top: Processed motor nerve recording. Bottom: CFR histogram triggered on bout onset recorded at motor nerve. B: Probability of CFR during bouts across graded OMR-inducing drift velocities. Drift velocity did not substantially affect CFR probability during bouts in most cells with CFR bout responses (9/11 cells, binary logistic regression, $p < 0.05$) and in the remaining cells (bolded traces) explained less than 15% of the variance in CFR firing across bouts (Nagelkerke R^2). C: Comparison of CFR rate during bouts in Closed loop (CL), motor bouts in PB, and the visual consequences of bouts replayed during Playback (PB) trials. Cell exhibits CFR modulation during bouts in CL (shaded histogram) and in PB shows similar modulation to bouts (solid line, top), but not to the bout-driven visual consequences replayed from CL (solid line, bottom).

Although we detected no image velocity-related modulation of CFR bout responses or CFR responses to visual motion on the timescale of motor bouts, we did observe a number of

non-bout related visual signals in the CFR, indicating that it is not purely a motor signal. Consistent with a previous study (Hsieh et al. 2014), we observed increased CFR activity in response to luminance changes in many cells (23/31) (Fig. 4.9*A*). CFRs were also strongly modulated by onset of visual drift in most cells (21/29) (Fig. 4.9*B*). These drift-related CFR modulations were most often (20/21 cells) selectively driven by one of the two drift directions and were transient compared to the drift responses observed in the membrane potential and simple spike firing rates (Fig. 4.4*A*). Since fish rarely swam at the onset of head-to-tail motion, CFR responses to drift, in this direction at least, are unlikely to be due to motor signals.

Finally, the observation of transient CFR modulation in response to drift onset raise the question of why bout timescale visual responses were not observed in our closed loop data (Fig. 4.8*C*). Closer inspection revealed that most swim bouts reach completion before the average onset of CFR modulation due to drift (Fig. 4.9*C*). This suggests that these drift-related CFRs would not be triggered by drift on the timescale of a bout, consistent with our earlier observations.

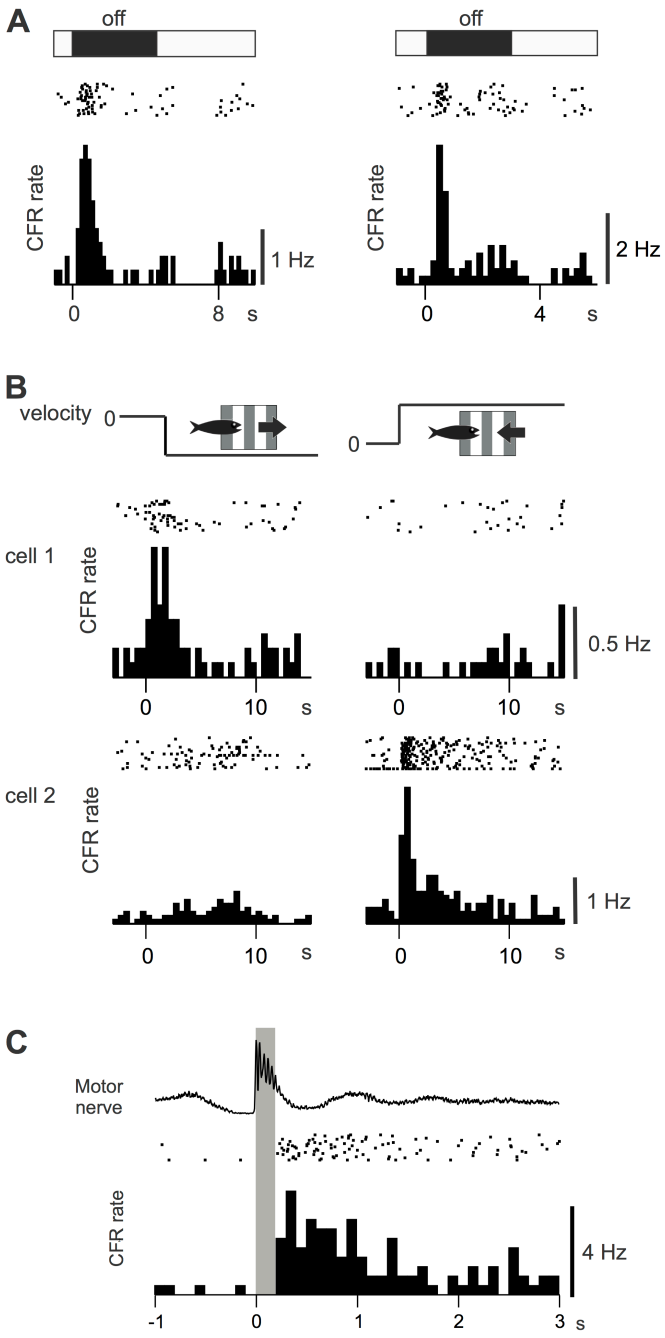

Figure 4.9 Visual modulation of climbing fiber response rate.
A: Example cells with transient increases in climbing fiber response (CFR) rate at light offset. *Top:* Time course of luminance states (*black fill indicates light off*). *Bottom:* Raster and histogram of CFR rates. *B:* Example cells with transient increases in CFR rate at onset of OMR-inducing or OMR-inhibiting drift *Top:* time course of OMR-inducing and OMR-inhibiting drift. *Middle:* Raster and histogram of CFR rates for cell with transient increase in CFR rate in OMR-inducing direction only. *Bottom:* Raster and histogram of CFR rates for cell with transient increase in CFR rate in OMR-suppressing direction only. *C:* Timing of onset of OMR-suppressing drift-related increase in CFR rate (*bottom*) relative to time course of average bout recorded at motor nerve (*top*). Bouts are rapid compared to increase in CFR rate, consistent with the lack of observed CFR modulation during transient drift in the OMR-suppressing direction during Playback of visual stimulus driven by bouts in Closed loop (Fig 8C).

