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Dedication

I would like to dedicate this thesis to my family who has continually provided support throughout my training and in particular to my parents for fostering my inquisitive nature from an early age.

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Abstract

The cerebellum is a major motor control structure with a highly ordered circuitry. The parallel fibers (PFs) are a dominant element of the cerebellar circuitry. Parallel fibers are the bifurcated axons of the granule cells (GCs) that project across the surface of the cerebellar cortex and synapse on the major output neuron, the Purkinje cell (PC).

However, the role of PFs in the cerebellum has long been controversial and a matter of intense debate. Early studies inspired the "beam" hypothesis whereby GC activation results in PF driven, post-synaptic excitation of beams of PCs. However, the "radial" hypothesis postulates that the ascending limb of the GC axon provides the dominant input to PCs and generates patch-like PC responses. To address the beam versus patch controversy and PF function in the cerebellar cortex, this thesis used optical imaging and single PCs recordings in the mouse cerebellar cortex, both in normal mice and in a murine model of a P/Q-type Ca^{2+} channelopathy. The results provide the first demonstration of beam-like activation of PCs in the cerebellar cortex to peripheral input in normal mice. Furthermore, the pattern of PC responses depends on extracellular glutamate and its local regulation by excitatory amino acid transporters. The findings account for the contradictions of previous studies, clarifying why the responses in some regions of the cerebellar cortex are patch-like and other beam-like.

Altered GC-PF-PC synaptic transmission is hypothesized to produce cerebellar motor dysfunction. This thesis tests this hypothesis in the tottering (tg/tg) mouse that has mutation in the gene that codes for the α1A pore-forming subunit of the P/Q-type voltage
gated Ca\textsuperscript{2+} channel and is a model for human episodic ataxia type 2 (EA2). This channel is highly expressed on both GCs and PCs. Further, both EA2 patients and \textit{tg/tg} mice have cerebellar ataxia. The thesis shows that the GC-PF-PC synaptic transmission is reduced in the \textit{tg/tg} mouse and a main pharmacological therapy for EA2, 4-aminopyridine, rescues the deficits. The results strongly implicate decreased GC-PF-PC function in the baseline ataxia.

Both EA2 patients and \textit{tg/tg} mice have non-episodic neurologic dysfunction, such as the cerebellar ataxia, but also episodic dysfunction. The episodic abnormalities involve the cerebral cortex, including epilepsy, migraine headaches and cognitive dysfunction. The final component of the thesis examined whether episodic abnormalities are present in the cerebral cortex of the \textit{tg/tg} mouse. Optical imaging and single cell recording results demonstrate highly abnormal excitability changes throughout the cerebral cortex of \textit{tg/tg} mice consisting of transient low frequency oscillations (LFOs) very high power. The LFOs are mediated, at least in part, by neuronal activity. Unexpectedly, the LFOs are driven by reducing excitatory inputs to the cerebral cortex. Furthermore, the high power LFOs are decreased markedly by acetazolamide and 4-aminopyridine, the primary treatments for EA2, demonstrating disease relevance. The LFOs in the \textit{tg/tg} mouse represent an abnormal state involving decreased excitatory synaptic transmission and may underlie non-cerebellar symptoms that characterize P/Q-type Ca\textsuperscript{2+} channelopathies.
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CHAPTER I: BACKGROUND

Introduction

In this first chapter I will begin by providing a brief overview of the cellular organization and circuitry of the cerebellum. I will then provide background for the first aim of my thesis that examined the role of parallel fibers (PFs) in the cerebellar cortex. I also introduce the subject of the final two aims, the mutant tottering (tg/tg) mouse. I provide context for the investigation of how abnormalities in PF-Purkinje cell excitation might contribute to the gait ataxia exhibited by this mouse as well as for the question of whether abnormal episodic activity occurs in the cerebral cortex of the tg/tg mouse.

Cytoarchitecture and cortical circuitry of the cerebellum

The lattice cytoarchitecture of the cerebellar cortex, comprised of just seven different neuronal cell types, is beautifully deceiving, as this outwardly simple arrangement obscures the underlying complexity of the cerebellum. Detailed histological descriptions of the cerebellar cortex may be found elsewhere (Eccles et al., 1967; Palay and Chan-Palay, 1974; Mugnaini, 1972) and, therefore, only a brief overview is provided here. The cerebellar cortex is divided into three layers from superficial to deep: the molecular layer, the Purkinje cell layer and the granular layer. The molecular layer contains the PF axons of granule cells (GCs), stellate and basket cell interneurons, as well as the dendritic arbors of the principle neuron of the cerebellar cortex—the Purkinje cell
(PC). The PC layer is largely a monolayer of PC somata but also contains the interspersed somata of Bergmann and candelabrum glia (Sillitoe and Joyner, 2007). The granular layer is comprised primarily by the most numerous neuronal cell types in the brain, the GC. Golgi, Lugaro and unipolar brush cells (all inhibitory interneurons) are also found in the granular layer. The three layers of the cerebellar cortex overlay the white matter tracts that contain the afferent (climbing fiber and mossy fiber axons) and efferent (PC axons) cerebellar projections.

The cerebellar cortex receives excitatory glutamatergic input from mossy fibers and climbing fibers. The majority of afferent input to the cerebellar cortex is conveyed by the mossy fibers. Mossy fibers receive input from a combination of peripheral sensory nerves and numerous central structures. The specific inputs received by individual mossy fibers differ depending on the cerebellar cortical zone that they project to. Projections to the vestibulocerebellum arise from the vestibular nuclei while spinocerebellar pathways project to the vermis. Mossy fiber tracts conveying peripheral sensory information from the spinocerebellar tracts and from the motor cortex via the pontine nuclei project to the intermediate zone of the cerebellar cortex (Eccles et al., 1967; Llinas, 1981). The lateral zone of the cerebellar cortex receives mossy fiber projections arising from the basal pontine nuclei which provide information from nearly all cerebral cortical regions, but especially from premotor, supplementary motor areas, sensorimotor, parietal association, and visual cortices (Allen and Tsukahara, 1974; Glickstein et al., 1985).
As the sole output, PCs are the fundamental computational unit of the cerebellar cortex. Morphologically, PCs are one of the most recognizable neuronal cell types in the brain due to their massive and elaborate, planer dendritic arbors that extend into the molecular layer in perpendicular alignment to the folia and PFs. PCs are GABAergic inhibitory neurons (Ito et al., 1970) that massively converge (~25:1) on their targets in the deep cerebellar nuclei (DCN) and the vestibular nuclei (Caddy and Biscoe, 1979; Harvey and Napper, 1991; Heckroth and Abbott, 1994).

The output of the cerebellum is generated by the DCN and the vestibular nuclei. Somatotopic representations of the body are found in each of the nuclei (Allen et al., 1977; Allen et al., 1978; Asanuma et al., 1983d; Asanuma et al., 1983a; Asanuma et al., 1983b; Asanuma et al., 1983c; Thach et al., 1992) and the nuclei influence motor control over the ipsilateral musculature. Cerebellar output is conveyed to essentially all CNS motor areas including the spinal cord, the vestibular, reticular, and red nuclei, the superior colliculus, and, via the thalamus, the primary motor and premotor cortices, and the prefrontal cortex. In the quiescent state, DCN neurons maintain a consistent firing rate of 40-50 Hz (Fortier et al., 1989; Thach, 1978).

**Basic electrophysiological properties of the PC**

Neurons of the inferior olivary nucleus, located in the brainstem, project climbing fibers across the midline into the contralateral cerebellar cortex (Eccles et al., 1967). As
the climbing fibers enter the cerebellar cortex, they branch in the parasagittal plane and contact approximately 10 PCs with each PC receiving input from a single climbing fiber. A climbing fiber makes extensive synaptic connections with its target PCs (~1000s) such that climbing fiber activation strongly depolarizes the PC (ECCLES et al., 1964; Schmolesky et al., 2002). The action potential produced in the PC by the climbing fiber input is known as a complex spike which is a unique, prolonged depolarization consisting of a single action potential followed by a series of smaller spikelets (Eccles et al., 1966b; ECCLES et al., 1964; Eccles et al., 1967). The spontaneous complex spike rate in PCs is typically quite low and averages ~1 Hz (Armstrong and Rawson, 1979; Keating and Thach, 1995; Thach, 1968).

The other type of action potential exhibited by PCs is the simple spike (SS) and is characterized by the traditional action potential waveform. The average spontaneous SS rate in PCs is ~ 50 Hz but may reach a maximal rate of ~250 Hz (Eccles et al., 1966a; Thach, 1968; Thach, Jr., 1967; Bell and Grimm, 1969). PCs intrinsically fire action potentials, even in the absence of synaptic input (Raman and Bean, 1999; Hausser and Clark, 1997; Hausser et al., 2004). The intrinsic SS firing exhibited by PCs is driven by a unique resurgent Na⁺ current (Raman and Bean, 1997). Therefore, SS firing in PCs reflects the net total of the intrinsic PC activity plus the sum of their synaptic inputs.
Parasagittal organization of the cerebellum

Beyond the traditional histological description of the cerebellar cortex, anatomical and immunohistochemical examination reveals a further layer of complexity. Cerebellar patterns have been described based on molecular expression domains and by patterns of connectivity. For example, both classical anatomical techniques and gene expression studies led to the subdivision of the cerebellar cortex into transverse zones that divide the cerebellum along the anteroposterior axis (Voogd and Ruigrok, 1997; Ozol et al., 1999). The anterior zone encompasses vermician lobules I-V and the hemispheric simplex lobule in the mouse; the central zone VI-VII, Crus I and Crus II; the posterior zone VIII-IX, paramedian and cupula; and the nodular zone X, flocculus and paraflocculus (Apps and Hawkes, 2009; Larouche and Hawkes, 2006).

Parasagitally aligned stripes demarcated by the expression of molecular markers further subdivide the transverse zones. Zebrin II is the most extensively studied of these markers (Brochu et al., 1990) and, since the initial discovery, has been determined to be the glycolytic enzyme aldolase C (Ahn et al., 1994; Hawkes and Herrup, 1995). Zebrin II stripes are symmetrically distributed across the midline, highly consistent between individuals (Brochu et al., 1990; Hawkes et al., 1985; Hawkes and Leclerc, 1987; Fujita et al., 2014), and found in a variety of mammalian species (Sillitoe et al., 2005). PCs that express zebrin II (zebrin II+) comprise stripes of a few hundred to a few thousand PCs and alternate between zones in which all PCs are zebrin II+ (e.g., the central and nodular zones) and zones with alternating stripes of zebrin II+ and zebrin II negative (zebrin II-).
A number of other molecules are co-expressed with zebrin II including: zebrin I (Hawkes et al., 1985; Hawkes and Leclerc, 1987), sphingosine kinase 1a (Terada et al., 2004), phospholipase Cβ3 (Sarna et al., 2006), excitatory amino-acid transporter 4 (EAAT4) (Dehnes et al., 1998), metabotropic glutamate receptor 1α (mGlur1α) (Mateos et al., 2001), integrin β1 (Murase and Hayashi, 1996), protein kinase Cδ (Chen and Hillman, 1993; Barmack et al., 2000), and the GABA_B2 receptor (Chung et al., 2007). There are also molecular markers of zebrin II- PCs including phospholipase Cβ4 (Sarna et al., 2006), NMDA receptor (NR2C subunit) (Karavanova et al., 2007), and neuroplastin (Marzban et al., 2003). Importantly, many of these molecules are involved with synaptic transmission and intracellular signaling pathways, suggesting that excitability differs in zebrin II+ versus zebrin II- zones.

The parasagittal organization in the cerebellar cortex extends beyond the molecular phenotype of PCs. Molecular layer inhibitory interneurons (basket and stellate cells) are also organized in parasagittal zones (Szentagothai, 1965). Furthermore, the dendritic arbor of Golgi cells does not cross PC stripe boundaries (Sillitoe et al., 2008). Physiological studies have also determined that the inhibitory fields of the molecular layer interneurons are confined to zebrin II+ stripes and exert their action within this
parasagittal arrangement (Ekerot and Jorntell, 2001; Ekerot and Jorntell, 2003; Jorntell and Ekerot, 2002; Gao et al., 2006; Dizon and Khodakhah, 2011).

The climbing fiber projections from the inferior olive and the PC projections to the DCN are organized in parasagittal zones (Brodal and Kawamura, 1980; Voogd and Ruigrok, 2004; Buisseret-Delmas and Angaut, 1993; Voogd and Bigare, 1980). Mossy fibers terminate in the granule layer, exhibiting a reproducible, partially overlapping relationship with the overlying PC zebrin II+ zones (Wu et al., 1999; Ji and Hawkes, 1994; Voogd et al., 2003; Pijpers et al., 2006; Yaginuma and Matsushita, 1987; Voogd, 1969). Mapping of the mossy fibers and climbing fibers within a single parasagittal zone of PCs has demonstrated a high degree of overlap between the two inputs (Voogd et al., 2003; Pijpers et al., 2006; Garwicz et al., 1998). Physiological studies have provided further evidence for the convergence of mossy fibers and climbing fiber inputs within the same PC zone (Odeh et al., 2005). However, for both mossy fibers and climbing fibers there is not a perfect alignment between their terminals and zebrin II+ or zebrin II- PCs (Sugihara and Shinoda, 2004; Voogd et al., 2003).

The parasagittal compartmentalization extends to the cerebellar output, the DCN. DCN neurons receive inhibitory projections from PCs as well as collateral excitatory inputs from climbing fibers as they project to the cerebellar cortex (Shinoda et al., 1987; Sugihara, 2011). Tract tracing studies show that PCs located within the same zebrin II parasagittal zone, in adjacent or separate lobules, project to a restricted region
within the DCN (Sugihara et al., 2009; Voogd and Ruigrok, 2004; Pijpers et al., 2005; Apps and Garwicz, 2000). Furthermore, climbing fiber collaterals and axons from the PCs innervated by the same climbing fibers converge to the same area within the DCN (Sugihara et al., 2009). Despite the evidence that the parasagittal organization observed in the cerebellar cortex is maintained in the DCN output neurons, the functional consequences of this are not understood.

**Functional differences in parasagittal zones**

Excitability and information processing differs across parasagittal zones. For example, the proteins involved in glutamatergic neurotransmission are differentially expressed in parasagittal stripes of PCs. The mGluR1b splice variant is found on zebrin II- PCs (Mateos et al., 2000). While, as mentioned above, EAAT4 and protein kinase Cδ are expressed in zebrin II+ PCs (Chen and Hillman, 1993; Barmack et al., 2000). The parasagittal expression pattern of EAAT4 produces regional differences in the susceptibility of the PF-PC synapse to long term depression (Gao et al., 2003; Wadiche and Jahr, 2005).

Parasagittal zones also differ in the expression of long latency patches. Patches of elevated activity are evoked by high frequency PF stimulation. The patches appear along the PC beam evoked by PF stimulation at latencies of ~25 s (Wang et al., 2011), are mGluR1 dependent and are restricted to zebrin II+ PC zones. Furthermore, the patches
require intracellular Ca\textsuperscript{2+} release \textit{via} PLC\textbeta\ and ryanodine receptors (RyR). The patches also exhibit robust, mGluR1 dependent, long-term potentiation to high-frequency PF stimulation, another example of synaptic plasticity that differs among the PC zones (Wang et al., 2011; Wang et al., 2009). A possible mechanism is suggested by the observation that both mGluR1 receptors and the downstream signaling pathway in PCs are organized in parasagittal zones (Hartmann and Konnerth, 2009). As mentioned, the PLC\textbeta\3 isoform is found on zebrin II+ PCs while the PLC\textbeta\4 isoform and the mGluR1b splice variant are found on PCs in zebrin II- zones (Hartmann and Konnerth, 2009; Apps and Hawkes, 2009). The mGluR1b variant couples poorly to the PLC signaling pathway compared to the mGluR1a variant. Therefore, differences in signaling potency may underlie the expression and the unique properties of the long-latency patches (Wang et al., 2011).

Another example of functional differences in parasagittal zones is the amount of glutamate released per climbing fiber action potential. In cerebellar slice preparation the climbing fibers projecting to zebrin II+ zones release more glutamate per action potential than climbing fibers projecting to zebrin II- PC zones (Paukert et al., 2010). Enhanced glutamate release by climbing fibers in the zebrin II+ zones produces a longer-duration excitatory post-synaptic current, prolonging the complex spikes in zebrin II+ PCs. Longer duration complex spike produce greater amplitude long-term depression (Yang and Lisberger, 2014), therefore the prolonged complex spikes in zebrin II+ PCs likely
increases the susceptibility and/or the duration of plasticity at the climbing fiber-PC synapse in the zebrin II+ zones.

Finally, the PC zebrin II phenotype in the parasagittal zones influences susceptibility to cell death. PC death resulting from genetic neurodegeneration or toxicity often preferentially occurs in zebrin II+ or Zebrin II- PC zones (depending on the specific degenerative disease process or the toxin) rather than being randomly distributed across PCs in the cerebellar cortex (Sawada et al., 2009; Leclerc et al., 1992; Welsh et al., 2002; Sarna et al., 2003; Fletcher et al., 2001; Angner et al., 2000; Balaban and Severs, 1991; Balaban et al., 1988). Overall, one subpopulation of PCs does not appear to be more sensitive to genetic or toxic insult than the other (Sarna and Hawkes, 2003). In conclusion, the patterned loss of PCs may occur because the insult is localized to parasagittal zones or because all of the PCs receive the same level of insult but some PC populations are more susceptible to the particular injury.

In summary, parasagittal PC zones exhibit differences in response to PF and CF input, including in excitability and synaptic plasticity as well as susceptibility to cell death. Though the extent is unknown, these parasagittal differences add another layer of complexity to the basic computations performed by the cerebellar cortex.
The role of PFs in the cerebellar cortex

The cerebellar molecular layer is dominated by PF axons project mediolaterally across the cerebellar cortical surface (Palay and Chan-Palay, 1974; Haines et al., 1982; Voogd, 1967). The length of the PFs is species dependent, for example being 3-4 mm in the cat (Brand et al., 1976) and 1-2 mm in the mouse (Huang et al., 2006a). PFs make 100,000 - 200,000, glutamatergic synapses on PC dendrites. Given their massive numbers and striking medio-lateral geometry, PFs are widely thought to play a central role in the computations performed by the cerebellar cortex. Multiple theories have been proposed to explain the functional role of PFs in cerebellar cortical processing. One of the early theories is the “beam” hypothesis which postulates that MF activation of a cluster of GCs results in beam-like excitation of PCs (Eccles et al., 1967). Proponents of the beam hypothesis suggested that the PFs provide for spatial-temporal transformations, such that propagation of action potentials along the PFs sequentially activates PCs at precisely timed intervals (Braitenberg and Atwood, 1958; Braitenberg, 1961; Braitenberg et al., 1997). Consequently, the distance traveled by the action potentials would be coded by the timing of the PC responses. The PFs have also been hypothesized to coordinate the movements of different body parts across PC parasagittal zones (Thach et al., 1992).

In the Marr-Albus theory, the PF-PC synapse is the plastic element and posited to be central to the cerebellum’s role in motor learning (Marr, 1969; Albus, 1971; Ito, 2002; Ito, 2006; Hansel et al., 2001). Given that individual PFs synapse on 100’s of PCs and form the axonal element of the most abundant neuron type in the mammalian brain, understanding PF function is essential to understanding cerebellar cortical function.
Under the beam hypothesis, PFs serve a central role equal to their striking anatomical prominence in the cerebellar cortex. However, the beam hypothesis remains highly controversial. Simultaneous recordings of PCs separated by more than 100 μm in the medio-lateral direction have uncovered only scant evidence for temporal synchrony in their simple spike discharge (Ebner and Bloedel, 1981; Heck et al., 2007; Shin and De Schutter E., 2006; Bell and Grimm, 1969; Bosman et al., 2010; Garwicz and Andersson, 1992). Mapping of the responses to peripheral inputs reveal short-latency patches of PC activation, but little evidence for beam-like PC activation (Bower and Woolston, 1983; Santamaria et al., 2007; Shambes et al., 1978a; Cohen and Yarom, 1998; Rokni et al., 2007; Gao et al., 2006; Rokni et al., 2008).

The radial hypothesis is based on the radial organization of the PCs that receive synapses from the GC axon as it ascends to the molecular layer and synapses on PCs (Gundappa-Sulur et al., 1999; Lu et al., 2009; Napper and Harvey, 1988; Llinas, 1982). The radial hypothesis postulates that the ascending axon of GCs provides the dominant input to PCs and that PCs located laterally are only weakly, if at all, activated by the PFs (Gundappa-Sulur et al., 1999; Rokni et al., 2007; Rokni et al., 2008; Santamaria et al., 2002; Santamaria and Bower, 2005; Llinas, 1982). The synaptic connections made along the ascending limb have been argued to be stronger, have a higher number of connections on a PC, and result in more synchronous activation of PCs than that provided by PF synapses (Llinas, 1982; Sims and Hartell, 2006; Sims and Hartell, 2005; Isope and Barbour, 2002; Rokni et al., 2008). Modeling studies found almost no role for the PFs in
modulating PC simple spike discharge, suggesting instead that PFs provide a level of background tone (Santamaria et al., 2002; Santamaria et al., 2007; Santamaria and Bower, 2005).