Relationship between simple spikes and climbing fibers in individual Purkinje cells

Simple spikes and CFRs have distinct origins and likely play different functional roles. Though the relationship between simple spikes and complex spikes has been studied extensively in Purkinje cells in mammals under a variety of conditions, little is known in zebrafish (Hsieh et al. 2014). Though we cannot rule out systematic relationships between simples spike and CFRs, such relationships were not obvious in our data. A survey of our recordings showed that the strength or polarity of simple spike modulations did not obviously correlate with the presence or strength of CFR modulations in the same Purkinje cell. Indeed, no systematic relationship between changes in membrane potential/simple spike firing and changes in CFR rate was observed in any of our experimental paradigms. Similar bout-related increases in CFR rates were associated with simple spike decreases in some cells and increases in others (Fig. 4.10*A*). Similar non-direction selective drift-induced increases in simple spikes could be accompanied by highly direction-selective CFR responses (Fig. 4.10*B*). Finally, similar increases in CFR rate in response to a luminance decrease could coincide with either no change or an increase in with simple spike firing (Fig. 4.10*C*).

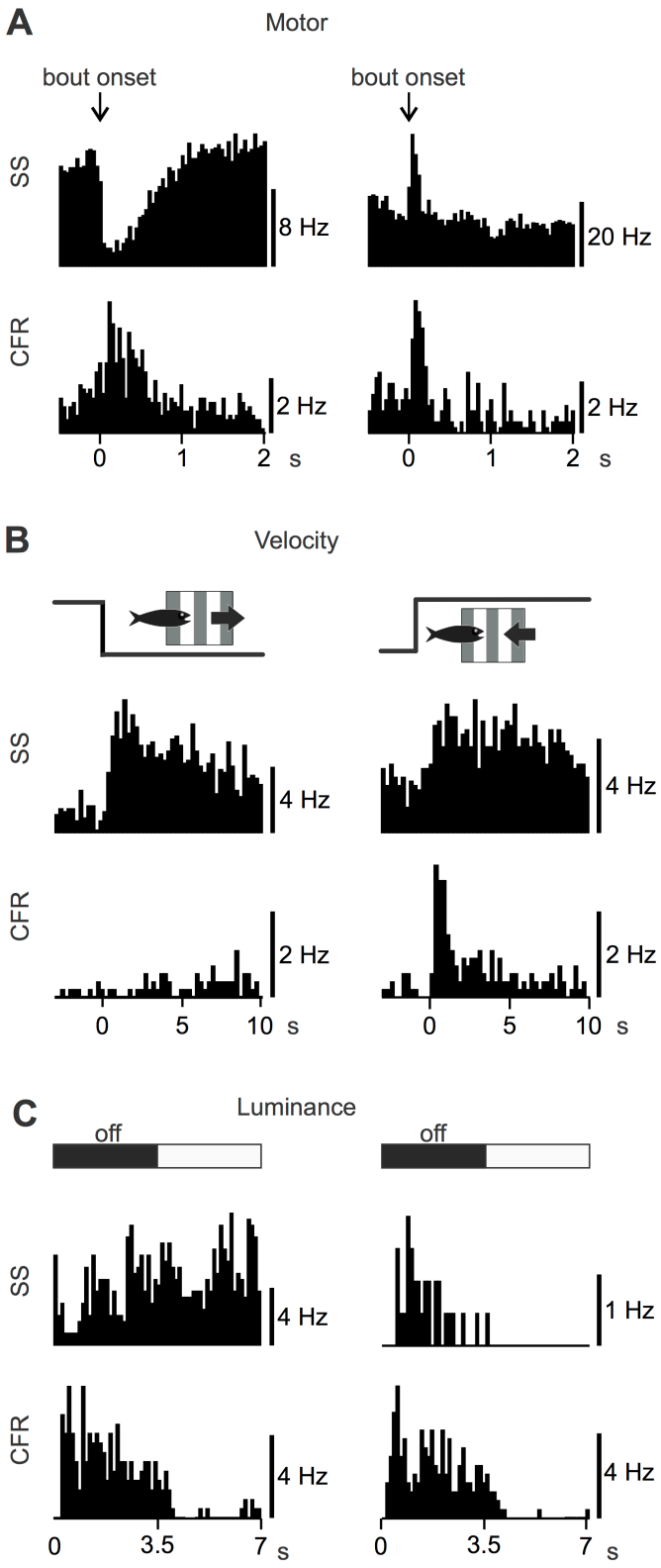

Figure 4.10 Climbing fiber response and simple spike response differences.

A: Inconsistent climbing fiber response (CFR) and simple spike (SS) response relationship during motor bouts. *Left*: Cell with opposite simple spike and CFR response polarity. *Right*: Cell with matching simple spike and CFR response polarity. *B:* Inconsistent CFR and SS response relationship during sustained grating drift. *Top*: simple spike rate is increased for drift in either direction. *Bottom*: CFR rate is only transiently modulated and only for one direction of drift. *C:* Inconsistent CFR and SS response relationship during luminance changes. *Left*: Cell has no simple spike response, but strong CFR response to light off. *Right:* Cell has strong simple spike as well as CFR response to light off.