An alternative hypothesis holds that the two segments of the GC axon serve different functional roles with the ascending limb providing an innate PC input while the PF limb provides a plastic or acquired PC input (Rokni et al., 2008). This view is based on the observation that the synapses between the granule cell ascending axon and PCs do not appear to exhibit plasticity (Sims and Hartell, 2006). In contrast, plasticity at the PF-PC synapse is a well established phenomenon (Jörntell and Ekerot, 2002; Wang et al., 2011; Gao et al., 2003; Ito and Kano, 1982; Lev-Ram et al., 2002; Salin et al., 1996).

However, despite there being more experimental support for the radial hypothesis than the beam hypothesis, major concerns also exist with the radial hypothesis. Ascending limb synapses provide only 3 - 6% of the total synapses on a PC (Napper and Harvey, 1988; Lu et al., 2009; Huang et al., 2006b), and not the 20% claimed in one study (Gundappa-Sulur et al., 1999). In the cerebellar slice, the strength of the synapses between the ascending axon and PCs and between PFs and PCs are equivalent and PFs activate PCs for at least several hundred μm along the PFs (Isope and Barbour, 2002; Walter and Khodakhah, 2009).
A variant of the radial hypothesis proposes that GABAergic inhibition generated by cerebellar interneurons prevents beam-like activation by PFs (Bower, 2002; Santamaria et al., 2007; Santamaria et al., 2002). Experimental support remains equivocal (Rokni et al., 2007; Santamaria et al., 2007). However, the response to PF activation can be highly modulated by local conditions, including locally elevated extracellular glutamate transients resulting from climbing fiber discharge (Dzubay and Jahr, 1999), PF-PC synaptic spillover (Carter and Regehr, 2000) and retrograde release of glutamate from PCs (Levenes et al., 2001; Crepel, 2007; Shin et al., 2008; Duguid et al., 2007). Increased glutamate both facilitates PF-PC synaptic transmission by temporary suppression of stellate cell inhibition (Carter and Regehr, 2000) as well as inhibits PF-PC synaptic transmission, (Levenes et al., 2001; Crepel, 2007) leading to the hypothesis that the response patterns in the cerebellar cortex are controlled at least in part, by excitatory amino acid transporters (EAATs).

EAATs provide the primary means for glutamate re-uptake following excitatory transmission mediated glutamate release. Therefore, the EAATs control the amplitude and duration of excitatory post-synaptic currents (Takahashi et al., 1995; Takayasu et al., 2004), limit receptor desensitization, as well as ensure a high signal-to-noise ratio for glutamatergic synaptic transmission (Barbour et al., 1994; Marcaggi and Attwell, 2005). In the cerebellar cortex, four distinct EAAT4 isoforms (EAAT1-4) are expressed in the molecular layer (Takayasu et al., 2009). EAAT1 and EAAT2 are expressed on the surface membranes of Bergmann glial processes that surround the peri-synaptic spaces on
dendritic spines of PCs (Chaudhry et al., 1995; Lehre and Danbolt, 1998). At the PF-PC synapse, EAAT1 and EAAT2 re-uptake ~80% of synaptic glutamate while the remaining ~20% is taken up by the peri-synaptically localized, PC specific transporter EAAT4 (Dehnes et al., 1998; Tanaka et al., 1997; Otis et al., 2004). Despite not being the primary transporter responsible for glutamate uptake at the PF-PC synapse, EAAT4 is notable due to its non-uniform expression by PCs across the cerebellar cortex (as mentioned above) (Dehnes et al., 1998; Gincel et al., 2007) which influences parasagittal zone PC excitability.

Given that the PFs are a striking element of the cerebellum and that their functional significance to the cerebellar circuitry has been controversial, the first aim of this thesis investigated the role of PF in PC activation. Specifically, using optical imaging to re-evaluate the beam versus radial hypothesis and map the spatial patterns of cerebellar activation \textit{in vivo}. In addition, the aim tested the hypothesis that molecular layer inhibition modifies the granule cell-PF-PC response and investigated the role of parasagittal zonal differences in cerebellar activation to GC-PF input.

\textbf{Episodic ataxia type 2 and the mutant \textit{tottering} mouse}

The hallmark of episodic neurological dysfunction, including channelopathies, is that neurons and neural circuits transiently enter an abnormal excitability state resulting in an acute and self-limiting attack (Ryan and Ptacek, 2010; Kullmann, 2010). One
family of the Ca$^{2+}$ channelopathies is caused by autosomal dominant mutations of the CACNA1A gene that encodes the $\alpha_{1A}$, pore-forming subunit of the human Ca$^{2+}$,2.1 (P/Q-type) voltage gated Ca$^{2+}$ channel (Ophoff et al., 1996;Kramer et al., 1995). Clinical manifestations of CACNA1A mutations include episodic ataxia type 2 (EA2), familial hemiplegic migraine type 1 (FHM1), spinocerebellar ataxia type 6, or a combination of the three (Jen et al., 2004;Jen et al., 2007;Baloh et al., 1997;Rajakulendran et al., 2010). The spectrum of symptoms and severity of disease depends on the specific mutation.

Over 80 mutations of the CACNA1A that give rise to EA2 have been identified (Rajakulendran et al., 2012). Mutations that result in EA2 are predicted or have been shown to cause a loss or decrease of P/Q-type Ca$^{2+}$ channel function (Wappl et al., 2002;Spacey et al., 2004;Jen et al., 2004;Pietrobon, 2010). EA2 is characterized by paroxysmal attacks of cerebellar dysfunction including limb and gait ataxia, and nystagmus: episodes persist for hours to days. EA2 patients may also display paroxysmal dystonic symptoms (Spacey, 1993). Between episodes, patients exhibit progressive or (less commonly) static cerebellar dysfunction, including ataxia, and regional cerebellar atrophy (Baloh et al., 1997;Jen et al., 2007;Denier et al., 1999).

The tottering (tg/tg) mouse contains a recessive missense mutation in Cacna1a, ortholog of the human gene CACNA1A, resulting in a substitution of leucine for proline in the pore-forming region of the P/Q-type Ca$^{2+}$ channel (Fletcher et al., 1996). In the tg/tg mouse there is a 30-40% reduction in P/Q-type Ca$^{2+}$ current in PCs with little change in the kinetics of the channel (Wakamori et al., 1998). The phenotype of the tg/tg
mouse is characterized by paroxysmal dystonia, mild ataxia, and absence seizures, between dystonic episodes. The \( \text{tg/tg} \) mouse is the most widely used model of the human \( CACNA1A \) disorder EA2.

The P/Q-type Ca\(^{2+} \) channels are found in presynaptic terminals, soma, and dendrites of neurons (Mintz et al., 1992; Fletcher et al., 1996; Westenbroek et al., 1995). Importantly, P/Q-type Ca\(^{2+} \) channels are heavily expressed in the cerebellum, particularly in PCs and GCs (Mintz et al., 1992; Fletcher et al., 1996; Westenbroek et al., 1995). Due to efficient coupling with protein mediators of exocytosis, P/Q-type Ca\(^{2+} \) channels play a central role in action potential dependent neurotransmitter release (Pietrobon, 2010; Catterall, 1998). P/Q-type Ca\(^{2+} \) channels exhibit a great deal of functional variation that results from a multitude of alternative splice forms (Wykes et al., 2007; Kanumilli et al., 2006; Chaudhuri et al., 2004; Soong et al., 2002; Sandoz et al., 2001; Bourinet et al., 1999). Further variation of the P/Q-type Ca\(^{2+} \) channel results from a combination with various auxiliary subunits (Sandoz et al., 2001; De et al., 1994; Bichet et al., 2000; Hobom et al., 2000; Walker and De, 1998). These factors produce the wide spectrum of properties exhibited by the P/Q-type Ca\(^{2+} \) channels expressed throughout the brain and spinal cord (Mermelstein et al., 1999; Luvisetto et al., 2004). Despite being expressed by neurons throughout the CNS, P/Q-type Ca\(^{2+} \) channels are not functionally expressed by glia (Latour et al., 2003; D'Ascenzo et al., 2004).
In the cerebellum of the adult animal, P/Q-type Ca\textsuperscript{2+} channels are instrumental in synaptic transmission at most synapses including the excitatory, glutamatergic PF-PC and climbing fiber-PC synapses (Mintz et al., 1995) as well as inhibitory GABAergic synapses between PCs and their targets in the DCN (Iwasaki et al., 2000; Forti et al., 2000; Stephens et al., 2001). Consequently, any alteration in P/Q-type Ca\textsuperscript{2+} channel function could fundamentally alter synaptic transmission throughout the cerebellum and altered synaptic release has been hypothesized to underlie cerebellar dysfunction in the \textit{tg/tg} mouse (Matsushita et al., 2002; Zhou et al., 2003). However, the deficits in synaptic transmission in the cerebellar cortex of \textit{tg/tg} mice are not as pronounced as might be expected. There are no obvious deficits in climbing fiber-PC or PC-DCN synaptic transmission (Matsushita et al., 2002; Hoebeek et al., 2008). While some studies have described a reduction in PF-PC synaptic transmission (Matsushita et al., 2002; Chen et al., 2009), another investigation found PF-PC synaptic transmission intact (Zhou et al., 2003). Paired-pulse facilitation, a common functional measure of pre-synaptic release, is intact suggesting that PF-PC synaptic release is normal (Matsushita et al., 2002). An increase in N-type Ca\textsuperscript{2+} channels on the PFs partially compensates for the reduction in P/Q-type Ca\textsuperscript{2+} channel function (Zhou et al., 2003). At the hippocampus and neuromuscular junction, the loss of P/Q-type Ca\textsuperscript{2+} channels is completely compensated for by increase in N-type or R-type Ca\textsuperscript{2+} channels on the presynaptic terminals (Caddick et al., 1999; Qian and Noebels, 2000; Kaja et al., 2007b). Together these findings suggest that changes in pre-synaptic release are not the sole cause of dysfunction.
In addition to serving a central role in synaptic transmission throughout the CNS, P/Q-type Ca\textsuperscript{2+} channels account for approximately 70-80% of the total Ca\textsuperscript{2+} current in PCs (Usowicz et al., 1992; Mintz et al., 1992) and are fundamental to the spontaneous, intrinsic firing of PCs. The distribution of P/Q-type Ca\textsuperscript{2+} channels on PCs exhibits a spatial gradient of expression from relatively low levels at the PC soma to ~2.5 fold higher levels at the apical dendrites (Indriati et al., 2013). Within the plasma membrane, P/Q-type Ca\textsuperscript{2+} channels are positioned in close proximity to Ca\textsuperscript{2+} activated K\textsuperscript{+} channels (K\textsubscript{Ca}) (Indriati et al., 2013). The close association of P/Q-type Ca\textsuperscript{2+} channels with K\textsubscript{Ca} channels (both the SK and BK varieties) produces a functional coupling (Womack and Khodakhah, 2002; Womack and Khodakhah, 2003; Womack et al., 2009; Indriati et al., 2013). A reduction in P/Q-type Ca\textsuperscript{2+} current, as occurs in the \textit{tg/tg} mutation, increases the firing rate and irregularity of PC simple spike discharge. Similarly, blockade of P/Q-type Ca\textsuperscript{2+} channels in WT mice also results in irregular PC simple spike firing (Hoebeek et al., 2005; Walter et al., 2006).

In the \textit{tg/tg} mouse, abnormal Ca\textsuperscript{2+} dynamics extends beyond reduced P/Q-type Ca\textsuperscript{2+} channel function. For example, in the cerebellum there is increased expression of L-type Ca\textsuperscript{2+} channels in PCs but not in GCs or DCN neurons (Campbell and Hess, 1999; Erickson et al., 2007). Other CNS areas, except the nucleus of the brachium of the inferior colliculus, do not exhibit significant expression increases in L-type Ca\textsuperscript{2+} channels in the \textit{tg/tg} mouse.
The morphological and immunohistochemical changes in the \textit{tg/tg} mouse are subtle. The gross weight of the \textit{tg/tg} mouse brain is approximately the same as that of WT mice (Noebels and Sidman, 1979; Syapin, 1982). Many of the \textit{tg/tg} mouse CNS abnormalities are found in the cerebellum. For example, the weight of the cerebellum is reduced (Syapin, 1982) as well as the cerebellar volume, the thickness of the molecular layer, and the dimensions of the PC soma (Isaacs and Abbott, 1995). Loss of PCs does not appear to occur before about 1 year of age (Levitt, 1988; Isaacs and Abbott, 1995; Sawada et al., 2009). However, though prior to overt cell death, PCs exhibit abnormal axonal swellings (Rhyu et al., 1999), irregular microtubules, and an increase in cytoplasmic organelles (Meier and MacPike, 1971). Furthermore, \textit{tg/tg} mouse PFs contain enlarged varicosities and form multiple synapses on single PC dendritic spines (Rhyu et al., 1999). Examination of \textit{tg/tg} mouse PC terminals at their synapse on DCN neurons shows vacuolar enlargement of the axons and an increased number of mitochondria but no difference in density of axon-terminals or in the number of synapses (Hoebeek et al., 2008).

Noradrenergic projections from the locus coeruleus are increased to all brain regions (Levitt and Noebels, 1981) as are norepinephrine levels (Levitt and Noebels, 1981; Levitt et al., 1987) as well as increased tyrosine hydroxylase (TH) staining in the cerebellum (Hess and Wilson, 1991; Fletcher et al., 1996; Austin et al., 1992). The increased TH expression is spatially restricted to the soma and dendritic arbor of PCs but is distributed throughout the cerebellum. This TH expression profile contrasts with that of normal mice in which the expression in PCs is transient, with a small fraction of PCs
retaining TH expression into adulthood. Interestingly, the increased TH expression in the \(tg/tg\) mouse occurs in a similar parasagittal banding pattern as zebrin II, though zebrin II staining is unchanged (Hawkes and Herrup, 1995; Fletcher et al., 1996; Abbott et al., 1996). Both the mRNA and protein levels of neuronal nitric oxide synthase (nNOS) are elevated in the immature and adult \(tg/tg\) mouse cerebellum (Rhyu et al., 2003; Frank-Cannon et al., 2007).

\(\text{GABA}_A\) receptor changes are notable in the adult \(tg/tg\) mouse. There is a decrease in \(\text{GABA}_A\) receptor function in the cerebral cortex and changes in subunit composition and agonist binding properties that may contribute to the absence epilepsy (Tehrani and Barnes, Jr., 1990; Tehrani and Barnes, Jr., 1995). There is a reduction in the number of \(\text{GABA}_A\) receptors in the cerebellar granular layer largely due to a reduction in the expression of \(\alpha_6\) and \(\gamma_2\) subunits (Kaja et al., 2007a). Although the \(tg/tg\) mouse does exhibit some histological and histochemical abnormalities, they are not remarkable enough to explain the totality of the \(tg/tg\) mutant phenotype.

\textit{Mutant tottering mouse model of cerebellar ataxia}

Ataxia is a term applied to the uncoordinated movements resulting from damage of sensory systems or the cerebellum (Diener and Dichgans, 1992; Morton and Bastian, 2004; Bastian, 1997). In humans, cerebellar dysfunction may manifest via a multitude of signs and symptoms including limb and gait ataxia, nystagmus, oscillopsia, cognitive
impairment, locomotor dyscoordination, dysarthria, postural instability, and decreased reaction speed (Massaquoi, 2012; Marsden and Harris, 2011; Holmes, 1917; Holmes, 1939; Dow and Moruzzi, 1958). In contrast, the range of motor abnormalities that may manifest as a result of cerebellar dysfunction in the mouse is somewhat more limited. Therefore, in mouse models of cerebellar disease that exhibit impaired motor control, the term ataxia is used to primarily describe the motor abnormalities that manifest as an uncoordinated, wobbly gait (Ribar et al., 2000; Armbrust et al., 2014; Todorov et al., 2012; Glynn et al., 2005).

The cerebellar ataxia exhibited by the \(tg/tg\) mouse is relatively mild, as the impairment primarily involves the hindlimbs and tail. The animals also display uncoordinated head movements, have difficulty swimming, and cannot turn as well as WT mice (Green and Sidman, 1962; Syapin, 1982). In addition, \(tg/tg\) mice have deficient balance and do not perform well on the rotarod test (Syapin, 1982; Walter et al., 2006; Alvina and Khodakhah, 2010b; Alvina and Khodakhah, 2010a) and exhibit decreased walking velocities (Scholle et al., 2010). The \(tg/tg\) mouse also has eye movement deficits including basal upward deviation of the eyes, reduced gain of both the vestibulo-ocular and optokinetic reflex as well as reduced vestibulo-ocular reflex plasticity (Hoebeek et al., 2005; Stahl et al., 2006; Stahl and Thumser, 2013).

One proposed hypothesis attributes the ataxia of the \(tg/tg\) mouse to increased variability in the simple spike firing of PCs, termed “loss of the precision of pacemaking”
(Walter et al., 2006; Alvina and Khodakhah, 2010b; Alvina and Khodakhah, 2010a). Loss of pacemaking is also hypothesized to underlie impaired sensorimotor processing in the flocculus of the \textit{tg/tg} mouse, leading to eye movement deficits (Hoebeek et al., 2005). While it is established that \textit{tg/tg} PCs exhibit increased variability in simple spike firing relative to WT mice, the role of the increased irregularity in the abnormal motor behavior is controversial (Glasauer et al., 2011; Stahl and Thumser, 2013). Also, the pacemaking hypothesis is primarily based on intrinsic simple spike firing (i.e., with synaptic inputs blocked) measured \textit{in vitro} (Walter et al., 2006; Alvina and Khodakhah, 2010b; Alvina and Khodakhah, 2010a). However, \textit{in vivo}, the simple spike firing of PCs is known for extraordinary variability (Thach, 1968; Chadderton et al., 2004; Ebner and Bloedel, 1981; Yartsev et al., 2009).

An alternative hypothesis proposes that impaired synaptic transmission in the cerebellar cortex may underlie the ataxia in the \textit{tg/tg} mouse. P/Q-type Ca$^{2+}$ channels play an essential role in synaptic transmitter release at the PF-PC and climbing fiber-PC synapses (Mintz et al., 1995). PF-PC synaptic transmission is reduced in the \textit{tg/tg} mouse (Chen et al., 2009; Matsushita et al., 2002). Furthermore, in \textit{tg/tg} mice synaptic transmission is altered in the cerebral cortex (Ayata et al., 2000; Tehrani et al., 1997; Sasaki et al., 2006) and at the neuromuscular junction (Plomp et al., 2000). Therefore, decreased PC responsiveness to PF input has been hypothesized to underlie cerebellar dysfunction in the \textit{tg/tg} mouse, and possibly in EA2 (Matsushita et al., 2002).
To gain a better understanding of the mechanisms underlying the baseline ataxia, the second aim of my thesis examines components of the cerebellar cortical circuitry in the mutant tg/tg mouse. Using in vivo flavoprotein and Ca$^{2+}$ optical imaging as well as single PC extracellular recordings, I test the hypothesis that deficits are present in the GC-PF-PC circuit in the tg/tg mouse that are proposed to contribute to the baseline ataxia in this reduction-of-function model of EA2.

**Episodic neurological disorders and low frequency oscillations in the tottering mouse**

As mentioned above, the tg/tg mouse is the most widely used animal model of EA2. The episodic components of the tg/tg mouse phenotype include dystonia and absence seizures. The absence seizures are behaviorally characterized by abrupt movement arrest, twitching whiskers, and fixed stare. Electroencephalographic recordings (EEG) show bilateral, synchronous 6-7 per second cortical spike and wave polyspike discharges that occur simultaneously with the cessation of movement (Kaplan et al., 1979; Syapin, 1982; Noebels and Sidman, 1979; Heller et al., 1983). The tg/tg mouse exhibits frequent absence seizures, with ictal episodes occurring \( \geq 30 \) times per hour with each episode lasting 0.3-10 seconds (Noebels and Sidman, 1979; Kostopoulos et al., 1987).

The other episodic trait exhibited by the tg/tg mouse is generalized dystonia which characteristically begins in the animal’s posterior, with the first manifestation
being extension of the hindlimbs. As the attack evolves, the trunk musculature becomes involved, leading to an arched back. At the peak of the attack, the animal loses the ability to ambulate as the majority of its agonist and antagonist muscle groups undergo rhythmic contractions, including the facial muscles. A notable feature of the episodic dystonia is the progressive and stereotypic manner in which attacks evolve. As the dystonic period subsides, the rhythmic muscle contractions cease in the same order as the attack began. Attacks occur spontaneously 1-2 times per day and during the 30-60 min episodes the animal remains conscious (Green and Sidman, 1962; Campbell and Hess, 1998). As in EA2 patients (Jen et al., 2007; Baloh et al., 1997), physiological or psychological stress (e.g., restraint or entry into a novel environment), caffeine, and ethanol trigger the motor attacks in the tg/tg mouse (Fureman et al., 2002; Campbell and Hess, 1998).

The episodic motor attacks are a distinct phenotypic element from the absence epilepsy exhibited by the tg/tg mouse. EEG recordings during episodes of dystonia do not show any correlated epileptiform activity, though the characteristic absence seizure activity independently occurs during attacks (Kaplan et al., 1979). Furthermore, the absence seizures are ameliorated by antiepileptic drugs while the dystonic episodes are not (Noebels and Sidman, 1979; Kaplan et al., 1979; Fureman et al., 2002). Finally, lesioning the locus coeruleus reduces absence seizures but not the episodic dystonia (Noebels, 1984; Campbell et al., 1999).
Previous studies have well established that the cerebellum and PCs are necessary for the expression of the motor attacks. For example, dystonic attacks result in immediate early gene expression (c-fos) in the cerebellum, including PCs, GCs, the inferior olivary nucleus, and the DCN (Campbell et al., 1999; Raike et al., 2012; Raike et al., 2013). Expression of the \(tg/tg\) mutation on a PC degeneration mouse line background demonstrates that loss of PCs coincides with the loss of attacks (Campbell et al., 1999). Furthermore, lesions of the anterior cerebellum reduce the duration and frequency of dystonic episodes (Abbott et al., 1996), selective PC loss (Raike et al., 2012), and cerebellar ablation causes complete attack cessation (Neychev et al., 2008). As described above, the \(tg/tg\) mouse is largely free of gross CNS abnormalities, implying that the deficits are due to the abnormal functioning of neurons, synapses, and/or circuits.