Discussion

Subthreshold/simple spike responses in relation to visual motion

During OMR behavior fish adjust the direction and speed of their swimming based on the direction and speed of retinal image motion (Neuhauss et al. 1999; Orger et al. 2008). Hence one possible role for the zebrafish cerebellum would be to transform image velocity signals into swim commands appropriate to stabilize the fish's position. Such a role would be analogous to that played by the cerebellum in the VOR, during which Purkinje cells participate in transforming head velocity information (conveyed by mossy fibers) into motor commands to counter-rotate the eye (Ito 1982). Direction-selective visual responses have been observed in areas of the larval zebrafish brain, including the pretectum, and have been suggested to play a role in the OMR (Kubo et al. 2014; Portugues et al. 2014). Although we found that a drifting grating stimulus evoked sustained membrane potential depolarization and increases in simple spike firing in Purkinje cells, such responses did not grade with image velocity. Direction-selective subthreshold and simple spike responses were also uncommon in Purkinje cells. Hence our results do not support a role for the zebrafish cerebellum in adjusting the OMR based on visual signals analogous to the established role for the mammalian cerebellum in adjusting the VOR based on vestibular signals. However, our conclusion that Purkinje cells lack velocity sensitivity should be considered in light of the facts that we sampled a relatively small number of

cells, only within the corpus cerebelli, within a limited developmental time window, and using a restricted set of visual stimuli.

Subthreshold/simple spike responses in relation to fictive swim bouts

The majority of recorded Purkinje cells exhibited subthreshold and simple spike responses to fictive swim bouts under closed loop conditions. Our playback paradigm showed that these are mainly or entirely motor responses, rather than responses to the rapid changes in visual input that would normally accompany swim bouts. These results are consistent with a previous whole-brain calcium imaging study that found that more cerebellar neurons showed activity that was strongly correlated with motor output than visual input during the OMR (Ahrens et al. 2012).

Our electrophysiological recordings also allowed us to examine the timing of motor response in Purkinje cells and show that such responses are closely tied to individual motor bouts. Though some Purkinje cells responded before motor nerve bouts others responded at the same time or shortly after bout initiation. Hence while it is not clear from these results whether Purkinje cells are active early enough to participate in initiating bouts, they certainly could participate in shaping their amplitude or duration. Consistent with this latter possibility, a recent study has shown that optogenetic activation or silencing of larval Purkinje cells could modify, but not initiate, swimming movements during the OMR (Matsui et al. 2014).

Our results provide some initial insights into how motor signals related to swimming are encoded in zebrafish Purkinje cells. One subset of cells exhibited a hyperpolarization and/or reduction in simple spike firing that was aligned with the termination of motor bouts. These responses clearly graded with bout duration in the cases in which it could be examined. Another small subset of cells exhibited structured simple spike firing within each bout that appeared to

track tail beat frequency, an important contributor to swim speed in larval zebrafish. Overall, however, the responses we observed in individual Purkinje cells were heterogeneous and generally did not closely follow the time-course of individual bouts. These findings are intriguing in light of a recent study of Purkinje cell encoding of another type of ballistic movement—saccades (Herzfeld et al. 2015). The authors showed that while responses of individual Purkinje cells did not accurately encode saccade kinematics, appropriately chosen populations of Purkinje cell responses did. For example, pooling cells with increases and decreases in simple spike firing related to saccades resulted in a temporal profile of activity that matched saccade duration. Bout responses reported here for Purkinje cells are similar to those observed for saccades in that both increases and decreases in simple spike firing were observed in different cells and that simple spike modulations often outlasted bouts. Future studies could examine this issue in more detail in zebrafish with the added possibility of using voltage-clamp recordings from efferent cells to directly isolate the summed response of a local population of Purkinje cells.

Aside from any specific role in controlling the OMR, the strong responses we observed to fictive swim bouts suggest that Purkinje cells play a general role in encoding, controlling, and/or adjusting parameters of larval zebrafish swimming. Such a generalized role for the cerebellum in adapting motor output could be desirable if the need for changes in swim strength was not task- or context-specific (as in the case of the OMR), but due to changes in the motor plant, for example growth or injury of the organism. In the larval zebrafish such a capacity would seem to be particularly important as this developing creature's body changes dramatically on a rapid timescale. For example, larval zebrafish increase in length by 60% from 15 to 30 dpf and undergo dramatic changes in tail, anal, and dorsal fin development over this same time period

(Singleman and Holtzman 2014). Studies of "natural" motor adaptation in developing zebrafish may provide a valuable complement to the extensive literature on controlled laboratory studies of motor adaptation conducted mainly in humans and monkeys (Shmuelof and Krakauer 2011).

Climbing Fiber Responses

Studies of cerebellar involvement in behaviors such as the vestibulo-ocular reflex and smooth pursuit eye movements, have suggested that climbing fibers drive adaptive modification of motor gain and timing by instructing synaptic plasticity in cerebellar circuitry, e.g. at synapses between parallel fibers and Purkinje cells (Ito 1993; Ito and Kano 1982; Medina and Lisberger 2008; Simpson et al. 1996). In these contexts complex spikes have been shown to encode "error" signals relevant for improving performance, such as unexpected image motion or retinal slip (Simpson et al. 1996). Consistent with a role for CFRs in adapting motor performance in zebrafish, lesions of the olivocerebellar pathway disrupted the ability of larval zebrafish to adaptively modify OMR gain (Ahrens et al. 2012). If CFRs plays a role in adjusting simple spike responses to motor bouts, we might expect CFRs to occur selectively during bouts. Indeed, we did find that CFRs occur with significantly greater frequency during swim bouts in most cells. A key question however, is what, if any, "error" information CFRs encode in relation to bouts. During the OMR, image motion information indicates whether a bout was successful in stabilizing the fish's position relative to the visual environment. Net tail-to-head image motion indicates that motor output is on average too weak while net head-to-tail image motion indicates that motor output is on average too strong. We failed to find clear evidence that CFRs encoded the amount of drift that occurred during a bout. Climbing fiber probability during bouts did not increase with increasing open loop drift velocity.