To explain the dramatic motor phenotype exhibited by the \(tg/tg\) mouse one would expect highly aberrant activity in motor areas throughout the CNS, including the cerebellum. The Ebner laboratory has described abnormal low frequency oscillations (LFOs, 0.04-2 Hz) in the cerebellar cortex of the \(tg/tg\) mouse using flavoprotein optical imaging \textit{in vivo} (Chen et al., 2009). The oscillations are transient, propagate and are evoked by caffeine. The LFOs spontaneously increase in amplitude in a region, spread to neighboring regions and then subside over a period of 30-120 min. The vast majority of PCs as well as other cerebellar cortical neurons exhibit oscillatory firing patterns at the same low frequencies. Cerebellar and muscle activity become highly coherent during a motor attack. The LFOs appear to be intrinsically generated in the cerebellar cortex as
blocking glutamatergic inputs does not affect the oscillations (Chen et al., 2009). Removal of Ca\(^{2+}\) from the bathing solution significantly reduces both the power and area of the LFOs in the cerebellar cortex. Similarly, blocking L-type Ca\(^{2+}\) channels reduces the oscillations while L-type Ca\(^{2+}\) channel agonists facilitate them, demonstrating the central role L-type Ca\(^{2+}\) channels play in generating the oscillations. Furthermore, PF stimulation fails to evoke or modify the LFOs. In awake \(tg/tg\) mice, caffeine triggers attacks and accentuates the LFOs. This led to the hypothesis that the LFOs transiently disrupt cerebellar cortical function and provide the neural substrate for episodic dystonia (Chen et al., 2009).

Dysfunctional PC firing likely causes the LFOs that, in turn, drive the episodic dystonia in the \(tg/tg\) mouse. Evidence directly implicating PCs in the LFOs and the episodic dystonia is that L-type Ca\(^{2+}\) channels in the cerebellum are selectively upregulated in \(tg/tg\) PCs (Campbell and Hess, 1998). L-type Ca\(^{2+}\) channel agonists evoke the LFOs (Chen et al., 2009) and antagonists prevent the dystonia (Campbell and Hess, 1999). In PCs, Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels initiates the release of intracellular Ca\(^{2+}\) stores via ryanadine receptor (RyR) activation and/or inositol-triphasphate receptor activation, which in turn initiates oscillations in the PC membrane potential (Kano et al., 1995). Furthermore, RyR antagonists significantly reduce the frequency of attacks (Raike et al., 2013). Finally, in immature PCs, L-type Ca\(^{2+}\) channels are the dominant Ca\(^{2+}\) channel type and PCs exhibit LFOs similar to those in \(tg/tg\) mice (Liljelund et al., 2000). Therefore, the changes in the ratio of P/Q-type to L-type Ca\(^{2+}\) channels likely
push the PCs toward an aberrant excitability state represented by the LFOs. Triggers initiate the oscillations in PCs and disrupt the normal processing of information in the cerebellar cortex. The LFOs propagate to neighboring regions in the cerebellum and transmit abnormal signals downstream that drive the dystonic movements.

The LFO hypothesis is consistent with a number of studies investigating the role of cerebellar dysfunction in dystonia. In addition to the \( \text{tg/tg} \) mouse, cerebellar dysfunction in other spontaneous mutant mice and rats is known to produce dystonic syndromes (Neychev et al., 2011). Previous work has demonstrated hyper-excitation of the cerebellar cortex via glutamatergic agonists (Pizoli et al., 2002; Fan et al., 2012) or direct electrical stimulation (Raike et al., 2012) is sufficient to produce dystonia. Building upon this previous work, while utilizing the high degree of cell type specific stimulation afforded by optogenetics, recent studies have further demonstrated that the synchronous modulation of large populations of PCs is sufficient to cause muscle contractions and movement (Witter et al., 2013; Heiney et al., 2014).

**Triggers of episodic neurological dysfunction in EA2 and the tottering mouse**

Attacks in EA2 patients and in \( \text{tg/tg} \) mice are initiated by shared triggers. In \( \text{tg/tg} \) mice, PCs are necessary for the generation of attacks (Campbell et al., 1999; Erickson et al., 2007), and depend on L-type \( \text{Ca}^{2+} \) channels (Campbell and Hess, 1999) to compensate for the decreased P/Q-type \( \text{Ca}^{2+} \) current. The departure from the normal PC
Ca\textsuperscript{2+} channel expression profile suggests that intracellular Ca\textsuperscript{2+} homeostasis in PCs is fundamental to the attack mechanism. Xanthines, caffeine and theophylline, evoke dystonic attacks in \textit{tg/tg} mice (Fureman et al., 2002). Caffeine causes the release of intracellular Ca\textsuperscript{2+} \textit{via} RyR activation in PCs but not the PF axons of cerebellar GCs (Kano et al., 1995; Carter et al., 2002). The final common pathway of attack triggers is the activation of RyRs, causing mobilization of internal Ca\textsuperscript{2+} stores from the endoplasmic reticulum (Fureman et al., 2002; Fureman and Hess, 2005; Chen et al., 2009; Raike et al., 2013).

Physiological and psychological stressors are powerful attack triggers. Introduction to novel environments and physical restraint reliably evoke dystonic attacks in \textit{tg/tg} mice (Kaplan et al., 1979; Syapin, 1983; Campbell and Hess, 1998). Noradrenergic neurotransmission and the locus coeruleus are widely implicated in mediating the stress response (Sved et al., 2002; Berridge, 2008). Neurons in the locus coeruleus increase firing in response to stress (Abercrombie and Jacobs, 1987). Noradrenergic projections throughout the CNS, including the cerebellar cortex, originate in the locus coeruleus (Olson and Fuxe, 1971; Pickel et al., 1974). Direct electrical stimulation of the locus coeruleus produces prolonged inhibition of PC firing (Hoffer et al., 1973; Woodward et al., 1979). The effects of noradrenergic signaling in the cerebellar cortex are difficult to dissect because norepinephrine produces both excitatory and inhibitory responses in neurons depending on the specific adrenergic receptor and cell type involved (Kondo and Marty, 1997; Saitow et al., 2000; Hirono and Obata, 2006). The
α-adrenergic receptors are expressed by cerebellar interneurons and Bergmann glia (Kulik et al., 1999; Hirono and Obata, 2006). The frequency of dystonic attacks is reduced by blocking α-adrenergic receptors but the severity and duration of attacks is not altered (Fureman and Hess, 2005). In contrast, pharmacological manipulation of β-adrenergic receptors, though found on PCs and molecular layer inhibitory interneurons, has no apparent effect on the attacks (Kondo and Marty, 1997; Hirono and Obata, 2006). Lesions of the locus coeruleus, the source of adrenergic input to the cerebellar cortex, do not affect the episodic dystonia (Noebels, 1984; Campbell et al., 1999), implying that noradrenergic hyperinnervation does not underlie stress mediated triggering of episodic dystonia. Given the complex interplay of molecular signaling pathways and CNS circuitry, it is unsurprising that the mechanism(s) of psychological and physiological attack triggers remain to be determined.

Episodic neurological dysfunction beyond the cerebellum

In both EA2 patients and tg/tg mice the primary focus has been on the cerebellar manifestations. However, P/Q-type Ca\(^{2+}\) channels are found in presynaptic terminals, soma, and dendrites of neurons throughout the CNS (Mintz et al., 1992; Fletcher et al., 1996; Westenbroek et al., 1995). Consequently, alteration in P/Q-type Ca\(^{2+}\) channel function is likely to produce abnormal neuronal activity throughout the CNS including the cerebral cortex. As described above, EA2 patients exhibit non-cerebellar symptoms including migraine, epilepsy, episodic weakness, and cognitive impairment (Jen et al., 2001; Jouveneau et al., 2001; Van and Szliwowski, 1996; Baloh et al.,
EA2 patients also have abnormal EEG findings (Balogh, 2012) and elevated cerebral cortical excitability (Helmich et al., 2010). These symptoms and findings all suggest abnormalities in cerebral cortical function.

Similarly, the $tg/tg$ mouse manifests cerebral cortical dysfunction including absence seizures with rhythmic polyspike EEG bursts (Noebels and Sidman, 1979; Kostopoulos et al., 1987). Other cerebral cortical abnormalities include reduced glutamate and GABA release (Ayata et al., 2000), impaired GABA$_A$ receptor mediated inhibition (Tehrani and Barnes, Jr., 1995; Tehrani et al., 1997), decreased muscarinic acetylcholine receptor density (Liles et al., 1986), and increased noradrenergic projections (2-3 fold) from the locus ceruleus (Levitt and Noebels, 1981). The basal cAMP levels are elevated in the cerebral cortex of the $tg/tg$ mouse (Tehrani and Barnes, Jr., 1995).

Finally, evidence suggests that CNS structures outside of the cerebellum contribute to attacks of episodic dystonia. First, the attacks evolve in a highly reproducible and stereotypical progression reminiscent of a “Jacksonian march” (Jackson, 1870; Charcot J.M. and Pitres A., 1877), implying the systematic spread of abnormal neural activity across a region of the brain with a highly organized somatotopy, such as the motor cortex. The cerebellum is not known to contain a similar somatotopic organization (Shambes et al., 1978b; Shambes et al., 1978a; Kassel et al., 1984; Bower and Woolston, 1983; Rowland and Jaeger, 2005; Allen et al., 1977; Allen et al., 1978). Second,
a Ca\textit{cna1a} mouse line that expresses one mutant P/Q-type Ca\textsuperscript{2+} channel \textit{tg} allele and one null allele throughout the CNS (Tottering\textsuperscript{haplo} mice) exhibits heightened sensitivity to attack triggers compared to the Tottering\textsuperscript{PC-haplo} mouse that expresses one \textit{tg} allele and one null allele in PCs but is otherwise heterozygous at the P/Q-type Ca\textsuperscript{2+} channel gene locus (Raike et al., 2013). The difference in trigger sensitivity between these two mouse lines suggests that structures outside the cerebellum contribute to the motor attack.

Previously, we described episodic, LFOs in the cerebellar cortex of \textit{tg/tg} mice that are coupled to the dystonic attacks (Chen et al., 2009). Based on the evidence described above, the third aim of my thesis tests whether similar LFOs occur in the cerebral cortex of the \textit{tg/tg} mouse.
CHAPTER II: RE-EVALUATION OF THE BEAM AND RADIAL HYPOTHESIS OF PARALLEL FIBER ACTION IN THE CEREBELLAR CORTEX

Introduction

Parallel fibers make 100,000 - 200,000, excitatory glutamatergic synapses on the dendritic trees of PCs (Eccles et al., 1967). In spite of their massive numbers and striking medio-lateral geometry, there are two markedly different views of the role of PFs in cerebellar cortical function. The classic “beam” hypothesis postulates that mossy fiber (MF) activation of GCs results in beam-like excitation of PCs (Braitenberg and Atwood, 1958; Braitenberg, 1961). In support of this hypothesis, MF input evokes field potentials in the molecular layer well outside of the MF termination area (Garwicz and Andersson, 1992), whisker stimulation co-modulates transversely located PCs (Bosman et al., 2010), and the presence of synchrony in the simple spike firing during natural inputs (De Zeeuw et al., 1997). However, the beam hypothesis is controversial as others reported that peripheral or MF inputs evoke only patches of PC or molecular layer activity (Bower and Woolston, 1983; Santamaria et al., 2007; Cohen and Yarom, 1998; Rokni et al., 2007). Others found little temporal synchrony in the simple spike discharge of PCs separated transversely by more than 100 μm (Ebner and Bloedel, 1981; Heck et al., 2007; Shin and De Schutter E., 2006; Bell and Grimm, 1969).

The alternative hypothesis is based on the radial organization of GC axons as they ascend to the molecular layer and make synaptic contacts on PCs (Gundappa-Sulur et al.,...
This “radial” hypothesis postulates that GC ascending axons provide the dominant input to PCs and that PCs located laterally are very weakly activated by PFs. Compared to PF synapses, the synaptic connections made along the ascending limb are argued to be stronger, have a higher number of connections on a PC, and result in more synchronous activation of PCs (Llinas, 1982; Sims and Hartell, 2005; Isope and Barbour, 2002). Modeling studies found little role for PFs in modulating PC simple spike discharge, suggesting instead PFs provide background tone (Santamaria et al., 2007; Santamaria and Bower, 2005).

There are major concerns with the radial hypothesis. The ascending limb provides only 3 - 6% of the total synapses on a PC (Napper and Harvey, 1988; Lu et al., 2009; Huang et al., 2006b). In the cerebellar slice, synaptic strength between ascending GC axons and PCs, and between PFs and PCs is equivalent, and PFs activate PCs for several hundred μms (Isope and Barbour, 2002; Walter and Khodakhah, 2009). A variant of the radial hypothesis proposes that GABAergic, molecular layer inhibition prevents beam-like activation by PFs (Santamaria et al., 2007; Santamaria et al., 2002).

The present study re-evaluated the beam versus radial hypothesis, using optical imaging to map spatial patterns of activation in vivo. The results show beam-like activation in response to MF, GC, and peripheral stimulation. Furthermore, GABAergic inhibition does not prevent beam-like responses. Instead, the non-uniform distribution of
EAAT4 on PCs (Nagao et al., 1997; Dehnes et al., 1998) and regulation of extracellular glutamate concentration may contribute to the pattern of PC activation.

**Methods**

*Animal preparation*

Both FVB (Charles River Laboratories, Wilmington MA) and EAAT4 reporter mice on a C57BL/6 background were used for experiments in this study (Gincel et al., 2007). For both lines, mice of either sex were used. All animal experimentation was approved by and conducted in conformity with the Institutional Animal Care and Use Committee of the University of Minnesota. Detailed descriptions of the preparation of animals for optical imaging have been described in previous publications (Gao et al., 2006; Reinert et al., 2004) and are only described briefly. Adult mice ages 3-8 months were anesthetized by an initial intramuscular injection of 2 mg/kg acepromazine followed by an intraperitoneal injection of 2 mg/kg urethane and supplemented with 1.5 mg/kg urethane as needed. Animals were placed in a stereotaxic frame, mechanically ventilated and their body temperature feedback regulated. Depth of anesthesia was monitored via electrocardiogram and testing for responses to somatosensory stimuli. A craniotomy exposed Crus I and II and then a watertight acrylic chamber was constructed around the exposed folia and filled with Ringer’s solution gassed with 95% O2 and 5% CO2.
Drug administration

Bicuculline [1(s), 9(R)-(−)-bicuculline methochloride], GABAzine (SR-95531), glycine (L-glycine), and NMDA (N-Methyl-D-aspartic acid) were purchased from Sigma (St. Louis, MO). TBOA (DL-threo-β-benzyloxyaspartic acid), DNQX (6,7-dinitroquinoxaline-2,3-dione), LY 367385 [(S)-(+)−α-amino-4-carboxy-2-methylbenzeneacetic acid], and APV (D-(−)-2-amino-5-phosphonopentanoic acid) were purchased from Tocris Bioscience (Bristol, UK). All drugs were dissolved in normal Ringer’s solution and applied to the cerebellar surface by replacing the solution in the optical chamber except for DNQX/APV and NMDA/glycine which were microinjected into the cerebellar cortex.

Simulation, microinjection, lesioning, and electrophysiological techniques

A paralyene-coated tungsten microelectrode (2-5 MΩ, Fredrick Haer Co., Bowdoin, ME) was used to activate MF afferents in the cerebellar white matter (WM). Typical parameters for MF stimulation consisted of six pulses of 100-150 µA, 100-200 µs at 100 Hz. PF stimulation consisted of ten pulses of 100-125 µA, 100 µsec at 100 Hz. Peripheral responses were evoked via bipolar stimulation with two electrodes placed ~1 mm apart either surrounding the ipsilateral C3 vibrissa follicle or in the medial and lateral walking pads on the ventral aspect of the forepaw using 20 V, 300 µs pulses at 10 Hz for 10 s (Gao et al., 2006) or 10-20 V, 300 µs pulses at 100 Hz for 50-100 ms, respectively.
Microinjections of NMDA/glycine or DNQX/APV into the cerebellar cortex were performed by lowering a glass micropipette (1-5 MΩ) beneath the cerebellar surface and triggering single ejection pulses (75-100 kPa, 100-400 ms) using a pico-injection system (PLI-100; Medical Systems, Greenville, NY). The injection parameters were set to deliver as small a volume as possible over a brief duration. The volume of the injection was calculated by collecting an image of the droplet produced upon injection. The image allowed the diameter of the droplet to be measured and the volume (assumed to be spherical) calculated.

In some experiments lesions were generated in the molecular layer. After evoking a beam with PF stimulation, a tungsten microelectrode was used to produce electrolytic lesions across the beam. Typically, lesions were made at 3-5 locations (50 µA, 1 s) to just span the width of the beam. After generating the lesions, the response to PF stimulation was evoked to assess the effectiveness and extent of the lesion, followed by histological verification (see below).

Single unit extracellular recordings of cerebellar neurons used glass-coated, platinum iridium microelectrodes (1-2 MΩ Alpha Omega, Nazareth, Israel) and conventional electrophysiological techniques (Chen et al., 2009). Recordings were restricted to the molecular layer where PCs were identified by the presence of spontaneous simple spikes and complex spikes (Gao et al., 2006). All other neurons were
classified as unidentified cerebellar neurons. Recordings were digitized at 32 KHz and stored online for offline analysis.

Optical imaging

The anesthetized animal in the stereotaxic frame was placed on an x-y stage mounted on a modified Nikon (Tokyo, Japan) epifluorescence microscope fitted with a 4X objective. Images of Crus I and Crus II were acquired with a Quantix cooled charge coupled device camera with 12 bit digitization (Roper Scientific, Tucson, AZ). A 100 W mercury-xenon lamp (Hamamatsu Photonics, Shizouka, Japan) with direct current (DC) controlled power supply (Opti Quip, Highland Mills, NY) was used as the light source. Images were binned 2 X 2 to yield images of 256 X 256 pixels with a resolution of ~ 10 X 10 µm per pixel.

Ca\(^{2+}\) imaging was performed by first loading the Ca\(^{2+}\) dye via a series of microinjections into the imaging field. The solution consisted of 10 mM Oregon Green 488 BAPTA-1/AM (Invitrogen, Carlsbad, CA) dissolved in DMSO plus 20% Pluronic F-127 solution (Invitrogen) and diluted 20 times in normal Ringer’s solution (Stosiek et al., 2003; Sullivan et al., 2005; Gao et al., 2006). A glass micropipette (resistance 1-5 MΩ) was filled with the dye solution and lowered to a depth of ~150-300 µm in the cerebellar cortex. Injections were made in approximately 15 locations to uniformly stain the folium of interest and the preparation was then incubated for 30 min to allow the Ca\(^{2+}\) dye to equilibrate. Images were captured using a custom Ca\(^{2+}\) filter set with excitation at 490-
510 nm, a long-pass dichroic mirror of 515 nm, and emission at 520-530 nm.

Flavoprotein autofluorescence imaging used a bandpass excitation filter (455 ± 35 nm), a dichroic mirror (500 nm), and a >515 nm long-pass emission filter (Gao et al., 2006; Reinert et al., 2004).

Optical imaging data analysis

A series of Ca$^{2+}$ or flavoprotein images consisting of 40-310, 200 ms frames (5 frames/sec) was acquired. Difference images were then generated by subtracting the average of nine control frames (control average) from each control and experimental frame. These difference images were then divided by the control average, yielding images in which the intensity of each pixel reflects the $\Delta F/F$ change in fluorescence relative to the control average.

As shown in Figures 1 and 2, MF stimulation and microinjection of NMDA/glycine resulted in beam-like responses with a centrally located increase in Ca$^{2+}$ fluorescence. Quantification aimed to capture both aspects of the responses. Therefore, regions of interest (ROIs) were placed on the beam-like responses medial and lateral to the stimulation/microinjection (Beam component). A rectangular region (area of 2500 µm$^2$) centered over the stimulation/microinjection site (Center component) was also measured. This approach was also applied to the beam-like response evoked in Crus II by vibrissal stimulation in the presence of TBOA and to the beam responses evoked by direct PF stimulation (Fig. 10). The Center ROI was placed on the patch-like region
evoked in the absence of TBOA, and the beam regions placed on the areas of increased fluorescence that extended medially and laterally from the patch in the presence of TBOA. The average ΔF/F response corresponding to peak activation (3 frames) was determined for each ROI and averaged for each series.

For peripheral, WM, and GC stimuli, we used the following method to quantify the length of the evoked responses parallel to the long axis of the folium, referred to as mediolateral axis (ML), and along the perpendicular axis, referred to as rostrocaudal axis, (RC). The dimensions of the evoked responses were measured by first generating images of the significant evoked responses by first low-pass filtering (3 X 3) the ΔF/F image, and then determining the mean and SD of a control region. The pixels above or below this mean ± 2 SD were pseudocolored, aligned, and superimposed on an image of the folia using a custom program written in Matlab (MathWorks, Natick, MA). Based on this thresholding, a 100 X 100 µm grid was overlaid on the response region and the response dimensions were then measured along the RC and the ML axes of the folium. The ML and RC lengths were determined for each 100 µm section of the grid and the ratio of the average ML and RC lengths (ML/RC) was computed.