While we did not find evidence that CFRs encoded error signals appropriate for adjusting the OMR, our results do suggest that CFRs can be driven by visual as well as motor signals. CFRs often showed transient responses to visual motion onset that were direction-selective and were also often responsive to luminance changes. Therefore, CFRs appear to encode both sensory and motor information. Clearly, more work is required to understand what signals CFRs encode and what functions they serve.

Relationship between simple spikes and climbing fibers in individual Purkinje cells

We observed diverse relationships between simple spikes and CFRs across Purkinje cells. This was true for both visual and motor related responses. As shown in Figure 4.10, the strength, polarity, or timecourse of simple spike modulations did not necessarily correspond to that of CFR modulations. A recent electrophysiological study of larval zebrafish Purkinje cells reported that CFRs evoked sustained depolarizations and bursts of simple spike firing in a subset of Purkinje cells (Sengupta and Thirumalai 2015). Though we did not attempt to quantify this behavior, we also sometimes observed bursts of simple spikes and transitions between quiescence and bursting coincident with CFRs. However, as noted above we also observed many cases in which depolarizations and simple spike occurred in the absence of CFRs. Diverse relationships between simple and complex spikes have also been reported in mammalian Purkinje cells (see e.g. Yakusheva et al. 2010). However, this observation has special importance for studies of zebrafish cerebellum given the amenability of this system to calcium imaging. Calcium imaging studies of mammalian Purkinje cells have focused almost exclusively on calcium transients related to complex spikes and there have been no clear indications that simple spike firing rates can be recovered from calcium imaging data (Gaffield et al. 2015; Kitamura

and Hausser 2011; Ozden et al. 2009; Schultz et al. 2009; Sullivan et al. 2005). Studies aimed at directly determining how calcium responses relate to simple and complex spikes in larval zebrafish Purkinje cells will be needed to take full advantage of the potential power of calcium imaging for studying the zebrafish cerebellum.

Conclusion

The mammalian cerebellum has been intensively studied for many years and though much is known, there is no general consensus regarding the core function it performs or even whether such a core function exists. Understanding the cerebellum will likely require a variety of approaches applied to a variety of systems. In this regard, the larval zebrafish cerebellum is an attractive candidate for study given its small size and accessibility to population imaging, visualized electrophysiological recordings, and genetic manipulations. Key to such efforts will be defining the inputs to the cerebellum and understanding how they are transformed within cerebellar circuitry in the context of cerebellar-dependent behavior. The present description of the responses of Purkinje cells to visual and motor signals during the OMR provides an initial step towards this goal. Though we found little evidence for signals encoded in simple spikes and/or CFRs that are specifically suited for controlling or adjusting the OMR, our recordings clearly demonstrate that Purkinje cells encode motor signals related to swim bouts. Hence the zebrafish cerebellum may play a general role in controlling and/or adjusting swim behavior.

Moreover, the rapid and dramatic developmental changes in the motor systems and behavior of larval zebrafish may offer a tractable and ethologically-grounded approach to understanding how the cerebellum contributes to adapting and optimizing motor performance based on experience.

CHAPTER 5
FUTURE DIRECTIONS FOR CEREBELLUM IN ZEBRAFISH

CHAPTER 5: FUTURE DIRECTIONS FOR CEREBELLUM IN ZEBRAFISH

Introduction

In the preceding chapter, I provided an initial characterization of PC activity during optomotor behavior in larval zebrafish. The foundation of cerebellar knowledge in zebrafish, to which this characterization contributes, is at an infantile stage compared to the vast quantities of data amassed about cerebellar function in other model systems. The justification for catch-up efforts lies in the smaller number of neurons in the cerebellum and motor control apparatus and their compatibility with powerful genetic and imaging tools that could make possible approaches not previously available in cerebellar research. However, to realize the full command of these tools, they must be customized for cerebellum specific studies and deployed from an informed vantage point that includes at least a basic characterization of connectivity and physiology of the zebrafish cerebellum and its role in a cerebellum-dependent behavior.

In the rest of this chapter, I will first itemize the current state of cerebellar methods and foundational knowledge in larval zebrafish and the remaining ground to cover. I will then proceed to discuss some of the methodological advantages that a fully realized zebrafish cerebellar paradigm could offer in the context of the types of experimental questions they could help to address. Specifically, I will explore the potential for (1) Broad and unbiased sampling of population activity. (2) Optogenetic access to all cells in cerebellum, including output cells. (3) Potential to monitor molecular correlates of learning in real time.

Status of cerebellum-specific methods in zebrafish

Genetic targeting of material to label, monitor, and manipulate cerebellar cell types

Genetically labeling neuronal cell types, monitoring their activity with calcium sensitive indicators, exciting them with optogentic tools like channelrhodopsin and inhibiting them with with optogentic tools like archaerhodopsin are four of the critical capabilities available for taking advantage of the optogenetic accessibility of zebrafish. All of these techniques have been applied in zebrafish, but have so far have been effective in different cerebellar cell types to different degrees. Achieving these manipulations in GCs, PCs, and efferent cells, as well as ideally in some of the major inhibitory interneurons such as Golgi cells and stellate cells, would provide great flexibility with which to approach cerebellar experimental design.

All four of these manipulations have been reported recently in PCs (Matsui et al. 2014). Recently published Gal4 lines for targeting arbitrary reporters to GCs and efferent cells also exist, but have so far been used only to express GFP (Takeuchi et al. 2015). Theoretically, crossing these lines with UAS GCaMP, channelrhodopsin and archaerhodopsin (Arrenberg et al. 2009; Chen et al. 2013; Umeda et al. 2013) should be straight-forward, but it has not yet been reported. As yet there are no known reports of cell-specific expression in zebrafish inhibitory interneurons other than PCs.

In the interim, monitoring neuronal activity in cell types without cell specific expression of GCaMP may be possible using fish with pan-neuronal GCaMP expression driven by the panneuronal elavl3/HuC promoter (Ahrens et al. 2013; Higashijima et al. 2003) if cell location and morphology can be used to identify cell types. Recently developed HuC:Gal4FF/UAS:GCaMP6s larvae have sparser mosaic GCaMP6 expression (Chen et al. 2013) that may facilitate this. In practice though, similar cell body size and mixing of cell types in the layers of cerebellum would likely make identification difficult in most cells, though typically larger Golgi cells may be an exception.

Critically, as was found in chapter 2, loading cells with calcium indicator does not guarantee that spiking activity can be read out in fluorescence signals. Electrophysiological recording could be used to calibrate the relationship between spiking and fluorescence signals. And, in the event that indicator was not sensitive enough to read out spiking, electrophysiological recordings could be used to sample from a large percentage of the relatively small number of cells of any given cell types in larval zebrafish cerebellum, albeit not with the efficiency of imaging.

Electrophysiological recording in identified cerebellar cell types

Electrophysiological recording from identified cerebellar cell types will be important for calibrating any spiking activity visualized with calcium sensitive indicator. Separately, it also represents a means of acquiring subthreshold and higher resolution temporal information. Therefore, methods for recording in GCs, PCs, efferent cells, Golgi cells and stellate cells are important tools to establish.

Recording in PCs has been demonstrated herein and in two recently published studies (Hsieh et al. 2014; Sengupta and Thirumalai 2015). No electrophysiological recordings in other zebrafish cerebellar cell types have been reported. However, from unpublished observations I believe recording from efferent cells and GCs should be relatively straight-forward. I obtained a handful of putative efferent cell recordings in the course of recording PCs, identified based on their location, lack of climbing fiber response, and morphology when filled. I have also observed proof-of-principal recording of a GFP-labeled GC.

Characterizing inputs to and targets of cerebellum

Understanding the complete input-output transformation of information through zebrafish cerebellum will require knowledge of the nature of these inputs and outputs. Visual and likely motor inputs must contact cerebellum to drive the motor and visual activity observed in PCs during the OMR. Other potential sensory inputs, to which behavioral responses have been successfully engaged experimentally in larval zebrafish, include lateral line, vestibular, auditory, and olfactory inputs (Bhandiwad et al. 2013; Kermen et al. 2013; Kohashi et al. 2012; Levi et al. 2015). Only two sources of mossy fiber inputs have so far been identified anatomically, one from the dorsal tegmental nuclei (Bae et al. 2009) and the other from pretectal nuclei (Volkmann et al. 2010). Tracer techniques could be used to identify additional mossy fibers regions of origin.

Experiments in a number of teleost species have shown a wide array of downstream targets of efferent cells ranging from the diencephalon to the medulla based on tracer experiments (Ikenaga et al. 2006). More recently, two papers using genetically labeled efferent cells have elucidated specific downstream targets in zebrafish. One paper identified two distinct subsets of efferent cells terminating in tectum and thalamus (Heap et al. 2013), while another observed termination in the tegmentum and rostral hindbrain, where the oculomotor nucleus and reticular formation, amongst other structures, are situated (Takeuchi et al. 2015).

Describing cerebellum-dependent behavior

Marr-Albus based models of cerebellar function are premised on the idea that cerebellum serves to learn relationships between current state information and some desired output. Therefore to determine whether and how the cerebellum carries out such algorithm will require a learning behavior in zebrafish with a demonstrated cerebellar-dependency.

OMR gain adaptation represents one possible such behavior. As described in chapter 3, in a set of experiments in which the inferior olive was lesioned, zebrafish lost the ability to instigate OMR gain adaptations in a fictive closed loop paradigm (Ahrens et al. 2012). Given that the olive is the source of all CF inputs to the cerebellum, the cerebellum could be the means by which the olive's role in gain change adaptation is carried out. However, lesion manipulations are an imprecise means of selectively eliminating a structure's contribution to behavior as processes from other brain regions can pass through the lesioned area and be unintentionally inactivated as well.

A more precise evaluation of the contribution of the cerebellum to OMR adaptation could be achieved using archaerhodopsin to silence efferent cell output in a gain change learning paradigm. Fish that were able to learn gain changes under control conditions, but not during archaerhodopsin inactivation of efferent cell activity would provide strong evidence that the cerebellum is involved in OMR gain change adaptation.

Of note, after considerable effort I was not able to reliably initiate OMR learning in zebrafish using my fictive OMR paradigm. This may have been due to intrinsic variability between fish or it may have been due to differences between my experimental setup or animals as compared to those used in published studies of OMR gain-adaptation (Ahrens et al. 2012; Portugues and Engert 2011). Most notably, my fish were of a different genetic background (AB vs. WIK) and had their brains exposed to an artificial cerebrospinal fluid for electrophysiological recordings. These two major variables would not be necessary in the archaerhodopsin experiment described above.