The quantification of the beam-like response in Crus I followed a similar procedure as that described for the NMDA/glycine microinjection and MF stimulation experiments. However, multiple ROIs were defined corresponding to the medial and lateral portion of the beam-like response. The region of the folium where the molecular
layer was lesioned was omitted from analyses (see example ROIs in Fig. 5). This procedure was repeated post lesion in order to compare the peripherally evoked $\Delta F/F$ response on-beam before and after the PF disruption.

Example images displayed in Figures 1, 2, and 3 were constructed by averaging 4-32 trials pixel by pixel and then averaging the 5-10 frames encompassing the maximum of the response of interest. Resultant images were scaled to $\pm 1.5\% \Delta F/F$ for grayscale display using Metamorph (Molecular Devices, Downington, PA).

To quantify the spatial relationship between the peripherally evoked optical responses and the distribution of EAAT4, an ROI for the region with the response was identified by thresholding the $\Delta F/F$ response to define the portion of the folia exhibiting the peak evoked fluorescence. The $\Delta F/F$ along the ROI was determined for both the $\text{Ca}^{2+}$ indicator responses and the background fluorescence, which are related to the EAAT4 expression level, across the folium. The correlation coefficient ($\rho$) between these two $\Delta F/F$ spatial profiles was computed.

*Electrophysiology analysis*

Analysis of single unit recordings consisted of constructing peri-stimulus time histograms (1 ms bins) of spike firing 10 ms prior to stimulus onset and 40 ms post-stimulus using Spike 2 (Cambridge Electronic Design Limited, Cambridge, England). The number of trials ranged from 100-200. Cells were counted as responding to the
stimulus if $\geq 3$ consecutive post-stimulus bins contained firing rates greater or less than the pre-stimulus baseline mean $\pm 2$ SDs. The latency of the response was defined as the bin with the first significant increase or decrease (onset) and the end of the response (offset) was the bin at which the firing no longer differed significantly from baseline. The duration of the response was defined as the time between onset and the offset. Response amplitude was defined as the average change in firing during the response compared to mean baseline firing. Percent change in firing from baseline was used to normalize for different firing rates among neurons.

Histology

Post experiment, animals were transcardially perfused with 1% phosphate buffered saline followed by 4% paraformaldehyde in phosphate buffer (pH = 7.4). Brains were then extracted, post-fixed in paraformaldehyde for 2 hours and cryoprotected in 30% sucrose. Cryosectioning of the cerebellum was performed to obtain 40 $\mu$m thick, coronal sections that were thionine stained for lesion identification.

Statistical analysis

The statistical analysis was performed using SAS (SAS Institute, Cary, NC). The effects of various drugs, pre-and post-lesion responses, and on versus off patch single-unit response latency were statistically evaluated using a paired Student’s $t$ test. $\Delta F/F$ responses evoked by microinjection of NMDA/glycine at various depths below the cerebellar cortex were statistically evaluated with ANOVA (within-subject design with
repeated measures) followed by Bonferroni post hoc test (P < 0.01). In the text and figures all values are reported as means ± SD. When describing the results of an experiment, "n" refers to the number of animals used.

Results

WM stimulation evokes a beam-like response

The initial in vivo experiment used Ca$^{2+}$ imaging to examine the spatial distribution of cerebellar cortical activation in response to MF input. To directly activate MFs, a microelectrode aligned approximately perpendicular to the surface of Crus II was lowered to a depth of 450-500 µm and stimuli were delivered to the WM. Note that in the FVB mouse the molecular layer, PC layer and granular layer combined are ~440 µm thick (Oz et al., 2011; Serinagaoglu et al., 2007). The example response reveals beam-like activation of the cerebellar cortex along the entire folium with a centrally located, patch-like response above the stimulation site (Fig. 1A). Similar responses were observed in each mouse tested (n = 7), consistent with activation of a group of GCs by MF afferents that in turn leads to PF activation of PCs. The results also are consistent with a strong local activation above the site of WM stimulation that likely includes excitation of PCs by ascending GC axons (Cohen and Yarom, 1998; Linas, 1982; Isope et al., 2002; Walter et al., 2009).
Figure 1. WM stimulation evokes beam-like responses. A Left is background fluorescence image of Crus II after injection of Oregon Green. Also shown is electrode placement within the folium and ROIs used to quantify the Center (red) and Beam (blue) components of the fluorescence response. Microelectrode was lowered to a depth of 500 µm below the surface. Middle image shows the beam-like Ca\(^{2+}\) response to WM stimulation (200 µA, 100 µs pulses at 100 Hz for 100 ms). Right image shows the beam response is largely blocked by i+mGluR antagonists (100 µM DNQX, 250 µM APV, and 200 µM LY367385). B Summary data (mean ± SD, n = 4 mice) for the Center and Beam response components before and after block by GluR antagonists (* = p < 0.05 for this and all figures). C Summary ML/RC data for WM stimulation (open bar) and after (filled gray bar) application of i+mGluR antagonists (n = 4 mice).

To determine the pre- versus postsynaptic nature of the response, a cocktail of ionotropic and metabotropic glutamate receptor (i+mGluR) antagonists (100 µM DNQX, 200 µM LY 367385, and 250 µM APV to antagonize AMPA, mGluR1, and NMDA receptors, respectively) was added to the optical chamber Ringers. The example images show a large reduction in the response in the presence of the i+mGluR antagonists (Fig. 1A). Although both the Center and Beam components were reduced significantly compared to baseline (Center \(t_3 = 87.31, P < 0.0001\) and Beam \(t_3 = 9.31, P = 0.0026\), Fig.
1B), the Beam component had the larger decrease (83 ± 16%). This finding demonstrates that the beam-like response is primarily post-synaptic. A small beam-like response remains that likely represents a less than 100% block of synaptic transmission. In the presence of i+mGlur antagonists, 51 ± 45% of the Center response remains. Both direct activation of CF afferents (pre-synaptic component) and antidromic activation of PCs likely contribute to the remaining response. In fact, the Center response has a parasagittal shape consistent with the projection pattern of the CF afferents (Sasaki et al., 1989; Chen et al., 1996). To quantify the geometry of the response evoked by WM stimulation, we measured the length of the response along the ML and RC axes of the folium and determined the ratio of the ML/RC axes. The ML/RC ratio is also used in subsequent experiments in order to compare the geometry of the responses evoked by different stimuli. The ML/RC ratio for WM stimulation was 3.00 ± 0.79 (Fig. 1C). Upon application of i+mGlur antagonists, the ratio decreased significantly to 1.84 ± 0.80 ($t_3 = 7.42$, $P = 0.0051$, Fig. 1C). Importantly, this initial experiment demonstrates that MF stimulation evokes beam-like responses \textit{in vivo}, in contrast to previous results in the isolated guinea pig cerebellum (Rokni et al., 2007; Cohen and Yarom, 1998) but in agreement with an \textit{in vitro} autofluorescence optical imaging study (Coutinho et al., 2004).

\textit{NMDA activation of GCs evokes a beam-like response}

To obtain greater target selectivity than that afforded by electrical stimulation and to confirm the initial results showing beam-like activity in response to MF-PF-PC circuit
activation, we employed a pharmacological approach to activate GCs. We microinjected NMDA (1.20 ± 0.20 nl, 500 µM) and its co-agonist, glycine (10 µM), into the cerebellar cortex bathed in a 0 Mg²⁺ Ringer’s solution. NMDA receptors are expressed on GCs and account for ~30% of the excitatory response to MF input (D'Angelo et al., 1995). Conversely, adult mice do not have functioning NMDA receptors on PFs (Lonchamp et al., 2012; Piochon et al., 2007; Shin and Linden, 2005), although these receptors are expressed on PCs (Renzi et al., 2007; Piochon et al., 2007), suggesting that the NMDA will primarily activate GC dendrites and a patch of PCs and/or interneurons in the immediate vicinity of the injection. However, local activation of PCs and/or interneurons will not generate a beam-like response. NMDA/glycine microinjection evokes a beam-like activation pattern, with the largest amplitude responses evoked by injections within the GC layer between 200-350 µm below the cortical surface (Fig. 2A and C). Also, a patch-like region of activation occurs above the site of injection. The ΔF/F responses evoked by NMDA/glycine for the Beam region were 0.36 ± 0.22% at 50-150 µm, 0.75 ± 0.41% at 200-350 µm, and 0.02 ± 0.17% at 400-500 µm (Fig. 2C, n = 7 mice). The greatest magnitude response occurred when NMDA/glycine was injected at the 200-350 µm depth range and the responses were significantly different at each depth (F₁₅,₁₈₆ = 24.55, P < 0.0001 followed by post hoc test). The response amplitudes (ΔF/F) in the Central region were 0.61 ± 0.41% at 50-150 µm, 0.69 ± 0.50% at 200-350 µm, and 0.11 ± 0.20% at 400-500 µm. The largest amplitude responses occurred at both the 50-150 µm and 200-350 µm ranges (F₁₅,₁₈₆ = 32.37, P < 0.0001 followed by post hoc test). As noted, this is expected as the NMDA will activate neurons locally at the injection site in
both the molecular and GC layers. The lack of a response at the greatest depths below the GC layer (> 400 µm) adds further support that the responses are evoked by GC activation. The smaller response amplitude at greater depths is consistent with the mouse GC layer being ~250 µm thick beneath the ~190 µm molecular and PC layers (Oz et al., 2011; Serinagaoglu et al., 2007). At the 50-150 and 200-350 µm ranges, the responses also had the largest ML/RC ratios; 4.01 ± 2.58 and 4.60 ± 1.74, respectively ($F_{8,19} = 4.72$, $P = 0.0026$, followed by post hoc test, Fig. 2C).

To verify the responses were NMDA receptor-mediated, in three mice NMDA injections were repeated at the depth (200-350 µm) evoking a maximal response in the 0 Mg$^{2+}$ Ringer’s solution after reintroduction of 2 mM Mg$^{2+}$ into the optical chamber bath (Fig. 2B). In the presence of Mg$^{2+}$, the amplitude of the ΔF/F response was significantly reduced from 0.61 ± 0.19 % to 0.16 ± 0.12 % ($t_2 = 4.50$, $P = 0.045$, n = 3, Fig. 2D). Similarly, the Central response was significantly reduced from 0.95 ± 0.34 % to 0.21 ± 0.08 % ($t_2 = 4.84$, $P = 0.040$). In the presence of 2 mM Mg$^{2+}$, the ML/RC ratio decreased markedly to 2.02 ± 0.89 which was significantly different from the ratio obtained by NMDA injections at 200-350 µm ($t_2 = 22.28$, $P = 0.0020$, Fig. 2D). Therefore, electrical stimulation of MF afferents and glutamate and NMDA microinjection in the GC layer evoke beam-like responses, implying that strong stimulation of a group of MFs or GCs activate PFs that, in turn, activate PCs.
Figure 2. Granular layer stimulation by NMDA evokes beam-like response. A, Cerebellar activation evoked by NMDA/glycine microinjection. Left, Background fluorescence of Crus II followed by a series of grayscale images to the right showing typical Ca$^{2+}$ responses to NMDA/glycine microinjections at 50, 200, and 400 μm below the cerebellar surface. Peak beam-like activity is evoked when NMDA/glycine is microinjected at 200–350 μm below the cortical surface, depths corresponding to the granular layer. B, To verify that the responses were NMDA receptor-mediated, NMDA injections were performed at a depth evoking a maximal response in the 0 mm Mg$^{2+}$ Ringer’s solution and repeated upon reintroduction of 2 mm Mg$^{2+}$ into the optical chamber bath. C, Fluorescence responses (ΔF/F on left axis) are summarized for the center and beam components (Fig. 1A, ROI example) as well as the ML/RC ratios (right axis) for NMDA/glycine injection within three depth ranges below the cerebellar surface (n = 9 mice). The *, □, ◊ values are significantly different from all other values in the same group (i.e., control, beam, ML/RC). The ◊ indicates the ML/RC ratio for injection at 400–500 μm is significantly different from the ML/RC ratio for injections at either 50–150 or 200–130 μm. D, Summary fluorescence responses and ML/RC ratios for NMDA/glycine injections with and without Mg$^{2+}$. The *, □, ◊ values are significantly different from all other values in the same group (i.e., control, beam, ML/RC). The ◊ indicates the ML/RC ratio for injection at 400–500 μm is significantly different from the ML/RC ratio for injections at either 50–150 or 200–130 μm.
Peripheral stimulation evokes a beam-like response in Crus I

While the first two experiments demonstrate MF and GC stimulation result in beam-like activation \textit{in vivo}, it can be argued neither stimulus is physiological (Bower, 2002; Bower, 2010). We estimate that the 150 \( \mu \)A stimulation in the WM activated axons of passage within a volume of \( 4 \times 10^{-3} \) mm\(^3\), a sphere with a radius of \( \sim 100 \mu \)m (Ranck, Jr., 1975). Based on the volume (\( \sim 1.2 \) nl) of the NMDA injections and the density of GC somata (\( 4 \times 10^6 \) cells per mm\(^3\)), we estimate that at least 4000 GCs were activated (Korbo et al., 1993). It remains to be determined whether peripherally driven MF inputs produce beam-like activation patterns. In response to ipsilateral forelimb stimulation, \( \text{Ca}^{2+} \) imaging reveals beam-like responses in Crus I (Fig. 3). Although some areas of patchy activation are also evident and suggestive of localized MF input, beam-like responses extend the entire length of the exposed folium. The ML/RC ratio of the responses evoked in Crus I by forelimb stimulation was 3.48 \( \pm \) 1.70, a value consistent with a beam-like pattern. The population ratio was obtained from the 19 mice in which the responses in Crus I to forepaw stimulation were determined (see Figs. 3, 4 and 5).
Figure 3. Ipsilateral forelimb stimulation evokes beam-like responses in Crus I. A-D Four examples of the beam-like Ca\(^{2+}\) responses evoked by bipolar electrical stimulation applied to the ipsilateral forepaw (10-20 V, 300 µs pulses at 100 Hz for 50-100 ms). Each example is from a different mouse. The Ca\(^{2+}\) images are shown as ΔF/F obtained by subtraction of averaged baseline images from images obtained during the stimulus (see Methods) and displayed with grayscale.

Although forelimb stimulation evokes beam-like patterns in Crus I, the contribution of PFs to the response remains unknown. This question was tested using two different approaches. First, single unit activity was recorded in the molecular layer of Crus I inside and outside of the thresholded Ca\(^{2+}\) response area as shown for an example experiment (Fig. 4A and B). For PCs recorded within the response region, simple spike activity increases significantly to the forelimb stimulation (Fig. 4C). Conversely, simple spike discharge for PCs recorded outside of the region is not modulated (Fig. 4D). In 7 mice, 108 PCs and 28 unidentified neurons were recorded. The simple spike firing of 59% (42/70) of the PCs recorded within the beam-like response region responded to the peripheral stimulus while only 3% (1/38) of the PCs
outside of the region were significantly modulated. These results are consistent with our previous studies that ~90% of the flavoprotein signal is due to post-synaptic activation (Wang et al., 2011; Wang et al., 2009; Reinert et al., 2011). The simple spike responses within the beam-like response regions had a latency of 27.7 ± 5.0 ms and duration of 16.9 ± 9.1 ms. The latencies are comparable to those reported for forelimb stimulation in the rat (Holtzman et al., 2006). Similarly, 76% (16/21) of the unidentified cells recorded within the beam-like region and 0% of the unidentified cells recorded outside of the beam region exhibited a significant change in firing.

In the second approach to testing the PF contribution to the response, discrete lesions in the molecular layer were made to assess changes in the peripheral responses medial and lateral to the lesion. Figure 5 shows the results of two experiments. Forelimb stimulation evoked beam-like Ca²⁺ responses that parallel the rostral border of Crus I (left images of Fig. 5A and B) similar to the responses evoked by PF stimulation (right images of Fig. 5A and B). The electrolytic lesions (50 µA, 1 sec, 3-5 locations) were made across the response defined by PF stimulation (green ROIs in Fig. 5C and D). Following the lesioning, PF stimulation shows that the responses proximal to the lesion were relatively intact and either blocked or greatly reduced distal to the lesion (right images in Fig. 5C and D). Importantly, the electrolytic lesions were discrete and limited to the molecular layer (Fig. 5E). The abrupt transection of the response to PF stimulation and the small lesions demonstrate that the damage to the cerebellar cortex was restricted. The extent of the lesion was further estimated in 7 experiments by directly stimulating PFs on
both the medial and lateral sides of the lesion and measuring the width of absent/decreased optical response. The average region of decreased responsiveness was $203 \pm 71 \, \mu m$, suggesting that beyond the transection damage is limited and consistent with the histology (Fig. 5E). Further, these results establish the PFs and cerebellar cortex are responsive and functioning on either side of the lesioned area.

Comparing the cerebellar responses to forelimb stimulation before and after lesioning shows the decrease in activation either lateral (Fig. 5C) or medial (Fig. 5D) to the lesion. The population data from 7 mice shows the amplitude of the on-beam response to forelimb stimulation (red ROIs in Fig. 5C and D) is significantly reduced to $52 \pm 26\%$ of the baseline response following the lesioning (Fig. 5F, $t_6 = 7.28$, $P = 0.0003$). Therefore, PFs contribute to the beam-like responses in Crus I.
Figure 4. On and off beam PC simple spike responses in Crus I. A Example of the Ca\textsuperscript{2+} response defined by statistical thresholding (see Methods) evoked by ipsilateral forepaw stimulation overlayed on an image of the background fluorescence. B Recording locations for PCs recorded within (On Beam) or outside (Off Beam) of the forelimb-evoked response region in A. C and D Simple spike firing histograms corresponding to the PCs shown in B. Histograms were constructed from 100-200 trials of 300 μs, 20 v pulses. Stimulus onset at t = 0 ms and 0 ms bin is blank due to the stimulus artifact.
Figure 5. PFs contribute to the beam-like response in Crus I. A and B Example images from two mice showing the fluorescence responses evoked by forelimb stimulation (left) or direct PF stimulation (right). C and D After electrolytic lesioning of the molecular layer (see green ROIs in inset below), the peripherally evoked beam-like response (left) is reduced. Also, the response to direct PF stimulation is disrupted (right). Bottom images in C and D show the ROIs used to quantify the On Beam response (red) and the location of the lesion (green). E Consecutive 40 µm sections showing the lesion location and depth in the molecular layer (red arrow). F Normalized On Beam response to forelimb stimulation is significantly reduced post PF lesioning, (n = 7 mice).
Optical and single cell responses to peripheral stimulation in Crus II

Numerous studies report that peripheral inputs primarily evoke patch-like activation in the cerebellar cortex, particularly in Crus II (Santamaria et al., 2002; Bower and Woolston, 1983). These findings are in conflict with the beam-like activation found for peripheral stimulation in Crus I described above. In fact, stimulation of the ipsilateral C3 vibrissa evokes patch-like activation in Crus II as assessed by flavoprotein imaging (Gao et al., 2006). However, our initial study did not examine the responses of single cells to this peripheral input nor did it assess whether the flavoprotein imaging has the sensitivity to detect beam-like activity. Therefore, the next experiment combined monitoring the responses to vibrissal stimulation with both flavoprotein imaging and single cell recordings restricted to the molecular layer of Crus II within and outside of the response area. Both PCs and unidentified cells were recorded. As demonstrated previously (Gao et al., 2006), the flavoprotein responses to vibrissal stimulation are patch-like (Fig. 6A). Similar patch-like responses are found with Ca\(^{2+}\) imaging (see Figs 8, 9 and 10). The example data show that the PC simple spike responses are consistent with the optical responses. PCs recorded within the region of increased fluorescence (± 2 SD above background) respond with a significant increase in simple spike firing and cells recorded outside of the region do not respond (Fig. 6A and B). The correspondence between the simple spike and optical responses is also evident in the averaged simple spike responses for the 80 PCs recorded within or outside of the optical responses (Fig. 6D). Of the 33 PCs recorded within the region of increased flavoprotein fluorescence, the simple spike firing of 30 (91%) was modulated significantly by vibrissal stimulation.
Of the 47 PCs recorded outside the optical response region, the discharge of only 7 (15%) was modulated significantly. None of the PCs responded with an increase in complex spike discharge. Similar to PCs, the firing of 22 (96%) of the 23 unidentified cells located within the flavoprotein response region was significantly modulated by vibrissal stimulation. The firing of only 2 (17%) of the unidentified cells recorded outside the border was modulated. Both the simple spike response duration and response amplitude are greater for PCs recorded within versus outside of the optical response (Fig. 6C). The simple spike responses within the region of increased fluorescence have a latency of 9.8 ± 1.5 ms and duration of 10.2 ± 5.5 ms. The unidentified cells recorded within the response region exhibit a similar latency of 10.1 ± 2.5 ms and duration of 10.0 ± 5.3 ms.