It is possible that it will turn out that the cerebellum is not clearly involved in OMR adaptation. In this case it would be necessary to delineate a different behavior in which

cerebellum is critically involved. One promising study, also described in chapter 3, suggests that the cerebellum plays a role in an associative conditioning task in which a conditioned visual stimulus drives an enhanced motor response after pairing with an unconditioned touch stimulus (Aizenberg and Schuman 2011). This behavioral change was paralleled by facilitation of luminance responses in cerebellar neurons after conditioning. Interestingly, laser ablation of the cerebellum arrested acquisition and extinction (dependent on when during training the lesion was made) but not retention of the learned response. As noted above laser ablations allow for the possibility that non-cerebellar processes in the vicinity can be affected. Nevertheless, this paradigm is a promising one given its similarity to mammalian cerebellar-dependent classical eyelid conditioning paradigms (Thompson and Steinmetz 2009) and its cerebellar dependence could be explored more precisely using optogenetic silencing.

Another interesting behavioral paradigms, prey capture, has not been evaluated for cerebellar dependence, but it is easy to imagine that the sensorimotor demands of this behavior that requires precisely time movements could make use of cerebellar apparatus (Bianco et al. 2011; Gahtan et al. 2005).

Monitoring molecular correlates of learning in cerebellum

As discussed in chapter 1, disentangling the roles of diverse sites of plasticity in the cerebellum has been a long-standing challenge. Inactivating sites of plasticity with good specificity is difficult, and even if accomplished may alter the dynamics of plasticity compared to their interplay in an unmanipulated state. A recently published method demonstrating the ability to monitor internalization of GFP-labeled AMPA receptors in mouse barrel cortex vivo (Zhang et al. 2015) could make it possible to directly monitor plasticity in naive animals in real

time. Such a method is not yet established in zebrafish but should be feasible using similar methods of in utero electroporation (Hoegler and Horne 2010). The optical accessibility of the zebrafish makes it particularly amenable to this approach as all relevant synapses could likely be visualized, in comparison to mice where synapses onto the deep cerebellar nuclei could not be accessed noninvasively.

Zebrafish experimental possibilities on the horizon

In the remainder of this chapter I discuss some areas of cerebellar research that could particularly benefit from the methodological capabilities that are made possible by the larval zebrafish's small size and genetic tractability. Because the particulars of any of these experiments will depend on the success of development of the tools outlined above, I will not go into great detail about how to carry out actual experiments. Instead I focus on the nature of the challenges in each area of inquiry and why the types of methodological approaches that can be realized in zebrafish could offer a powerful means of contending with them.

Accessibility of population data

The comparatively small number of neurons in the zebrafish cerebellum makes it possible to acquire data from complete populations using imaging, and to obtain higher resolution visualized electrophysiological recordings with an expectation of unbiased and comprehensive sampling. This potential for broad sampling affords a number of advantages. It can obviate concerns that conflicting data from different experiments stem from sampling of different populations. It also allows the role of network dynamics to be examined directly rather than inferred from observations of small subsets of neurons.

The dramatic coding expansion that occurs in GCs, discussed in chapter 2, exemplifies a region where broad sampling could provide insights about the information and processing these cells supply to PCs. Indeed population characterization of sensory responses in these cells has already begun. In the first report of imaging in GCs (Sylvester et al. 2011), researchers found GC activity in response to visual motion clustered into two groups, direction selective and non-selective. These findings are interesting in light of the surprising largely direction insensitive responses of PCs I observed during the OMR.

As noted, existing accounts of GC response properties from published whole-cell recordings in other organisms provide conflicting evidence for the combinatorial encoding that has long been theorized to occur in GCs. Recordings in mormyrid have indicated that individual granule cells do receive multimodal proprioceptive and corollary discharge inputs (Sawtell 2010), a finding that is complimented by anatomical work in mouse finding a convergence on granule cells of a pathway carrying upper body proprioceptive information and a basilar pontine pathway, thought to carry upper body motor corollary discharge information (Huang et al. 2013). Recently published whole cell recordings in mouse have similarly demonstrated GC integration of vestibular and visual information (Chabrol et al. 2015). On the other hand, whole cell recordings in cat have indicated that granule cells receive only unimodal cutaneous or joint-related information with similar receptive fields at all inputs (Jorntell and Ekerot 2006). It is possible that these divergent results stem from different properties in different GC populations. Sampling uniformly across the entire GC population in response to multiple sensory stimuli would allow assessment of whether, at least in one organism, multi-modal GCs are the exception, the rule, or somewhere in between.

Beyond addressing discrepancies that could result from under sampling, monitoring population activity could make it possible to address inherently population level questions that could be difficult to answer in larger systems. For example, combinatorial encoding in GCs has been theorized to result in sparse encoding that allows PCs to assign different significance to a given mossy fiber input depending on the identity of the other mossy fibers with which it is co-active. In population terms, overlapping patterns of mossy fiber inputs are separated into distinct GC patterns with stricter, and thus less frequently engaged, activation requirements. Sparse encoding thus allows specificity of learned responses, but it has been pointed out that a trade-off for sparseness could be a hindered ability to generalize learning across related contexts (Spanne and Jorntell 2015). Therefore, the ideal as well as actual proportion of activity in the granule cell population at any given time is an open question that is likely best addressed by sampling activity at a population level.

Even with massive expansion, it's likely not possible to represent all potential combinations of mossy fibers distinctly in GCs. A further question then is whether there is behaviorally-relevant logic to the combinations that are represented or if they are the result of random mixing (Barak et al. 2013). Broad sampling would be critical in addressing this question across the many types of inputs GCs can receive, which may be represented to different extents and mix with different degrees of randomness.