We also tested whether there was a correlation between the firing rate change and the change in flavoprotein fluorescence. The correlation coefficient (ρ) between the change in firing relative to baseline and the ΔF/F at the corresponding recording location was determined for all 115 cells across 8 animals. The correlation coefficient was 0.57 and provides further evidence that the flavoprotein signal reflects the underlying neuronal activity. Therefore, vibrissal stimulation evokes MF input into Crus II in a patch-like pattern, whether assessed by single cell recordings or flavoprotein imaging, with no evidence of a beam-like response.
**Figure 6.** Spatial relation between flavoprotein and PC simple spike responses to peripheral stimulation in Crus II. **A** Ipsilateral vibrissal pad stimulation evokes patch-like increase in fluorescence that was defined by the statistical thresholding. Also shown are the recording locations (colored circles) of PCs relative to the optical response (On Patch versus Off Patch). **B** Simple spike histograms for the On Patch (left) and Off Patch (right) PCs shown in A. Each histogram was generated from 100 vibrissal stimuli. Other conventions as in Fig. 4. **C** Duration and amplitude (mean ± SD) of the significant simple spike modulation for the population of 80 PCs recorded within and outside of the response region (n = 8 animals). **D** Population histograms of simple spike firing for the 80 PCs recorded On Patch (left) and Off Patch (right) of the statistically defined optical response.

**Molecular layer inhibition does not suppress the beam-like response**

The next experiments addressed possible mechanisms for the beam-like responses in Crus I versus the patch-like responses in Crus II. It was hypothesized that molecular layer inhibitory interneurons control the geometry of the PCs activated by PFs and produce the patch-like responses (Bower, 2002; Santamaria et al., 2007; Santamaria et al., 2007).
Application of GABA\textsubscript{A} antagonists was reported to result in beam-like responses in PCs (Santamaria et al., 2007). We re-examined this hypothesis monitoring the flavoprotein responses in Crus II evoked by stimulation of the ipsilateral C3 vibrissa under baseline conditions and in the presence of GABA\textsubscript{A} receptor antagonists (200 µM GABA\textsubscript{zine} or 100 µM bicuculline) (Gao et al., 2006). If molecular layer inhibition underlies the patch-like geometry of cerebellar cortical activation to GC input, blocking the action of GABAergic interneurons is expected to convert the patch-like response into a more beam-like response (Santamaria et al., 2007). As shown in the example experiments, the application of GABA\textsubscript{A} receptor antagonists increases the amplitude and overall area of activation but does not result in conversion to a beam-like response (Fig. 7A). Furthermore, we measured the ML/RC ratios; if the response becomes more beam-like with blocking GABAergic transmission the ratio should increase. However, the ML/RC ratios were 1.74 ± 0.62 for the baseline, 1.87 ± 0.40 upon bicuculline application (n = 6 mice), and 1.89 ± 0.46 with GABA\textsubscript{zine} (n = 3 mice). The ratios were not significantly different for either antagonist (P > 0.05 for both drugs, paired Student’s \textit{t}-test, Fig. 7B). These results are in agreement with voltage-sensitive dye imaging studies \textit{in vitro} in which blocking GABA\textsubscript{A} receptors failed to generate beam-like responses to MF stimulation (Rokni et al., 2007). Therefore, although molecular layer inhibition controls the amplitude and spatial extent of the responses (Gao et al., 2006), it does not prevent beam-like activation \textit{in vivo}. 
Figure 7. Blocking molecular layer inhibition does not result in beam-like responses. A Left image demonstrates the patch-like response in Crus II to stimulation of the ipsilateral vibrissal pad. Insert in upper right in A shows thresholded response region covered by grid used to measure the RC and ML extent of the response. Statistical thresholded flavoprotein response is superimposed on the background fluorescence. Right image shows application of GABAzine (SR-95531, 200 µM) increases the amplitude and area of the response but does not result in beam-like activation. Also shown in both images is the PF stimulating electrode used in all experiments to test the integrity of the cerebellar cortex. B Summary data of the ratio of the extent of the response in the mediolateral (ML) and rostrocaudal (RC) directions before (baseline) and after application of the GABA_A antagonists, 200 µM GABAzine or 100 µM Bicuculline (n = 9 mice).

Role of excitatory amino acid transporters in the spatial pattern of the responses to peripheral input

Unresolved is why peripheral inputs evoke beam-like responses in Crus I (Figs. 3, 4 and 5) and patch-like activation in Crus II (Figs. 6 and 7). Because molecular layer inhibition does not appear to be responsible, we hypothesized that the differential
EAAT4 is the predominant glutamate transporter on PCs, is PC-specific and is expressed in parasagittal bands of zebrin-positive PCs in Crus II (Dehnes et al., 1998; Gincel et al., 2007; Nagao et al., 1997). The parasagittal distribution of EAAT4s is known to differentially modulate PC responses to both PF and climbing fiber inputs (Wadiche and Jahr, 2005; Paukert et al., 2010; Tsai et al., 2012). In contrast, EAAT4 is expressed uniformly in Crus I.

**Figure 8.** Spatial correspondence between EAAT4 expression in PCs and the response to peripheral input in Crus II. A Example images of the patch-like Ca\(^{2+}\) responses in Crus II evoked by stimulation of the ipsilateral vibrissae using the EAAT4 reporter mouse that expresses GFP under the control of the EAAT4 promoter. The responses align with parasagittal regions expressing lower levels of the EAAT4 transporter. B Evoked Ca\(^{2+}\) fluorescence responses (black trace) and background EAAT4 fluorescence in the same folium (gray trace) corresponding to the images in A. Correlation coefficient (\(\rho\)) between the background fluorescence and the evoked response is shown.
To test this hypothesis, the first experiment used a reporter mouse that expresses green fluorescent protein (GFP) under the control of the EAAT4 promoter (Gincel et al., 2007). In Crus II, vibrissal stimulation evokes patch-like increases in Ca\(^{2+}\) fluorescence similar to those observed with flavoprotein imaging (compare Figs. 7 and 8). As shown for the two example experiments, the Ca\(^{2+}\) mediated responses to vibrissal stimulation are centered between the parasagittal zones with the higher expression levels of EAAT4 (Fig. 8A). Also, note the lack of a parasagittal distribution of EAAT4 in Crus I (Gincel et al., 2007). To quantify the spatial relationship between the optical responses and the distribution of EAAT4, a rectangular ROI (800 µm along the mediolateral axis of the folium and 10 µm along the rostrocaudal axis of the folium) was defined that encompassed both the responsive and non-responsive regions. The fluorescence change along the ROI was determined for both the Ca\(^{2+}\) responses and the background fluorescence of EAAT4 expression and the correlation coefficient between these two profiles was computed. The fluorescence profiles for the two example experiments show the inverse relationship between EAAT4 expression and the responses to C3 vibrissa stimulation (Fig. 8B) and the negative correlation coefficient (\(\rho = -0.43\) and \(\rho = -0.91\), \(P < 0.0001\)). In each of the 6 mice studied, a significant inverse relationship exists between the regions responding to the peripheral stimulus and EAAT4 expression (average \(\rho = -0.70\), range -0.43 to -0.91).
Figure 9. Blocking EAATs converts the patch-like response in Crus II to a beam-like response.  A Statistically thresholded image of the Ca\textsuperscript{2+} response in Crus II to ipsilateral vibrissal stimulation (10-20 V, 300 µs pulses at 100 Hz for 50-100 ms) prior to (Vibrissa) and upon bath application of 300 µM TBOA in an FVB mouse (Vibrissa + TBOA).  Also note the increased fluorescence response on or surrounding several blood vessels.  This reflects the increased blood flow that accompanies activation of the cerebellar cortex (Yang et al., 1999;Mathiesen et al., 1998).  Blood vessel related activation was commonly observed.  B Same experiment as in A in an EAAT4 reporter mouse.  C Ratio of ML to RC dimensions of the evoked responses in the baseline condition (white) and in the presence of TBOA (grey) based on 10 FVB and 2 EAAT4 mice.
We reasoned that if the higher level of EAAT4 in the parasagittal zones reduces the responses of PCs to MF inputs then blocking EAATs should convert the patch-like activation of Crus II into a beam-like response. Therefore, the EAAT blocker, TBOA was applied to the Ringer’s solution in the optical chamber (Shimamoto et al., 1998; Dehnes et al., 1998). In both FVB (Fig. 9A) and EAAT4 reporter mice (Fig. 9B), vibrissal stimulation evokes the typical patch-like responses in Crus II. Application of TBOA increases the response amplitude and extent of the patch components, but also results in a beam-like profile. For both examples in the presence of TBOA, there are two patches of increased fluorescence embedded in a beam-like response that extends the length of the folium. The conversion to a beam-like profile was observed in all 12 mice tested (10 FVB and 2 EAAT4) and quantified using the analysis described for evaluating the effect of the GABAergic antagonists (see Fig. 7). The ML/RC ratio was 1.46 ± 0.64 for the patch-like response and increased significantly to 3.24 ± 1.44 upon application of TBOA ($t_{11} = -4.73$, $P = 0.0006$; Fig. 9C).

If the beam-like response in the presence of TBOA is driven by a general increase of excitability at the level of the granular layer, local inhibition of MF-GC synaptic transmission should affect a correspondingly local region of the beam-like response. Conversely, if the TBOA is acting at the level of the PF-PC synapse, local block of MF-GC synaptic transmission should decrease the activation along the beam-like response due to a reduction in PF activation. To test this hypothesis, the AMPA and NMDA iGluR antagonists (100 µM DNQX and 250 µM APV, 5.95 ± 2.37 nl) were injected into
the granular layer (depth of 250 µm) with the TBOA application, targeting the patch region of increased fluorescence evoked by vibrissal stimulation. Similar to Fig. 9, the two examples in Fig. 10 A and B demonstrate the conversion of the peripherally evoked patch-like response to a beam-like profile by TBOA (300 µM). With injection of the iGluR antagonists, the beam-like responses to vibrissal stimulation are reduced, both medially and laterally. For the 6 mice tested, the evoked fluorescence changes were quantified as for the WM stimulation and NMDA injection experiments (see Figs. 1 and 2). For the Beam region the average ΔF/F was 0.21 ± 0.09 % for vibrissal stimulation alone, 0.71 ± 0.31 % in the presence of TBOA and 0.45 ± 26 % for microinjection of iGluR antagonists in the presence of TBOA ($F_{17, 398} = 88.6, P < 0.0001$ followed by post hoc test). Similarly, the ΔF/F response for the Central region was 0.38 ± 0.16 % for vibrissal stimulation, 1.18 ± 0.59 % upon bath application of TBOA and 0.52 ± 0.48 % for microinjection of iGluR antagonists with TBOA ($F_{17, 398} = 190.7, P < 0.0001$ followed by post hoc test). The ML/RC ratio was 1.02 ± 0.17 for the patch-like response, increased significantly to 2.18 ± 0.63 ($t_{10} = -4.32, P = 0.0015$) upon application of TBOA, and decreased to 0.86 ± 0.55 ($t_{10} = 3.85, P = 0.0032$) with injection of the iGluR antagonists. Therefore, a focal reduction in GC activity results in decreased activation along the beam and converts a beam-like response into a patch-like response, consistent with PF mediated activation along the transverse axis of Crus II.

The ~6.0 nl injection volume of DNQX/APV is a 180 µm diameter sphere. To assess the spatial extent of the action of the injected iGluR antagonists a control
experiment was performed. Two beams were evoked by direct PF stimulation in Crus II (see example in Fig. 10D) and the iGluR antagonists were injected at a depth of ~250 µm below the cerebellar surface between the beams. We reasoned the extent to which the response to PF stimulation in the molecular layer was altered would provide an estimate of the spread of the iGluR antagonists. As shown in the example (Fig. 10D), injecting iGluR antagonists into the granular layer had no obvious effect on the response to PF stimulation demonstrating the spread into the molecular layer, and therefore, into the GC layer, is limited. The ΔF/F responses were quantified for 3 mice using the same approach as the WM stimulation experiment (see Fig. 1). The Center ROI was placed directly over the injection and the Beam ROI were measured medial and lateral to the injection site. The ΔF/F response for the Beam region was 0.96 ± 0.12 % for the baseline and 0.97 ± 0.11 % with injection of the iGluR antagonists (t_{95} = 0.82, P = 0.4149; Fig. 10E). The ΔF/F response for the Central region was 1.44 ± 0.29 % for the baseline and 1.36 ± 0.21 % with injection of the iGluR antagonists (t_{95} = 1.66, P = 0.1005; Fig. 10E). This experiment demonstrates that microinjection of iGluR antagonists into the granular layer acts to locally block the MF-GC synapse. The iGluR antagonists do not diffuse sufficiently into the molecular layer to affect PF-PC synaptic transmission. Therefore, the results obtained from the EAAT4 reporter mice and TBOA experiments strongly suggest that EAATs, including EAAT4s on PCs, are involved in modulating the spatial pattern of the response to MF inputs in the cerebellar cortex.
Figure 10. Microinjection of glutamate receptor antagonists into the granular layer disrupts beam-like response in Crus II. A and B Statistically thresholded images from two mice of the Ca\textsuperscript{2+} response evoked by ipsilateral vibrissal pad stimulation (Vibrissa), with bath application of 300 µM TBOA (Vibrissa + TBOA), and with microinjection of iGluR antagonists (100 µM DNQX and 250 µM APV, 5.95 ± 2.37 nl) into the granular layer (depth of ~250 µm). Blue arrow in the middle images points to the injection site. In both examples focal injection of iGluR antagonists into the granular layer reduces the beam-like response in the presence of TBOA. The PF stimulating electrode is evident in the experiment shown in A. Also, blood vessel related increases in fluorescence are present in both A and B (see Fig. 9). C Summary data (n = 6 mice) for the Center and Beam response components for the baseline with TBOA alone and TBOA with microinjection of GluR antagonists. D Statistically thresholded images of the Ca\textsuperscript{2+} response in Crus II evoked by direct PF stimulation with two electrodes before (Baseline) and after microinjection of iGluR antagonists (DNQX/APV) at a depth of 250 µm (blue arrow). E Summary data (n = 3 mice) for the Center and Beam responses for the Baseline and DNQX/APV injection.
Discussion

The first major finding is that MF or GC stimulation activates beam-like responses in the cerebellar cortex in vivo. There is also a large amplitude response directly above the site of MF or GC stimulation, consistent with the GC ascending limb axons exerting a strong influence on PCs. With electrical stimulation there remains some possibility of direct PF activation, for example, spread of current along the electrode-tissue interface. Importantly, microinjection of NMDA into the granular layer evokes a post-synaptic beam-like response. Given the lack of functioning NMDA receptors on PFs in adult mice (Shin and Linden, 2005; Qiu and Knopfel, 2007; Piochon et al., 2010), NMDA provides a highly selective method of activating GCs without activating PFs directly. PCs and cerebellar interneurons express NMDA receptors (Renzi et al., 2007; Piochon et al., 2007; Bilak et al., 1995); however, activation by focal NMDA injection would result in a patch response and not the observed beam-like activation pattern.

To our knowledge, the observation that forelimb stimulation evokes beam-like responses in Crus I is the first optical imaging evidence for the beam hypothesis. Single cell recordings confirm PC simple spike activation along the beam. Importantly, focal lesioning of PFs reduces the activation lateral and medial to the transection, strongly suggesting that PFs contribute to the transverse response. If the beam-like response was solely due to GC ascending axons, the decrease would be limited to the lesion site.
Molecular layer inhibition does not control beam-like responses

The next major finding is that molecular layer inhibition does not prevent beam-like activation of PCs (Santamaria et al., 2007; Bower, 2010). Application of GABA_A antagonists does not convert patch-like responses into beam-like responses, and instead the response amplitude and extent along both the mediolateral and rostrocaudal axes increases. Conversion of the patch-like response into a beam-like response by TBOA demonstrates that optical imaging has the sensitivity to detect such changes in the activation pattern (see Figs. 9 and 10). The single cell recordings establish that the flavoprotein autofluorescence accurately represents the spatial extent of the underlying neuronal responses. Possibly the flavoprotein imaging may have failed to detect beam-like responses in PCs consisting of very brief duration excitation followed by prolonged inhibition. However, this type of response is highly unlikely given that block of GABA_A receptors increases the duration of the excitation and blocks the inhibition (Bao et al., 2010; Jaeger and Bower, 1994). Therefore, present and previous results fail to support the hypothesis that molecular layer inhibition prevents beam-like responses.

Role of parasagittal zones and glutamate extracellular concentration in response pattern

The finding of patch-like responses in Crus II and beam-like responses in Crus I raises the question of the mechanism underlying the regional differences. Intriguingly, a recent study examining whisker inputs into Crus I and II observed a similar phenomenon (Bosman et al., 2010). Co-modulation in the simple spike discharge occurs in transversally located pairs of PCs in Crus I but not in Crus II. One likely explanation is
the known differences in parasagittal zonation. Crus II has zebrin-positive and -negative zones that reflect differential expression of a host of molecules on PCs (Apps and Hawkes, 2009), many of which control PC excitability and plasticity (Brasnjo and Otis, 2001; Paukert et al., 2010; Wadiche and Jahr, 2005; Wang et al., 2011; Gao et al., 2006). However, Crus I lacks this parasagittal zonation (see Figs. 8 and 9) (Sillitoe et al., 2003; Sarna et al., 2006).

One possible contributor to the patch-like responses is EAAT4. The level of EAAT4 expression on PCs is inversely correlated with glutamate concentration and extra-synaptic GluR activation (Tsai et al., 2012). As we show, the responses to peripheral input in Crus II are inversely related to EAAT4 expression. There are a number of possible explanations. However, differential release of glutamate does not appear to contribute as release is similar in regions with low and high EAAT4 levels (Tsai et al., 2012). We propose the low levels of EAAT4 play a role in generating the patch-like responses. Another possibility is that the MF projections mediating the vibrissae response preferentially terminate between zebrin II parasagittal bands and contribute to the patch-like activation. As recently reviewed (Apps and Hawkes, 2009), MFs show some but not perfect alignment with the overlying zebrin banding pattern or longitudinal zones (Ji and Hawkes, 1994; Wu et al., 1999; Voogd et al., 2003; Pijpers et al., 2006). Nor is there evidence that MF inputs selectively innervate regions of low EAAT4 expression and sparingly innervate regions of high EAAT4. For example, granular layer responses to peripheral and central inputs show activation throughout Crus I and II.
(Shambes et al., 1978b; Kassell, 1980; Kassell et al., 1984). Similar maps in Crus II and lobule IX show granular layer responses to the same input that extend across overlaying zebrin positive and negative zones (Hallem et al., 1999; Chockkan and Hawkes, 1994). Therefore, it is unlikely the differential responses in relation to EAAT4 zonation are solely due to variation in the strength of the MF projections. There are other mechanisms that could contribute to patch-like versus beam-like responses, for example the recently described differential transmitter release probability at PF-PC synapses during GC high frequency bursts (van Beugen et al., 2013). Still, the present results show that PFs are activated and glutamate clearance plays an important role in whether PCs are activated in patches or beams.

The TBOA experiments support the interpretation that EAATs shape the response pattern, with foci of activation that become beam-like in the presence of TBOA (Figs. 9 and 10). What the TBOA results show is that by increasing extracellular glutamate concentration, PC excitability increases and the PF contribution becomes apparent. The change to a beam-like pattern is unlikely to reflect a generalized increase in excitability, as GABA_A receptor antagonists increase excitability without producing a beam (see Fig. 7). We hypothesized that the patch-like regions are a major source of the activated PFs responsible for the beam-like response. The finding that focal injection of iGluR antagonists into the granular layer reduces the beam-like responses strongly supports this hypothesis.
Direct PF, MF or GC stimulation evokes beam-like responses in Crus II that may seem at odds with the patch-like responses to peripheral stimulation. However, parasagittal differences in EAAT4 levels do not affect electrically evoked post synaptic responses on PC spines (Tsai et al., 2012), accounting for the beam-like responses to direct electrical or NMDA stimulation. We also suggest that PF, MF or GC stimulation produces a larger and more synchronized PF input that can overcome local mechanisms controlling excitability. In contrast, peripheral stimulation produces smaller amplitude and less synchronous activation of MFs, and in turn GCs, allowing EAAT4 to have a greater role in shaping the spatial pattern of the responses. While we can only speculate on functional implications the results suggest differential requirements for processing of PF inputs, with greater responsiveness in zones expressing lower levels of EAAT4. A corollary is that the width of such processing zones is defined by EAAT4 expression levels. This is consistent with growing evidence that the parasagittal zones have differential physiological and functional properties (Apps and Hawkes, 2009). Clearly, additional studies are needed to determine if the parasagittal distribution of EAAT4 on PCs has similar effects on the response patterns in different folia.

Reconciling previous observations

The present results explain some of the reported discrepancies on the response patterns in Crus I versus II. Several studies supporting the radial hypothesis based their conclusions on patch-like PC responses to peripheral stimuli in Crus II (Santamaria et al., 2007; Bower and Woolston, 1983). There is also less evidence for PF-mediated
synchrony in Crus II than in Crus I (Wise et al., 2010; Bosman et al., 2010). Furthermore, in the cat anterior lobe, responses to various stimuli occur along several mm of a folium (Yu et al., 1985; Valle et al., 2008; Garwicz and Andersson, 1992) and these folia have narrower and more widely spaced zebrin II zones (Sillitoe et al., 2003). While we can only infer that the physiological responses in previous reports are related to EAAT4 expression levels, the evidence suggests the present findings generalize beyond Crus I and II. The results do not necessarily explain why WM stimulation in the isolated cerebellum preparation primarily evoked patch-like responses (Cohen and Yarom, 1998; Rokni et al., 2007). An anatomical comparison is not possible as these earlier studies did not report the lobules evaluated. However, differences between the previous and present studies including using an isolated preparation versus intact animal and voltage-sensitive dye versus Ca\(^{2+}\) imaging, respectively, likely explain the differences.