For instance, there is evidence in mormyrid that parallel fibers provide randomly mixed proprioceptive and corollary discharge signals, although at least some of this mixing occurs presynaptic to GCs (Requarth et al. 2014). The paper in which these results were published makes a good case for why such mixing would be desirable for downstream processing. However, mixing of other inputs might be less plausibly useful. For example, in chapter 2, I

discussed a second but not at all mutually exclusive theory of GC processing that GC and interneuronal circuitry provide temporally expanded representations of transient outputs to which delayed responses might be desired. There is evidence that such temporal expansion occurs in mormyrid granule cells, where multimodal encoding has also been demonstrated (Kennedy et al. 2014; Sawtell 2010). Therefore a question is whether temporal expansion and multimodal encoding occur in the same cells and if so whether sensory information from different time windows is randomly mixed. Natural stimuli often engage more than one sense so it seems likely that combining multimodal sensory information conveying information from the same time window would be more useful than combining information from two random delays. It's possible then that we would see a blend in GC encoding of random mixing of modality with ordered mixing of temporal delay.

Concrete theories exist regarding the benefits that combinatorial or temporal expansion in GCs, and related levels of sparseness, could provide in terms of learning capabilities. In larval zebrafish, population data regarding granule cell activity in various sensorimotor contexts could be used in models with biologically accurate numbers of interconnected neurons to assess the computational power and trade-offs of observed granule cell encoding in terms of such criteria as accuracy and generalization of pattern learning.

Accessibility of all cell types

The layered structure of the cerebellum means different stages of processing are different amounts accessible to imaging and optogenetic stimulation in other organisms. The ability to visually access every cell in the zebrafish brain confers depth of sampling as well as breadth. In particular, in mammals cerebellar efferent cells are located in the deep cerebellar nuclei, well

beneath the limits of optical access without surgical manipulation. In zebrafish these cells are quite accessible. Furthermore in teleost cerebellums, cerebellar efferent cells are scattered amongst the PCs that innervate them, making simultaneous monitoring of activity in connected cells feasible through both imaging and electrophysiological approaches.

As discussed in chapter 1, the significance in the processing that occurs downstream of PCs has become a hotly debated topic since the early days when they were treated largely as relay neurons that simply conferred a sign flip. Though PCs do inhibit efferent cells, efferent cell activity does not necessarily look like an inversion of PC activity see e.g. (McDevitt et al. 1987). In the vestibular nucleus, linear summation of PC input rates by vestibular neurons has been demonstrated (Medina and Lisberger 2009). Accordingly, in dynamic clamp studies, simulation of asynchronous PC input inhibited cells of the deep cerebellar nuclei in a fairly straight-forward manner (Person and Raman 2012). However, simulation of synchronous inputs to these cells resulted in entrainment of nuclear cell firing to their synchronous firing. Furthermore, partial synchrony drove entrainment with the subset of synchronized Purkinje cells, revealing a synchrony-based nonlinear interaction between population PC activity and its effect on efferent cell firing. In this same study, in vivo stimulation trains in the molecular layer could also entrain nuclear cell firing.

Synchronous pausing of PC activity has also been implicated in another type of non-linear encoding in efferent cells: rebound spiking triggered by strong inhibition (Aizenman and Linden 1999; Gardette et al. 1985a; b; Tadayonnejad et al. 2010). Rebound spiking has been theorized to provide a timing signal that can be used for various purposes (Steuber and Jaeger 2013). Though while rebound firing has been demonstrated robustly in vitro, its prevalence in vivo is not clear and the purpose and conditions under which it occurs are under active debate

(Alvina et al. 2008; Bengtsson et al. 2011; Tadayonnejad et al. 2010; Tadayonnejad et al. 2009). Rebound discharge requires sudden release from strong inhibition, which would likely require synchronous pausing across groups of innervating PCs. Complex spikes in PCs are followed by a pause in simple spiking, so it's been theorized that synchronized complex spike inputs across groups of PCs could be a mechanism by which rebound discharge is triggered (Bengtsson et al. 2011; Steuber and Jaeger 2013).

In short, synchrony of activity across groups of PCs could provide strong modulation of efferent cell activity, but it is not clear whether such a mechanism is regularly engaged in vivo and to what effect. The optical accessibility of efferent cells in larval zebrafish could allow direct monitoring of efferent cell activity concurrent with PC activity. Furthermore paired patching of these two cell types would allow direct assessment of the effect of an individual PC's spiking output on efferent cell activity. The effects of synchronous PC pauses could be explored by optogenetically driving direct transient inactivation of PCs, or indirect inactivation via activation of climbing fiber inputs from the inferior olive. Of note, while optogenetic activation of PCs is possible in mouse, and could conceivably be combined with electrophysiological recording from cells in the deep cerebellar nucleus, only a subset of PCs can be stimulated by non-invasive measures due to light attenuation caused by tissue fissures and obstruction of parts of the cerebellum by other parts of the brain (Tsubota 2013).

As interesting as is the effect exerted by PCs on efferent cells, the effect exerted by efferent cells on cerebellar targets is arguably an even more critical piece of the puzzle, as it represents the net outcome of the various levels of processing in the cerebellum. The ability to monitor and manipulate efferent cell outputs in larval zebrafish, along with their small number (well below the approximately 300 Purkinje cells amongst which they are interspersed) and the

relatively simple motor apparatus in this organism justifies optimism that some of the effects of efferent cells on motor behavior could be untangled in this system.

Efferent cells in the zebrafish have been characterized as targeting a number of brain regions (Heap et al. 2013; Matsui et al. 2014; Takeuchi et al. 2015), including the nMLF (Matsui et al. 2014). As noted in chapter 1, the nMLF consists of a cluster of about 20 neurons that is part of the reticulospinal cell population that projects to spinal cord. Particular cells (MeLc and MeLr) have been implicated in sensorimotor behavior, such as prey capture and high speed optomotor responses (Gahtan et al. 2005; Severi et al. 2014). Interestingly activity in these cells appears to correspond differentially to tail beat frequency and bout duration. Given the tail beat frequency and bout duration information I observed in PC recordings, it would be interesting to assess the relationship between efferent cell outputs and these identified neurons both anatomically and by driving activity in efferent cells optogenetically and observing effects in these cells. The effects of efferent cell activation on tail beat frequency and bout duration could also be evaluated directly by observation of swim statistics in the presence or absence of optogenetic manipulation.

Ability to monitor molecular correlates of learning in real time

As discussed in chapter 1, climbing fiber mediated learning is not likely to be the only mechanism of learning in the cerebellum. Dissecting the different and likely overlapping contributions of diverse sites of plasticity in the cerebellum has faced a number of challenges. The difficulty of monitoring neural activity over long periods of time has meant that often inferences about plasticity have had to be made based on comparison of activity before and after training rather than being able to monitor the time course with which these changes happen. To

limit the possible routes by which the observed changes could occur, manipulations of sites of plasticity has been attempted. However such manipulations can be fraught with non-specific effects -- for example disruption of molecules involved in signaling cascades for plasticity can also disrupt signaling cascades that are important for other pathways as well (Boyden et al. 2004; Schonewille et al. 2011) – and must also contend with the possibility that that mechanisms of plasticity might substitute for one another when disruption occurs, taking on roles that don't reflect normally plasticity in the unmanipulated condition.

In the larval zebrafish it may be possible not only to monitor the time course of changes in activity noninvasively during learning, but also to monitor the actual molecular components of plasticity as it occurs during learning in unmanipulated systems. A recently published study demonstrated the ability to monitor internalization of GFP-labeled AMPA receptors in mouse barrel cortex vivo (Zhang et al. 2015). If such a method can be established in zebrafish, the optical accessibility of PCs and efferent cells could allow direct observation of subcellular level AMPA trafficking processes underlying the existence and timescales of LTP and LTD at parallel fibers to PCs vs parallel fibers to efferent cells for example.

Uncertainty about the roles of different locations of plasticity exists even across different inputs to the same cell and could also be resolved by direct observation of plasticity. For example, many modern Marr-Albus based models of cerebellum share central computational features with an engineering control theory mechanism called an adaptive-filter (Dean et al. 2010; Fujita 1982). In essence, a basis of component signals extracted from mossy fiber inputs are conveyed to PCs. An error signal then weakens any inputs with which it is correlated, while any inputs not correlated with this error signal have their weights increased. Assuming these correlations reflect causation, this approach to sculpting a PC output driven by contextual inputs

that are maximally decorrelated with error will result in optimal PC performance. In engineering applications however, the weights used in simple adaptive filters can be both positive and negative. Such a capacity could not be achieved by LTD of parallel fiber inputs alone, but Dean and Porrill propose that if the weights of parallel fiber innervations of inhibitory stellate and basket cells that synapse on PCs could be adjusted by CF activity in the opposite direction from its effect on parallel fiber inputs, they could offer a sign-flip that would effectively provide indirect negative weights from parallel fibers onto PCs via inhibitory interneurons. Indeed CFs contact and can influence stellate cell activity (Barbour 1993; Jorntell and Ekerot 2002; Sugihara et al. 1999)

An interesting set of in vivo experiments demonstrated a role of climbing fiber to stellate cell plasticity by co-opting the CFs teaching signal to drive artificial learning in stellate cells. Using stimulating electrodes to co-activate parallel fibers and CFs, the authors caused the cutaneous receptive fields of stellate cells expand, suggesting strengthening of a broader range of cutaneous mossy fiber inputs onto stellate cells (Jorntell and Ekerot 2002). After this manipulation, electrical recordings from stellate cells demonstrated increased active parallel fiber synapses (Jorntell and Ekerot 2003). These results credibly suggest that stellate cells input to PCs can provide negative weighting of parallel fiber information to PCs, but how concurrent weakening of the parallel directly onto PCs might interact with stellate cells carrying the same information with a flipped sign is not known.

Given evidence that in some systems gain up and gain down learning appear to proceed with different time courses and by different mechanisms (Boyden and Raymond 2003; Miles and Eighmy 1980; Robinson et al. 2003; Straube et al. 1997), it's possible that stellate cell inputs to PCs could learn at a different rate from direct parallel fiber inputs, resulting in a more nuanced

relationship between stellate cell and parallel fiber counterparts than simple extension of the spectrum of possible weights into a negative domain. The ability to visually monitor plasticity could be helpful in dissecting this relationship.

Concluding Remarks

There are any number of questions that remain about the canonical algorithm of the cerebellum. In many ways the minimal, optically accessible and genetically tractable cerebellar circuit of the larval zebrafish is ideally suited to get at some of them. However, as the rapid development of these methods has only recently pushed the larval zebrafish forward as a natural model for studying cerebellum, our relevant knowledge is still quite basic. With this dissertation I aim to outline some of our current knowledge as well as to contribute a preliminary characterization of Purkinje cell activity during optomotor behavior to the foundational knowledge that will be necessary to fully capitalize on the promise of the larval zebrafish as a model to understand cerebellar function.

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