**Summary**

The present study demonstrates that, in response to peripheral inputs, PFs are activated and modulate PC activity as predicted by the beam hypothesis. However, the hypothesis also envisioned sequential activation along the PFs (Braitenberg and Atwood, 1958; Braitenberg, 1961; Braitenberg et al., 1997). Detecting sequential activity is problematic given that physiological inputs, particularly during behavior, produce complex patterns of MF inputs in space and time (Figs. 3, 8 and 9). The non-homogenous properties of the cerebellar cortex add to the complexity (Apps and Hawkes, 2009), and these factors are likely to obscure any hypothesized timing differences in PC
activity along the PFs. The present findings also demonstrate that synapses along the GC ascending limb modulate PC activity. Both the beam and patch-like elements must be integrated into our understanding of cerebellar cortical function.
CHAPTER III: *IN VIVO* OPTICAL IMAGING DEMONSTRATES ALTERED CEREBELLAR CIRCUITRY IN THE *TOTTERING* MOUSE

**Introduction**

One family of the Ca$^{2+}$ channelopathies is caused by autosomal mutations of the *CACNA1A* gene that encodes the $\alpha_{1A}$, pore-forming subunit of the human Ca$_{v}$2.1 (P/Q-type) voltage gated Ca$^{2+}$ channel (Ophoff et al., 1996; Kramer et al., 1995). Clinical manifestations of *CACNA1A* mutations encompass a broad spectrum of symptoms subclassified into three groups: EA2, familial hemiplegic migraine type 1, or spinocerebellar ataxia type 6 (Jen et al., 2004; Jen et al., 2007; Baloh et al., 1997; Rajakulendran et al., 2010). The symptom complex and severity of disease depends on the specific mutation. EA2 is characterized by paroxysmal attacks of cerebellar dysfunction, including limb dysmetria, gait ataxia, and nystagmus that persist for hours to days. Between episodes, patients typically exhibit progressive cerebellar dysfunction, ataxia and regional cerebellar atrophy (Baloh et al., 1997; Jen et al., 2007; Denier et al., 1999). Importantly, P/Q-type Ca$^{2+}$ channels are heavily expressed in the cerebellum, particularly in Purkinje cells (PCs) and granule cells (GCs) (Mintz et al., 1992; Fletcher et al., 1996; Westenbroek et al., 1995). Mutations in *CACNA1A* that give rise to EA2 have been shown or are predicted to cause a loss or decrease of P/Q-type Ca$^{2+}$ channel function (Wappl et al., 2002; Spacey et al., 2004; Jen et al., 2004; Pietrobon, 2010).
The mutant \textit{tg/tg} mouse contains a recessive missense mutation in the pore-forming region of the P/Q-type Ca$^{2+}$ channel \textit{Cacna1a}, orthologue of the human gene \textit{CACNA1A} (Fletcher et al., 1996). The phenotype of the \textit{tg/tg} mouse consists of paroxysmal dystonia, ataxia, and absence seizures. One non-episodic element of the \textit{tg/tg} mouse phenotype is the uncoordinated, ataxic gait. Similar to EA2 patients, the \textit{tg/tg} mouse exhibits a reduction-in-function with a 30-40\% loss of P/Q-type Ca$^{2+}$ channel current in PCs. Cerebellar histopathological findings in the \textit{tg/tg} mouse are very limited and overt loss of PCs is only observed in animals beyond 1 year of age (Levitt, 1988; Isaacs and Abbott, 1995; Sawada et al., 2009). While frank neurodegeneration (primarily of PCs) often underlies severe ataxia in many cerebellar disorders, milder ataxia occurs in the absence of neuronal loss (for review see (Reeber et al., 2013)). In the absence of neurodegeneration, abnormalities in the cerebellar circuitry such as impaired synaptic transmission, developmental changes or altered excitability may give rise to the baseline ataxia (Ikeda et al., 2006; Matsumoto et al., 1996; Sato et al., 2009; Barnes et al., 2011; Ebner et al., 2013; Armbrust et al., 2014).

Two hypotheses have been put forward to explain the ataxia of the \textit{tg/tg} mouse. The first attributes the ataxia to increased variability in the simple spike firing of PCs, termed “loss of the precision of pacemaking” (Walter et al., 2006; Alvina and Khodakhah, 2010b; Alvina and Khodakhah, 2010a). Similarly, loss of pacemaking is hypothesized to underlie impaired sensorimotor processing in the flocculus and the corresponding abnormal eye movements exhibited by the \textit{tg/tg} mouse (Hoebeek et al., 2005). While it is
established \( tg/tg \) mouse PCs exhibit increased variability in simple spike firing, the role of the increased irregularity in the abnormal motor behavior of the \( tg/tg \) mouse is controversial (Glasauer et al., 2011; Stahl and Thumser, 2013; Stahl and Thumser, 2014).

Alternatively, impaired synaptic transmission in the cerebellar cortex may underlie the ataxia in the \( tg/tg \) mouse. P/Q-type \( Ca^{2+} \) channels play an essential role in synaptic transmitter release in the central nervous system (CNS) including in the cerebellar cortex (for review see (Ebner and Chen, 2012; Pietrobon, 2010; Catterall, 1998)). Parallel fiber (PF)-PC synaptic transmission is reduced in the \( tg/tg \) mouse (Chen et al., 2009; Matsushita et al., 2002). Furthermore, in \( tg/tg \) mice synaptic transmission is altered in the cerebral cortex (Ayata et al., 2000; Tehrani et al., 1997; Sasaki et al., 2006) and at the neuromuscular junction (Plomp et al., 2000). Therefore, decreased PC responsiveness to PF input has been hypothesized to underlie cerebellar dysfunction in the \( tg/tg \) mouse, and possibly in EA2 (Matsushita et al., 2002).

To gain a better understating of the mechanisms underlying the baseline ataxia exhibited by the \( tg/tg \) mouse, this study examines several components of the cerebellar cortical circuitry in the mutant \( tg/tg \) mouse \textit{in vivo}. Using flavoprotein and \( Ca^{2+} \) optical imaging as well as single PC extracellular recordings, we demonstrate decreased function in the GC-PF-PC circuit. In contrast, the climbing fiber-PC circuit is relatively intact. Importantly, 4-aminopyridine, one of the effective pharmacological therapies in EA2, corrects the GC-PF-PC deficit. We propose that the abnormalities in the GC-PF-PC
circuit contribute significantly to the baseline ataxia in this reduction-of-function mouse model of a \textit{CACNA1A} channelopathy.

\textbf{Methods}

\textit{Animal preparation}

All animal experimentation was approved by and conducted in conformity with the Institutional Animal Care and Use Committee of the University of Minnesota. Male and female \textit{tg/tg} mice on a C57BL/6 background as well as male and female C57BL/6 control mice were used for experiments in this study. Homozygous \textit{tg/tg} mice were obtained by crossing a line of mice that have the \textit{tg} and the semi-dominant \textit{Os} allele, which causes oligosyndactylism; \textit{tg/tg} homozygotes were identified at birth by the absence of oligosyndactylism. Putative \textit{tg/tg} homozygotes were challenged with caffeine (15 mg/kg, i.p.) or psychological stress (e.g. placement in a novel cage) to verify the expression of episodic dystonia. Detailed descriptions of the preparation of animals for optical imaging have been described in previous publications (Gao et al., 2006; Reinert et al., 2004) and therefore, are described briefly. Adult mice ages 3-8 months were anesthetized by an initial intramuscular injection of 2 mg/kg acepromazine followed by an intraperitoneal injection of 2 mg/kg urethane and supplemented with 1.5 mg/kg urethane as needed. Animals were placed in a stereotaxic frame, mechanically ventilated and their body temperature feedback regulated. Depth of anesthesia was monitored via electrocardiogram and testing for responses to somatosensory stimuli. A craniotomy
exposed Crus I and II and then a watertight acrylic chamber was constructed around the exposed folia and filled with Ringer’s solution gassed with 95% O₂ and 5% CO₂.

Drug administration

Glycine (L-glycine) and NMDA (N-Methyl-D-aspartic acid) were purchased from Tocris Bioscience (Bristol, UK) and were microinjected into the cerebellar cortex. 4-aminopyridine (4-AP) was also purchased form Tocris Bioscience, dissolved in Ringer’s and applied to the exposed cerebellar cortex by replacing the solution in the optical chamber. Caffeine was purchased from Sigma (St. Louis, MO), dissolved in normal saline and administered intraperitoneally.

Stimulation and electrophysiological techniques

A parylene-coated tungsten microelectrode (2-5 MΩ, Fredrick Haer Co., Bowdoin, ME) was used to activate PFs in the cerebellar cortex (Cramer et al., 2013). The parameters for PF stimulation consisted of 10 pulses of 150 µA, 100 µs at 10 or 100 Hz. Peripheral responses were evoked via bipolar stimulation with two electrodes placed ~1 mm apart on either side of the ipsilateral C3 vibrissal pad 10-20 V, 300 µs pulses at 100 Hz for 100 ms (Cramer et al., 2013;Gao et al., 2006).

To activate climbing fibers (CFs), a tungsten microelectrode (see above) was stereotaxically placed in the contralateral inferior olive (Barnes et al., 2011). The principal olive was targeted to activate CFs projecting to the cerebellar hemisphere. As
the electrode was lowered, stimulation was performed at 200 μm intervals to a final location that evoked the largest responses. The stimulation protocol used 100 μA, 100 μsec pulses at 10 Hz for 1 s.

Microinjections of NMDA/glycine into the cerebellar cortex were performed by lowering a glass micropipette (1-5 MΩ) beneath the cerebellar surface and triggering single ejection pulses (75-100 kPa, 200 ms) using a pico-injection system (PLI-100; Medical Systems, Greenville, NY) (Cramer et al., 2013). The injection parameters were set to deliver as small a volume as possible over a brief duration. The volume of the injection was calculated by collecting an image of the droplet (assumed spherical) produced upon injection and measuring the diameter.

Single unit extracellular recordings of cerebellar neurons used glass-coated, platinum iridium microelectrodes (1-2 MΩ Alpha Omega, Nazareth, Israel) and conventional electrophysiological techniques (Chen et al., 2009). Recordings were restricted to the molecular layer where PCs were identified by the presence of spontaneous simple spikes and complex spikes (Gao et al., 2006; Cramer et al., 2013). All other neurons were classified as unidentified cerebellar neurons. Recordings were digitized at 32 KHz and stored online for offline analysis.
**Optical imaging**

The anesthetized animal in the stereotaxic frame was placed on an x-y stage mounted on a modified Nikon (Tokyo, Japan) epifluorescence microscope fitted with a 4X objective. Images of Crus I and Crus II were acquired with a Quantix cooled charge coupled device camera with 12 bit digitization (Roper Scientific, Tucson, AZ). A 100 W mercury-xenon lamp (Hamamatsu Photonics, Shizouka, Japan) with direct current (DC) controlled power supply (Opti Quip, Highland Mills, NY) was used as the light source. Images were binned 2 X 2 to yield images of 256 X 256 pixels with a resolution of ~ 10 X 10 µm per pixel.

Ca²⁺ imaging was performed by first loading the Ca²⁺ dye via a series of microinjections into the imaging field as described previously (Gao et al., 2006; Cramer et al., 2013). Images were captured using a custom Ca²⁺ filter set with excitation at 490-510 nm, a long-pass dichroic mirror of 515 nm, and emission at 520-530 nm. Flavoprotein autofluorescence imaging used a bandpass excitation filter (455 ± 35 nm), a dichroic mirror (500 nm), and a >515 nm long-pass emission filter (Gao et al., 2006; Reinert et al., 2004).

**Optical imaging data analysis**

A series of Ca²⁺ or flavoprotein images consisting of 40-310, 200 ms frames were acquired. Difference images were then generated by subtracting the average of nine control frames (control average) from each control and experimental frame. These
difference images were then divided by the control average, yielding images in which the intensity of each pixel reflects the ΔF/F change in fluorescence relative to the control average.

As previously published (Cramer et al., 2013) and shown in Figure 2, microinjection of NMDA/glycine results in a beam-like response and a centrally located increase in Ca^{2+} fluorescence. Quantification aimed to capture both components of the responses. Therefore, regions of interest (ROIs) were placed on the beam-like responses medial and lateral to the microinjection (Beam component). A rectangular region (area of 2500 µm²) centered over the microinjection site (Center component) was also measured. The average ΔF/F response corresponding to peak activation (3 frames) was determined for each ROI and averaged for each series.

The dimensions of the peripherally evoked responses in Crus II were measured by first generating images of the significant evoked responses by first low-pass filtering (3 X 3) the ΔF/F image, and then determining the mean and SD of a control region. The pixels above or below this mean ± 2 SD were pseudocolored, aligned, and superimposed on an image of the folia using a custom program written in Matlab (MathWorks, Natick, MA). Based on this thresholding, the response dimensions were then measured along the rostrocaudal (RC) and the mediolateral (ML) axes of the folium to obtain the ML/RC ratio.
The response to inferior olive stimulation was measured by defining an ROI aligned over the simplex, Crus I, Crus II, and paramedian lobule. The pixel intensities within the ROI were obtained from an average of 5 difference images prior to the stimulus and 5 difference images during the peak response. The number of pixels that exhibited intensity values different from the baseline was divided by the total pixel count of the ROI to obtain the percentage of pixels that differed from the baseline during the climbing fiber response.

_Electrophysiology analysis_

Analysis of single unit recordings consisted of constructing peri-stimulus time histograms (1 ms bins) of spike firing 10 ms prior to stimulus onset and 40 ms post-stimulus using Spike 2 (Cambridge Electronic Design Limited, Cambridge, England). The number of trials ranged from 100-200. Cells were counted as responding to the stimulus if ≥ 3 consecutive post-stimulus bins contained firing rates greater or less than the pre-stimulus baseline mean ± 2 SDs. The latency of the response was defined as the bin with the first significant increase or decrease (onset) and the end of the response (offset) was the bin at which the firing no longer differed significantly from baseline. The duration of the response was defined as the time between onset and the offset. Response amplitude was defined as the average change in firing during the response compared to mean baseline firing. Percent change in firing from baseline was used to normalize for different firing rates among neurons.
Statistical analysis

The statistical analysis was performed using SAS (SAS Institute, Cary, NC). The ML/RC ratios of the response evoked by NMDA/Glycine microinjection were statistically evaluated using a Student’s t test. The amplitude of the evoked fluorescence response to NMDA/Glycine, PF stimulation, and vibrissae stimulation as well as the ML/RC ratios and climbing fiber responses were evaluated using an ANOVA (within-subject design with repeated measures). For the evaluation of the 4-AP experiments, ANOVA was followed by Bonferroni post hoc test (p < 0.01). A \( \chi^2 \) test was used evaluate the proportion of responsive and non-responsive PCs recorded within the patch response in WT and \( tg/tg \) mice. In the text and figures all values are reported as means ± SD unless otherwise noted. When describing the results of an experiment, "n" refers to the number of animals used.

Results

Reduced beam-like response evoked by direct PF stimulation

Our previous study documented spontaneous, episodic low frequency oscillations in the cerebellar cortex of the \( tg/tg \) mouse (Chen et al., 2009). We hypothesized that the oscillations are involved in the dramatic episodic dystonia that is one of the hallmark phenotypes of the \( tg/tg \) mouse. The goal of the present study was on the non-episodic, baseline ataxia observed in these mice. Therefore, the experiments were performed when these low frequency oscillations in the cerebellar cortex were not present.
Stimulation of PFs evokes a characteristic transverse beam of increased PC activity and has been extensively studied using electrophysiological and optical imaging techniques (Chen et al., 1998; Cohen and Yarom, 1999; Eccles et al., 1966a) including flavoprotein and Ca$^{2+}$ imaging in Crus II (Reinert et al., 2004; Gao et al., 2006; Wang et al., 2011; Cramer et al., 2013). Flavoprotein imaging studies demonstrate that ~90% of the increase in flavoprotein fluorescence (the “light phase”) of the evoked beam derives from the post-synaptic activation of PCs (Reinert et al., 2004; Reinert et al., 2007; Wang et al., 2011; Wang et al., 2009). Therefore, we tested PF-PC synaptic transmission by measuring the beam response evoked by PF stimulation in $tg/tg$ and WT mice.

This initial experiment determined the flavoprotein response in Crus II to PF stimulation at two different stimulus frequencies, 100 and 10 Hz. The response to PF stimulation is severely diminished in the $tg/tg$ mice (Fig. 1). At both 100 Hz (Fig. 1A and B) and 10 Hz (Fig. 1E and F) stimulation, the example images show that the response amplitude and mediolateral spread are diminished compared to control mice. Parallel fiber stimulation at 100 Hz evokes a 0.54 ± 0.15% change in flavoprotein fluorescence in the WT mouse ($n = 8$). In contrast, the same PF stimulus in $tg/tg$ mice produces a 0.17 ± 0.10% fluorescence response ($n = 8$), a 70% reduction in the amplitude of the WT response ($F_{1,14} = 3854.6; p < 0.0001$, Fig. 1C). Similarly, PF stimulation at 10 Hz evokes a 0.40 ± 0.15% fluorescence increase in WT versus a 0.12 ± 0.09% response in $tg/tg$ mice and is significantly less than the WT response ($F_{1,11} = -2268, p < 0.0001$, $n = 8$ $tg/tg$, $n = 5$ WT; Fig 1G).
A second measure of the response to PF stimulation is the geometry of the evoked fluorescence beam. The ML/RC ratio is calculated by measuring the extent of the ML distance of the response and dividing that quantity by the RC extent of the response. A ML/RC ratio >>1 corresponds to a response that is beam-like while ratios near 1 are more patch-like (Cramer et al., 2013). The geometry of the PF beam evoked by the PF stimulation in the \( tg/tg \) mouse is markedly abnormal compared to that of the WT mouse (Fig. 1A-B and E-F). At 10 Hz stimulation, the beam is truncated with a ML/RC ratio of 1.07 ± 0.41, markedly less than the WT ratio of 6.05 ± 5.67 (\( F_{1,11} = 228.2, \ p < 0.0001, \ n = 8 \ tg/tg, \ n = 5 \ WT; \ Fig. \ 1H \)). The ML/RC ratio of the response evoked by 100 Hz PF stimulation in the \( tg/tg \) mouse is also reduced compared to WT (\( F_{1,11} = 150.3, \ p < 0.0001, \ n = 8 \ tg/tg, \ n = 5 \ WT; \ Fig \ 1D \)). These observations confirm the earlier \textit{in vitro} and \textit{in vivo} reports of a decrease in responses to PF stimulation (Chen et al., 2009;Matsushita et al., 2002). These newer results establish that not only is the response amplitude reduced but also the medial-lateral extent to which PFs activate PCs.
Figure 11. Beam response to direct PF stimulation is reduced in the tg/tg mouse. A and B Example of the flavoprotein fluorescence response evoked by direct electrical stimulation of the PFs (150 µA, 100 µs pulses at 10 or 100 Hz, 10 pulses) in the WT and tg/tg mouse. C Summary of the beam fluorescence response for the WT (white) and the tg/tg (gray) mouse (n = 8 WT, n = 8 tg/tg). D Summary ML/RC ratios for the beam response evoked by 100 Hz stimulation in the WT (white) and tg/tg (gray) mice (n = 12 WT, n = 9 tg/tg). E and F Examples of fluorescence responses evoked in WT and tg/tg mice to direct PF stimulation at 10 Hz. G and H Summaries of the fluorescence response and the ML/RC ratios evoked by 10 Hz PF stimulation for WT and tg/tg mice (n = 5 WT, n = 8 tg/tg). Note that for all figures, bars show mean ± SD and * denotes significant difference at p<0.05.

NM A activation of granule cells evokes an attenuated beam-like response

To more fully evaluate the GC-PF-PC circuitry, the next experiment pharmacologically activated GCs to test PC responses to both the ascending limb and the PF component of the GC axon (Cramer et al., 2013; Sims and Hartell, 2005). As detailed previously, nano-injections of NMDA/Glycine (~ 1 nl) into the granular layer of the cerebellar cortex bathed in 0 Mg²⁺ Ringer’s solution provides for selective activation of granular cells (Cramer et al., 2013). Two components of the NMDA/Glycine evoked Ca²⁺ response were measured: the central response region over the injection site, and the beam component consisting of the response medial and lateral to the central response.
region (see Methods). Both the central and beam components of the response are significantly reduced in the \textit{tg/tg} mouse as shown for example experiments (Fig. 2A and B) and the population data ($F_{3,12} = 201.1, p <0.0001, n = 4$; Fig. 2C). Further, the ML/RC ratio of the response in the \textit{tg/tg} mouse ($1.45 \pm 0.25$) is significantly smaller than in the WT mouse ($2.90 \pm 1.10$; $t_6 = -2.54, p = 0.04, n = 4$; Fig. 2D). Therefore, both the amplitude and ML/RC geometry of the NMDA/Glycine evoked beam response are significantly reduced in the \textit{tg/tg} mouse as found for PF stimulation. These results suggest a fundamental abnormality in the GC-PF-PC circuitry in the \textit{tg/tg} mouse.

![Figure 12](image1.png)

**Figure 12.** NMDA (NMDA/Glycine) evoked granular layer response is reduced in the \textit{tg/tg} mouse. A and B Example of the Ca$^{2+}$ fluorescence beam evoked by microinjection of NMDA and glycine into the granular layer of WT and \textit{tg/tg} mice. C Summary of the fluorescence with NMDA (NMDA/Glycine) application for the center and beam response components in WT (white) and \textit{tg/tg} (gray) mice ($n = 4$). D Summary ML/RC ratios for WT (gray) and \textit{tg/tg} (white) mice ($n = 4$).

_Peripherally evoked patch response is reduced in the \textit{tg/tg} mouse cerebellar cortex_

The next experiment evaluated whether the response in the cerebellar cortex to a peripheral stimulus is also reduced in the \textit{tg/tg} mice. The response evoked by peripheral stimulation of the vibrissae is well characterized and described as a patch-like activation
of a cluster of PCs in the Crus II folium of the cerebellar cortex (Bower and Woolston, 1983; Santamaria et al., 2002; Gao et al., 2006; Cramer et al., 2013). We hypothesized that impairment of synaptic transmission in the tg/tg mouse would result in a reduction in the magnitude of the responses in the cerebellar cortex to peripheral stimulation. As hypothesized, both the example images (Fig. 3A and B) and population data (Fig. 3C) show the responses to C3 vibrissa stimulation are greatly attenuated in the tg/tg mouse compared to the WT response (0.15 ± 0.06% for the WT, n = 10 and 0.04 ± 0.05% in the tg/tg mouse, n = 5; $F_{1,13} = 446.7; p < 0.0001$).

Flavoprotein optical imaging is highly correlated with and sensitive to changes in PC simple spike firing (Cramer et al., 2013; Gao et al., 2006). To verify that the decreased responsiveness in tg/tg mice occurs in PCs, extracellular recordings were used to monitor changes in simple spike firing to C3 vibrissa stimulation. We recorded PCs within the optically defined patch region in WT and tg/tg mice. In WT mice (n = 5), 59 PCs were recorded within the patch response and of these, 44 (75%) cells exhibited an increase in simple spike firing to the vibrissa stimulus (Fig. 3D). In WT mice the peak increase in simple spike firing occurs at a latency of 9.8 ± 2.4 ms (Fig. 3G), consistent with our previous report of the simple spike latency for PCs recorded within the patch (Cramer et al., 2013). The simple spike discharge of 15 PCs (25%) was not significantly modulated by the sensory input in WT mice (Fig. 3D). In contrast, 75% (82 cells) of PCs recorded in tg/tg mice within the patch did not exhibit an increased simple spike discharge while 25% (28 cells) significantly increased their simple spike discharge rate.
Therefore, the percentage of modulated PCs in the patch is significantly different in the \( tg/tg \) mouse compared to WT (\( \chi^2(1) = 35.9, \ p < 0.0001 \)). The overall reduction in simple spike firing to vibrissa stimulation is evidenced in the population response histograms (Fig. 3E versus F). The reduced flavoprotein fluorescence and PC responses evoked by C3 vibrissa stimulation implies that synaptic transmission is impaired at one or more sites between the periphery and the PCs in the \( tg/tg \) mouse. Consistent with this assertion, the 25 PCs with significant simple spike responses to C3 stimulation exhibit an increased response latency of \( 11.8 \pm 3.5 \) ms compared to the \( 9.8 \pm 2.4 \) ms latency of WT PCs (\( F_{1,9} = 8.95, \ p = 0.004; \) Fig. 3G). Furthermore, the response duration is reduced (\( F_{1,9} = 36.75, \ p < 0.0001; \) Fig. 3H) as well as the amplitude of the evoked change in PC simple spike firing (\( F_{1,9} = 7.59, \ p = 0.008, \ n = 6 \) for \( tg/tg \), \( n = 5 \) for WT; Fig. 3I) in the \( tg/tg \) mouse compared to WT.
Figure 13. Patch response evoked by peripheral sensory input is reduced in the \(tg/tg\) mouse cerebellar cortex.  
A Example of the flavoprotein response defined by statistical thresholding evoked by ipsilateral vibrissal pad stimulation in the WT mouse versus the response B observed in the \(tg/tg\) mouse.  
C Summary of patch fluorescence response (\(\Delta F/F\%\)) for the WT (blue) and the \(tg/tg\) (orange) mouse.  Responses are shown as mean ± S.D. (\(n = 5\) WT, \(n = 10\) \(tg/tg\)).  
D Number of PCs recorded within the patch region that exhibited a significant increase in simple spikes (gray) in response to peripheral stimulation and the number of PCs that did not exhibit a response (white) in WT and \(tg/tg\) mice.  A total of 59 PCs were recorded from 5 WT mice and a total of 110 PCs were recorded in 6 \(tg/tg\) mice.  
E and F PC response duration and firing for the WT and \(tg/tg\) PCs that exhibited a significant increase in firing to the vibrissa stimulus (+) as well as the aggregate response of all PCs (all) recorded within the patch response region.  
G Summary response latency for the peak response in all PCs the significantly increased simple spike firing in WT (white) and \(tg/tg\) (gray) mice.  
H and I Summary histograms of all of the PCs recorded in WT (H) and \(tg/tg\) (I) mice.
Effects of 4-aminopyridine (4-AP) on responses to PF stimulation

A critical finding in support of the hypothesis that irregularity in the simple spike firing plays a causal role in the ataxia of the \textit{tg/tg} mouse is that the 4-AP normalizes the PC discharge (Walter et al., 2006; Alvina and Khodakhah, 2010b). However, 4-AP has multiple actions that alter neuronal excitability including increasing synaptic transmissions (Smith et al., 2000; Molgo et al., 1980; Lundh, 1978), including at the PF-PC synapse (Walter et al., 2006). These multiple actions open the possibility that the 4-AP may also act by rescuing the deficit in PF-PC synaptic transmission. We tested this possibility by applying 4-AP in relation to PF stimulation at 5, 10, and 250 $\mu$M concentrations.

Application of 4-AP to the chamber Ringer’s produces a marked improvement in both the amplitude and mediolateral extent of the response to PF stimulation at both 10 and 100 Hz (Fig. 4A and B). The amplitude of the response to PF stimulation is increased at both the 10 Hz stimulus frequency ($F_{3,16} = 1867.1$, $p < 0.0001$, $n = 4$) and 100 Hz ($F_{3,16} = 1616.1$, $p < 0.0001$, $n = 4$, Fig. 4C). Particularly impressive is the improvement in the ML/RC ratio in which 4-AP results in a beam-like response in the \textit{tg/tg} mouse at both the 10 Hz ($F_{3,17} = 100.25$, $p < 0.0001$, $n = 9$) and 100 Hz ($F_{3,17} = 20.17$, $p < 0.0001$, $n = 9$, Fig. 4D) stimulus frequencies. The increases in the amplitude of the response and ML/RC ratio by 4-AP are almost to WT levels. Further, the 4-AP changes in the response amplitude occur at concentrations as low as 5 $\mu$M. Therefore, the
improvement in the ataxia by 4-AP in tg/tg mice may be due to restoration PF-PC synaptic transmission (Alvina and Khodakhah, 2010b).

**Figure 14.** 4-AP rescues the beam response to PF stimulation in the tg/tg mouse. A and B Example flavoprotein fluorescence beam evoked by direct PF stimulation at 100 Hz (A) and 10 Hz (B) followed by bath application of 5, 10, or 250 μM 4-AP in tg/tg mice. C and D Population summaries of the fluorescence response (C) and the ML/RC ratios (D) of the beam response in the tg/tg mouse (n = 4).
Climbing fiber response is unaltered in the tg/tg mouse cerebellar cortex

Abnormal CF input to PCs is associated with motor impairments including ataxia (Barnes et al., 2011; Chen et al., 2010; Horn et al., 2013). Therefore we examined the responses evoked by electrical stimulation of the contralateral inferior olivary nucleus in the tg/tg mouse. The evoked response consisted of parasagittal bands of increased fluorescence in both tg/tg and WT mice as shown in the example images (Fig. 5A and B) and consistent with previous optical imaging studies (Barnes et al., 2011; Gao et al., 2003; Rokni et al., 2007). The response to CF stimulation is similar between WT and tg/tg mice ($F_{1,10} = 3.77$, $p = 0.055$, $n = 6$; Fig. 5C) consistent with a previous study (Matsushita et al., 2002).

![Figure 15](image.png)

**Figure 15.** Climbing fiber response is intact in the cerebellar cortex of the tg/tg mouse. A Example of the flavoprotein response evoked by electrical stimulation (100 μA, 100 μsec pulses at 10 Hz for 1 s) of the contralateral inferior olivary nucleus in the and B the tg/tg mouse. C Summary of the climbing fiber response shown as % change in pixel intensity between baseline and stimulus application in WT and tg/tg mice ($n = 6$).
Figure 16. Two mechanisms of cerebellar dysfunction in P/Q channelopathies. A proposes that irregular PC firing results in cerebellar dysfunction while B proposes that reduced PC responses produces cerebellar dysfunction and ataxia (note that the hypotheses are not mutually exclusive).

Discussion

The major finding in this study is the reduced responses in the cerebellar cortex to inputs mediated by the GC-PF circuitry in the \textit{tg/tg} mouse as assessed by \textit{in vivo} optical imaging and single unit electrophysiology. The responses to electrical stimulation of PFs or pharmacological activation of GCs are reduced. Not only is there a reduction in the amplitude but also in the beam-like geometry of the responses, demonstrating that the GC-PF-PC circuit is highly abnormal in the \textit{tg/tg} mice. Further, the response to whisker stimulation is reduced as demonstrated by both decreased flavoprotein activation and
modulation of simple spike firing. Previous *in vitro* investigations of PC response to PF inputs in the *tg/tg* mouse have been conflicting, with one reporting intact synaptic transmission (Zhou et al., 2003) and another reporting impaired transmission (Matsushita et al., 2002). An *in vivo* examination of the evoked PF response in the *tg/tg* mouse cerebellum reported a reduced response (Chen et al., 2009). The present results provide substantial evidence using multiple approaches that PF-PC synaptic transmission is reduced in the *tg/tg* mouse. Given the fundamental role this circuit is hypothesized to play in the cerebellar cortical function (Braitenberg, 1961;Thach et al., 1992;Ito, 2002;Marr, 1969;Hansel et al., 2001;Albus, 1971), and that GCs provide up to 200,000 inputs to a PC, we hypothesize that the reduced fidelity of GC-PF-PC synaptic transmission contributes to the baseline ataxic phenotype of the *tg/tg* mouse.

*Presynaptic or postsynaptic contributions to the reduced PC responses in the *tg/tg* mouse*

The reduced PC response to PF stimulation or GC activation, both the amplitude and mediolateral extent, shows that the excitability of either the PFs or PCs is impaired in the *tg/tg* mouse. Considering the large contribution of P/Q-type Ca\(^{2+}\) current in PF synaptic transmission (Mintz et al., 1995), a deficit in PF glutamate release might be expected in the *tg/tg* mouse. However, paired-pulse facilitation, a measure of presynaptic release, does not differ in *tg/tg* and WT mice (Matsushita et al., 2002;Zhou et al., 2003). In the *tg/tg* mouse, the loss of pre-synaptic P/Q-type Ca\(^{2+}\) channel function is
compensated for by other voltage-gated Ca\(^{2+}\) channels at numerous central synapses and neuromuscular junctions (Plomp et al., 2000), including on PFs and CFs (Matsushita et al., 2002). Based on these previous findings, the impaired response may be primarily due to postsynaptic dysfunction.

If as argued above, glutamate release from PFs is not the main mechanism for the decreased responsiveness, there are several other possibilities. First, PF or GC excitability could be reduced. To our knowledge, neither possibility has been investigated. P/Q-type Ca\(^{2+}\) receptors are found throughout cerebellar GCs and are particularly enriched at PF presynaptic varicosities (Kulik et al., 2004). Second, AMPA receptors on PCs could be reduced. In another \textit{CACNA1A} mutant, the \textit{rocker} mouse that exhibits reduced PF-PC transmission in the absence of impaired paired-pulse facilitation, it was found that AMPA receptors on PCs are diminished (Kodama et al., 2006). Again, in the \textit{tg/tg} mouse, whether AMPA receptors on PCs are reduced or have decreased responsiveness has not been studied to our knowledge.

The third possibility is a reduction in PC excitability due to reduced P/Q-type Ca\(^{2+}\) currents. This mechanism is highly likely given that P/Q-type Ca\(^{2+}\) channels account for 70-80\% of voltage-gated P/Q-type Ca\(^{2+}\) currents on WT PCs (Mintz et al., 1992; Llinas et al., 1992). Also, compensatory enhancement of L-type Ca\(^{2+}\) channel expression occurs in \textit{tg/tg} PCs (Campbell and Hess, 1999). Diminished and/or changes in the voltage-gated Ca\(^{2+}\) channels on PCs in \textit{tg/tg} mice could affect the integration of PF
inputs. Alterations in PC dendritic morphology have been described in the \textit{tg/tg} mouse, including ectopic dendritic spines and altered dendritic geometry (Rhyu et al., 1999), which could decrease somatic responses by altering current spread down the dendritic processes (Roth and Hausser, 2001; Vetter et al., 2001). This would not only be in the amplitude of the responses but also spatial information processing. Therefore, reduced P/Q-type Ca$^{2+}$ currents in PCs could explain the reduced mediolateral extent of PF-mediated activation and reduced responses to peripheral inputs.

\textit{Impaired synaptic transmission and ataxia}

Both the gait ataxia and eye movement abnormalities exhibited by the \textit{tg/tg} mouse have been attributed to increased variability in the simple spike firing of PCs (Walter et al., 2006; Alvina and Khodakhah, 2010b; Alvina and Khodakhah, 2010a; Hoebeek et al., 2005). While the irregularity of PC simple spike firing is established in the \textit{tg/tg} mouse, a causal role between the irregular firing and the abnormal motor behavior is not uniformly accepted (Glasauer et al., 2011; Stahl and Thumser, 2013; Stahl and Thumser, 2014). For example, the therapeutic efficacy of 4-AP in improving eye movement impairments in the \textit{tg/tg} mouse failed to demonstrate benefit (Stahl and Thumser, 2013). Regularization of \textit{tg/tg} mouse PC firing \textit{in vitro} with the voltage-gated K$^+$-channel blocker, 4-AP, provides critical evidence for the hypothesis that normalization of simple spike discharge accounts for the motor impairment observed with 4-AP administration \textit{in vivo} (Alvina and Khodakhah, 2010b). However, 4-AP has multiple actions that alter neuronal excitability including increasing synaptic transmission (Smith et al.,
In WT mice, low concentrations of 4-AP increase PF-PC synaptic transmission, but not the responses to white matter stimulation. In this study, 4-AP rescues both the decreased responses and mediolateral activation to PF stimulation found in the $tg/tg$ mouse. Therefore, the therapeutic action of 4-AP is likely to involve improving the response of PCs to PF inputs. While these observations do not rule out the contribution of the regularization of simple spike firing, clearly other mechanisms are important (see Fig. 16).

Alternatively, impaired GC-PF-PC circuitry in the $tg/tg$ mouse may contribute to the ataxia (Matsushita et al., 2002; Pietrobon, 2010). An important role for impaired synaptic transmission in this circuit is further supported by a recent study showing granule cell-specific deletion of the P/Q-type Ca$^{2+}$ channels recapitulates the three phenotypic elements of the $tg/tg$ mouse, including ataxia (Maejima et al., 2013). Therefore, deficient synaptic transmission at the PF-PC synapse, independent of PC dysfunction, can produce an ataxic phenotype. Reduced synaptic transmission at PF-PC synapses has been described in a number of diseases associated with impaired motor coordination and cerebellar ataxia independent of P/Q-type Ca$^{2+}$ channel alterations, including spinocerebellar ataxia types 5 (Ikeda et al., 2006; Armbrust et al., 2014) and 15 (Matsumoto et al., 1996) as well as dentatorubral-pallidoluysian atrophy (Sato et al., 2009).
The findings of reduced flavoprotein responses and a decrease in the number of modulated PCs to sensory input further supports that synaptic transmission is impaired in the tg/tg mouse cerebellar cortex and possibly at synapses outside of the cerebellum. Reduced cerebellar cortical activation to sensory input also demonstrates that a fundamental impairment of information processing occurs in the cerebellum of the tg/tg mouse. As discussed above, loss of information processing in the cerebellar cortex can produce abnormal motor control and ataxia. However, if the decrease in cerebellar responsiveness to sensory input is exacerbated by loss of fidelity in synaptic transmission at synapse(s) prior to entering the cerebellar cortex, information would be degraded to an even greater extent than if limited to the cerebellum. Disrupted proprioceptive sensory integration outside of the cerebellum can produce non-cerebellar ataxia (Sghirlanzoni et al., 2005; Spinazzi et al., 2010). Therefore, the ataxia in the tg/tg mouse may reflect the accumulation of subtle impairments in synaptic transmission to the cerebellum in addition to poor integration of these inputs in the cerebellar cortex resulting in deficits in motor control.

In conclusion, this first detailed in vivo assessment of the cerebellar cortical circuitry in the tg/tg mouse demonstrates impaired GC-PF-PC synaptic transmission and reduced cerebellar responses evoked by sensory input. Therefore, the ataxia of the tg/tg mouse is likely to involve pathological changes beyond irregular simple spike firing.
CHAPTER IV: ABNORMAL EXCITABILITY AND EPISODIC LOW FREQUENCY OSCILLATIONS IN THE CEREBRAL CORTEX OF THE TOTTERING MOUSE

Introduction

The hallmark of episodic channelopathies is that neural circuits transiently enter an abnormal excitability state resulting in paroxysmal neurological dysfunction (for reviews see (Ryan and Ptacek, 2010; Kullmann, 2010)). One family of Ca^{2+} channelopathies is caused by mutations of the *CACNA1A* gene that encodes the α_{1A}, pore-forming subunit of the Ca_{2.1} (P/Q-type) voltage gated Ca^{2+} channel (Ophoff et al., 1996; Kramer et al., 1995). Non-CAG expansion mutations of the *CACNA1A* gene include EA2 and familial hemiplegic migraine type 1 (FMH1) (Jen et al., 2004; Jen et al., 2007; Baloh et al., 1997; Rajakulendran et al., 2010). Typically, EA2 involves a decrease and FMH1 an increase of Ca_{2.1} function (Wappl et al., 2002; Spacey et al., 2004; Jen et al., 2004; Tottene et al., 2002).

Motor dysfunction is a prominent feature of EA2 with episodes of cerebellar dysfunction that include limb and gait ataxia and oscillopsia (Rajakulendran et al., 2012). Interictally patients may exhibit nystagmus and progressive cerebellar dysfunction linked to cerebellar atrophy (Baloh et al., 1997; Jen et al., 2007; Denier et al., 1999). The most widely studied model of EA2 is the *tg/tg* mouse that has a recessive mutation in the pore-forming region of the P/Q-type Ca^{2+} channel gene, *Caen1a*, ortholog of the human
CACNA1A gene (Fletcher et al., 1996). Similarly, the tg/tg motoric phenotype includes mild ataxia and a dramatic paroxysmal dystonia that are linked to cerebellar dysfunction (Campbell et al., 1999; Neychev et al., 2008; Raike et al., 2013; Walter et al., 2006; Chen et al., 2009; Hoebeek et al., 2005).

However, P/Q-type Ca\textsuperscript{2+} channels are distributed widely throughout the CNS, with high expression levels in the cerebral cortex and hippocampus as well as the cerebellum (Mintz et al., 1992; Fletcher et al., 1996; Westenbroek et al., 1995). Further, P/Q-type Ca\textsuperscript{2+} channels are central to neurotransmitter release (Pietrobon, 2010; Catterall, 1998). Therefore, it is not unexpected that EA2 patients and tg/tg mice have non-cerebellar dysfunction. Patients have a high incidence of migraine headaches and the associated transient aura of visual, motor, and somatosensory disturbances (Jen et al., 2004). Other non-cerebellar findings in EA2 include epilepsy, cognitive impairment, abnormal EEG, and elevated cortical excitability (Jen et al., 2001; Jouonceau et al., 2001; Van and Szliwowski, 1996; Rajakulendran et al., 2012; Baloh, 2012; Helmich et al., 2010). As for the ataxia, many of these clinical features are episodic. The tg/tg mouse has absence seizures with rhythmic polyspike EEG bursts (Noebels and Sidman, 1979; Kostopoulos et al., 1987). Cerebral cortical abnormalities in tg/tg mice include decreased glutamatergic and GABAergic signaling (Ayata et al., 2000; Tehrani et al., 1997), decreased muscarinic acetylcholine receptor density (Liles et al., 1986) and increased norepinephrine innervation (Levitt and Noebels, 1981).
Previously, we described episodic, LFOs in the cerebellar cortex of \(tg/tg\) mice that are coupled to the dystonic attacks (Chen et al., 2009). Therefore, we hypothesized that similar oscillations may occur in the cerebral cortex. This study demonstrates episodic LFOs of very high power throughout the cerebral cortex of anesthetized \(tg/tg\) mice. Representing a highly abnormal excitability state, the LFOs may contribute to non-cerebellar dysfunction in P/Q-type \(Ca^{2+}\) channelopathies.

**Methods**

*Animal preparation*

All of the animal studies were approved by and conducted in conformity with the Institutional Animal Care and Use Committee of the University of Minnesota. Male and female \(tg/tg\) mice on a C57BL/6 background as well as male and female C57BL/6 control mice (WT) were used in this study. Homozygous \(tg/tg\) mice were obtained by crossing a mouse line containing the \(tg\) allele and the semi-dominant allele \(Os\), which causes oligosyndactalism. The first step in identifying \(tg/tg\) homozygotes was by the absence of oligosyndactalism at birth. Putative \(tg/tg\) homozygotes were then challenged with caffeine (15 mg/kg, i.p.) or psychological stress (i.e., placement in a novel environment) to verify the expression of episodic dystonia prior to experimentation (Fureman and Hess, 2005).

Detailed descriptions of the anesthetized mouse preparation for optical imaging of the cerebellar cortex have been described in previous publications (Gao et al.,
The approach used here is similar except for the exposure and imaging of the cerebral cortex instead of the cerebellar cortex. Therefore, the preparation is only described briefly. Adult mice ages 3-8 months were anesthetized by an initial intramuscular injection of 2.0 mg/kg acepromazine followed by an intraperitoneal injection of 2.0 mg/kg urethane and supplemented with 1.5 mg/kg urethane as needed. Animals were placed in a stereotaxic frame, mechanically ventilated and their body temperature feedback regulated. Depth of anesthesia was monitored via electrocardiogram and testing for responses to somatosensory stimuli. A craniotomy (~7.00 mm x 6.75 mm) was performed to expose the cerebral cortex and the dura removed. A watertight acrylic chamber was constructed around the exposed cortex and filled with Ringer’s solution gassed with 95% O₂ and 5% CO₂.

**Drug administration**

Caffeine and acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) were purchased from Sigma-Aldrich (St. Louis, MO). DNQX (6,7-dinitroquinoxaline-2,3-dione), APV (D-(-)-2-amino-5-phosphonopentanoic acid), L-NAME (NG-nitro-L-arginine methyl ester hydrochloride), indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole), tetrodotoxin (octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol), GABAzine (6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide), MPEP (2-methyl-6-(phenylethynyl)pyridine hydrochloride), LY 367385 ((S)-(+)‐α‐amino‐4-carboxy‐2‐methylbenzeneacetic acid), ω-Agatoxin (ω-Agatoxin TK), and 4-AP (4-
aminopyridine) were purchased from Tocris Bioscience (Bristol, UK). All drugs, except caffeine, were dissolved in normal Ringer’s solution and applied to the surface of the exposed cerebral cortex by replacing the solution in the optical chamber. Caffeine was dissolved in normal saline and administered intraperitoneally.

**Optical imaging and neural activity acquisition and analysis**

The anesthetized animal in the stereotaxic frame was placed on an x-y stage mounted under a high-speed, cooled CCD macroscope (Nikon AZ-100) with a 1X (Fig. 10) or 2X (all other figures) objective with a 512 X 512 CCD chip at 16 bit digitization. Binning on the CCD chip was done to achieve a pixel resolution of ~35 X 35 µm (1X) or ~28 X 28 µm (2X).

A series of flavoprotein images (i.e., an imaging period) consisting of 625 frames (200 ms) each were acquired for all experiments except for those shown in Figure 10 which consisted of a series of 1500 ms frames. Difference images were then generated by subtracting the average of nine control frames (control average) from each control and experimental frame. These difference images were then divided by the control average, yielding images in which the intensity of each pixel reflects the change in fluorescence (ΔF/F) relative to the control period (Reinert et al., 2004; Gao et al., 2006). Images of the ΔF/F were used to illustrate the spontaneous oscillations in the *tg/tg* versus WT mice (see Fig. 1). Time points in the image series were selected and at each time point 10 frames (5 frames before and 5 frames after the time point) were averaged. Resultant images were
scaled to ± 5 % ΔF/F for pseudocolor display using Metamorph (Molecular Devices, Sunnyvale, CA).

The spectral content of the optical data was obtained as described previously (Chen et al., 2009) and therefore, is described only briefly. Images were low-pass filtered using a 13 X 13-pixel mean filter, then the optical signal for each pixel linearly detrended, and transformed into the frequency domain using a 2,048-point fast Fourier transform (FFT) algorithm. The power spectrum was computed using a 50% Hanning window with 25% overlap. The spectral content and power were analyzed in visually defined regions of interest (ROIs) over the left and right cerebral hemispheres. The ROIs were chosen to exclude non-cortical structures within the imaged field (i.e., the superior sagittal sinus, cranium, and acrylic chamber). This procedure was applied to each image acquisition period. As described in the Results, the average power spectrum from WT and \(tg/tg\) mice differed markedly between 0.035-0.11 Hz (see Fig. 2A) which was defined as the frequency band of interest. Subsequent analyses of the optical signals focused on this frequency band. Also as described in the Results, \(tg/tg\) mice exhibited very high power at these LFOs and values greater than 3 standard deviations above the mean WT power were defined as a high power state. In the population summaries of experimental conditions and genotype, the power within the ROIs with the frequency band of interest were averaged across multiple imaging periods.
Based on the spectral analysis, maps of the frequency, power, and phase shifts were generated for the ROIs as described previously (Chen et al., 2009). Within the frequency band of interest and for pixels with power above the high power state threshold, frequency maps represent the dominant frequencies at each pixel and the power maps illustrate the power of the oscillations at the dominant frequencies. The phase shift map indicates the degree to which oscillations are in or out of phase across the exposed cortex.

In some experiments flavoprotein optical imaging was acquired simultaneously with extracellular single-unit neural activity. Single-unit extracellular recordings of cerebral cortical neurons used glass-coated, platinum iridium microelectrodes (1–2 MΩ; Alpha Omega, Nazareth, Israel) and conventional electrophysiological techniques (Chen et al., 2009; Cramer et al., 2013; Haider et al., 2006). Recordings were conducted throughout the extent of the cerebral cortical layers. The single cell recordings were digitized at 32 kHz and stored online. Spikes were sorted offline using Spike2 (Cambridge Electronic Design, Cambridge, UK) and periods of spontaneous activity corresponding to temporal windows of image acquisition were exported for analysis in Matlab (The MathWorks, Natick, MA). The spike trains were converted into instantaneous firing rate with 200 ms binning using fractional intervals, a method used to determine the instantaneous firing rate in equal bins based on the inverse of the interspike intervals (Popa et al., 2012; Chen et al., 2009). The mean firing rate for each spike train period was subtracted from the instantaneous firing rate. The spectral content was
obtained using a 2,048-point FFT algorithm (Chen et al., 2009). The spectral content of the spontaneous flavoprotein response was determined within a 540 X 540 µm ROI visually aligned over the location of the single-unit recording (see examples in Fig. 5). The spectral content of the simultaneously acquired optical and single-unit activity between 0.035-0.11 Hz was then detrended and the correlation coefficient (r) between these power spectra computed using Matlab. For the examples comparing the power between single-unit firing and the optical activity, the power was normalized due to the widely different nature of the two signals.

**Optical imaging of responses to direct cortical and whisker stimulation**

In experiments examining cerebral cortical responses, direct cortical stimulation was delivered via a paralyene-coated tungsten microelectrode (2-5 MΩ, Frederick Haer, Bowdoin, ME) positioned ~1 mm lateral and ~1 mm rostral to bregma and lowered to a depth of ~265 µm below the cortical surface. Stimulation parameters consisted of 5 pulses of 200 µA, 100 µs at 5 Hz. Peripheral whisker stimulation was applied using a pico-injection system (PLI-100; Medical Systems) to deliver a series of air-puffs to the left C2 whisker. The whiskers on the left whisker pad were trimmed short except for whisker C2. The air-puff stimulus consisted of 5 pulses of 9 PSI, 120 ms at 5 Hz applied to the C2 whisker, 1 cm from its base (Devonshire et al., 2010). For both the intracortical and whisker stimulation, the experimental design was to avoid the LFO frequencies. After a 100 s baseline period of images were acquired, direct cortical or air-puff stimulation was delivered continuously, at random intervals between 6-8 s in duration.
The stimulus program was written in Spike2. During the 200 s imaging interval following the baseline period, 25-33 stimuli were delivered. The average (mean ± SD) number of stimuli applied per imaging period was 29 ± 1 for \( tg/tg \) mice and 28 ± 1 for WT. This inter-stimulus-interval corresponds to a frequency of 0.125-0.167 Hz, outside of the frequency band of the LFOs.

Analysis of the flavoprotein response to direct cortical or whisker stimulation consisted of determining the spectral content within a 700 µm X 700 µm ROI visually aligned over the cortical response region. For the response evoked by air-puff whisker stimulation, a single ROI was defined over the contralateral somatosensory cortex. For direct cortical stimulation, ROIs were defined over both the ipsilateral and contralateral response regions. The average power between 0.125-0.167 Hz (the range of the stimulus frequencies) was then computed for the ROI(s) per image acquisition period, similar to a previous report (Llano et al., 2009).

Western blotting

WT and \( tg/tg \) cerebral cortices were dissected and then homogenized in brain extraction buffer: 0.25 M Tris-HCl pH 7.5 with protease inhibitors (1183617001, Roche, Basal Switzerland). Protein concentration was determined with the Bradford method (Bradford regent and technique, Sigma-Aldrich) and 30 µg neuronal nitric oxide synthase (nNOS) or 60 µg endothelial nitric oxide synthase (eNOS) of total protein were denatured and run on a 4-15% gradient gel (BioRad, Hercules, CA) and blotted on mid-sized
nitrocellulose membrane. Membranes were blocked for ~ 1 hour with 5% blocking buffer (5% w/v milk in 1X PBS) with 0.1% v/v Tween-20. Membranes were incubated with nNOS (ab1376, AbCam Inc., Cambridge, UK) or eNOS (NBP1-19824, Novus Biologicals, Littleton, CO) antibody overnight at 4° C, washed three times with 1X PBS with 0.1% Tween-20 and incubated with anti-goat (nNOS) or anti-rabbit (eNOS) horse radish peroxidase secondary antibody for 2 hours. Samples were also probed with mouse anti-α-tubulin (T5168, Sigma-Aldrich) as loading control. Images of the membranes were acquired with ImageQuant (General Electric, Fairfield, CT) and protein levels quantified using ImageQuant densitometric analysis software.

Statistical analysis

The statistical analysis was performed using SAS (SAS Institute, Cary, NC). The effects of various drugs and evoked cortical responses were statistically evaluated with ANOVA (within-subject design with repeated measures). Statistical evaluation of protein expression levels between WT and tg/tg mice was performed using a Student’s t test. In the text and figures all values are reported as mean ± SD. When describing the results of an experiment, "n" refers to the number of animals used.

Results

Transient, low frequency oscillations in the cerebral cortex of tg/tg mice

In the initial experiments autofluorescence optical imaging was used to examine the spontaneous activity in the cerebral cortex of the anesthetized tg/tg mouse in vivo.
During the course of these experiments, oscillations in the flavoprotein fluorescence developed spontaneously throughout the exposed cerebral cortex. As shown for an example experiment, the oscillations in the \( tg/tg \) mouse consist of large amplitude fluorescence changes (\( \Delta F/F \)) across most of the cerebral cortex (Fig. 1A and B). The time courses of the \( \Delta F/F \) (Fig. 1B) based on four ROIs in the frontal and parietal cortices bilaterally (colored circles in Fig. 1A) reveal a periodicity of \( \sim 16 \text{ s} \) frequency (\( \sim 0.0625 \text{ Hz} \)), similar to the LFOs observed in the cerebellar cortex of the \( tg/tg \) mouse (Chen et al., 2009). The large amplitude of the changes in flavoprotein fluorescence (\( \pm 5 \Delta F/F \)) are consistent with extensive shifts in cortical neuronal activity (Reinert et al., 2004; Reinert et al., 2011; Cramer et al., 2013). In contrast, WT mice do not exhibit the large amplitude LFOs observed in \( tg/tg \) mice. As shown for a control animal (Fig. 1C and D), the spontaneous activity across the cerebral cortex consists of much smaller amplitude fluctuations (\( \pm 0.7 \% \Delta F/F \)) at higher frequencies (\( \sim 0.2-0.4 \text{ Hz} \)).
Figure 17. Spontaneous low frequency oscillations (LFOs) in the cerebral cortex of the 
tg/tg mouse.  A Background fluorescence image of the cerebral cortex with color coded 
ROIs used to quantify the fluorescence change over time. The red star indicates the 
location of bregma in this and all subsequent figures. The pseudocolored images show 
the spontaneous change in flavoprotein fluorescence (ΔF/F) in the cerebral cortex of a 
tg/tg mouse at 2 s intervals over a 16 s period. Note the large cyclical fluctuations at this 
periodicity throughout the exposed cortex. B Traces of the ΔF/F over time for the ROIs 
depicted in the background image in A demonstrate large amplitude, LFOs in both 
hemispheres. C Background image followed by pseudocolored images of the 
spontaneous change in flavoprotein activity in a WT mouse. D Traces of the ΔF/F over 
time for the ROIs depicted in the background image in C show only small amplitude 
fluctuations at higher frequencies.

Because the LFOs in the tg/tg mouse are spontaneous and transient, the first step 
was to quantitatively define this abnormal state based on the frequencies and magnitude 
of the oscillations. A two step spectral analysis of the autofluorescence signal was 
performed, similar to the approach used to quantify LFOs in the cerebellar cortex (Chen

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et al., 2009). In the first step, power as a function of frequency was determined for 947 imaging periods (each imaging period consisted of 125 s of consecutive images) of spontaneous activity in 34 tg/tg mice and in 308 sessions in 20 WT animals. Because the goal was to statistically define the occurrence of the episodic, large amplitude LFOs observed in tg/tg mice (Fig. 1A and B), the analysis is based on the activity in both hemispheres. Comparison of the average power versus frequency plots across all periods and animals establishes that strong oscillations occur in a frequency band between 0.035-0.11 Hz in the tg/tg mouse but not in the WT mouse (Fig. 2A). Therefore, we selected this range as the frequency band of interest.

In the second step, the occurrence rate of power in this frequency band was determined across all imaging periods. The power levels in tg/tg mice span three orders of magnitude, with a long tail extending into very high power levels (Fig. 2B, white bars). Therefore, we used a log10 scale for the x-axis (Fig. 2B). Imaging periods with these higher power values correspond to the LFOs shown for the example tg/tg mouse in Fig. 1A and B in which the average power between 0.035-0.11 Hz was 541 ± 754. In contrast, the power distribution in WT animals is confined to much lower values (Fig. 2B, gray bars) as shown for the example WT mouse in Fig. 2C and D in which the average LFO power was 24 ± 17. Therefore, the distributions show the existence of two states, low and high power. We defined the high power state as when the average power between 0.035-0.11 Hz exceeded the average power in the WT animal (16.3 ± 17.8) by three standard deviations (> 69.7). This threshold corresponds to a p-value < 0.003. The
high power state occurred spontaneously in 21 of 34 \(tg/tg\) mice and in 311 of 947 (32.8%) imaging periods. Conversely, using this threshold, there was only a single instance of a high power state in 1 of 20 WT animals out of 308 (0.003%) imaging periods. Based on this analysis, the power versus frequency plots show the marked differences in power between the high and low power state in \(tg/tg\) mice as well as between the high power state in \(tg/tg\) mice versus average power in WT mice (Fig. 2C).

Using the low frequency band of interest and the definition of a high power state, spectral analysis was used to generate maps of the frequency, power and phase superimposed onto a background image of the cerebral cortex. In these maps only pixels with power values greater than the threshold for a high power state are shown. For a \(tg/tg\) mouse in the high power state, maps of the spontaneous LFOs in the cerebral cortex (Fig. 3, top) show that the oscillation frequencies can be markedly uniform across the cortex; however, power typically varies greatly in different regions (see also Figs. 4, 9 and 11). For the \(tg/tg\) mouse in Fig. 3, a region of very high power is centered in the right hemisphere, ~1mm caudal to bregma and ~1mm lateral to the midline. The phase map shows regions with LFOs in phase both within and between the hemispheres. The phase map also illustrates the complex temporal relationships in the oscillations over the cerebral cortex. Conversely, in the example WT mouse (Fig. 3, bottom), there are only small regions within the occipital cortex that exceed threshold for the high power state. Note that because our definition of high power state is based on the average power across the cerebral cortex, it does not imply that individual pixels or regions do not exceed the
threshold in WT animals. The spectral maps illustrate the spatial extent and dynamic
term of the LFOs in the $tg/tg$ mouse during the high power state.

**Figure 18.** Transient, high power LFOs in the $tg/tg$ mouse cerebral cortex. **A** Power
versus frequency plot demonstrates the average power in the cerebral cortex of $tg/tg$
(black) and WT mice (gray) obtained from 947 imaging periods in $tg/tg$ mice ($n = 34$)
and 308 imaging periods in WT mice ($n = 20$). Spectral analysis demonstrates that the
greatest power in the $tg/tg$ mouse cerebral cortex is within a frequency band of 0.035-
0.11 Hz. Therefore, we focused on this frequency range for the remaining analysis. **B**
Occurrence rate (as a percentage of total number of imaging periods) is plotted versus the
log$_{10}$ of the average power histogram of 947 imaging periods in the $tg/tg$ mouse (white
bars) and 308 imaging periods in the WT mouse (gray bars). An imaging period was
defined as high power if the average power was $> 69.7$, a level corresponding to the mean
power exhibited by WT mice (gray trace, $16.3 \pm 17.8$) plus three standard deviations. **C**
Power versus frequency plots of the high power state (black trace) and low power state
(black dashes) averaged across all imaging periods from $tg/tg$ mice. Average power
versus frequency plots across all imaging periods from WT mice (gray trace).
Figure 19. Frequency, power and phase maps of the spontaneous activity in the cerebral cortex of \textit{tg/tg} and WT mice. Spectral analysis demonstrates the frequency (left), power (middle) and phase (right) maps for an example \textit{tg/tg} mouse (top) and the WT mouse (bottom) superimposed on background fluorescence images of the cerebral cortex.

\textbf{Neural contribution to low frequency oscillations in the cerebral cortex}

The spontaneous occurrence of the high power LFOs suggests the cerebral cortical network is not stable in the \textit{tg/tg} mouse and can enter an abnormal excitability state. A primary question is the neuronal contribution to this oscillatory activity. To evaluate the role of neuronal activity in the LFOs, 10 µM of tetrodotoxin (TTX) was bath applied to block voltage gated Na$^+$ channels and suppress action potentials (Kao, 1966). In the \textit{tg/tg} mice, the TTX was applied only after a minimum of 6 contiguous imaging periods confirmed the presence of the high power state in the cerebral cortex because we were specifically interested in the neural contribution to the high power LFOs. As shown
in the example experiment (Fig. 4), TTX suppresses the high power LFOs throughout much of the \(tg/tg\) cerebral cortex with only discrete, smaller regions still exhibiting high power oscillations. For the population, TTX significantly decreases the power in the LFOs by 40\% \((F_{1,8} = 58.8, \ p < 0.0001; \ n = 5, \ \text{Fig. 6E})\). This finding confirms a large neural contribution to the LFOs. The application of TTX in WT mice reduces the power \(~75\%\) \((F_{1,6} = 174.9; \ p < 0.0001, \ n = 4; \ \text{Fig. 4D})\) and demonstrates, as expected, that TTX greatly reduces excitability. However, TTX did not completely block the high power state in \(tg/tg\) mice. This observation suggests that once established, the high power LFOs are not solely dependent on action potential generation or intracortical connections.

**Figure 20.** TTX suppresses but does not block high power LFOs in the \(tg/tg\) mouse.  
A Example frequency, power, and phase maps of spontaneous, high power cerebral cortical activity in the \(tg/tg\) mouse.  
B Frequency, power, and phase maps of the same mouse after bath application of 10 \(\mu\)M TTX.  
C Summary of the average baseline power in \(tg/tg\) mice (gray; for all figures, bars show mean \(\pm\) SD and * denotes significant difference at \(p<0.05\)) and the resulting power in the presence of TTX (white, \(n = 5\)).  
D Summary of the average baseline power (gray) in WT mice and upon TTX application (white, \(n = 4\)).
In the next experiment we characterized the relationship between the LFOs and cerebral cortical neural activity in the \textit{tg/tg} mouse. Extracellular single-unit recordings of the spontaneous discharge of neurons in the cerebral cortex were obtained simultaneously with flavoprotein imaging. For the single-unit data, we recorded all neurons encountered on tracts beginning at the cortical surface to 860 µm, a depth corresponding to cortical layer VI in the mouse (DeFelipe et al., 2002). In 5 \textit{tg/tg} mice, 278 imaging periods (125 s duration) were acquired with recordings of 131 cerebral cortical neurons. A spectral analysis was performed on the simultaneously obtained optical and neuronal firing data for each imaging period. The analysis was restricted to a ~290 µm² ROI aligned over the recording electrode (see example ROIs in Fig. 5). As shown for several example cell recordings, the neuronal firing contains frequencies within the 0.035-0.11 Hz band of interest and the normalized power spectrum closely mirrors the spectral content in the flavoprotein signal in the same region (Fig. 5C and D). The firing of other cortical neurons had less power at these low frequencies or did not match the power in the optical signal (Fig. 5B and E). To quantify the neural-optical relationship, the correlation was computed between the power spectrum of the optical and neural data within the 0.035-0.11 Hz band of interest. The correlation coefficients (r-values) range from -0.82-0.99. However, for the vast majority of neurons the r-values are positive. Of the 278 recording periods, 148 (53\%) exhibited $r > 0.7$ and the overall mean r-value was $0.54 \pm 0.47$ (critical value for significance ($p \leq 0.05$) is $r_{15} \geq \pm 0.48$). Therefore, the firing of the majority of cortical neurons in \textit{tg/tg} mice oscillate at the same low frequencies observed
in the optical recordings. The results from application of TTX and the single-unit recordings show neuronal activity contributes to the LFOs.

Figure 21. Power comparison between simultaneously acquired flavoprotein fluorescence and single-unit extracellular recordings in the $tg/tg$ mouse. A Background image of the cerebral cortex with colored ROIs used to compute the power in the optical signal (see Methods) shown in the example traces in B-E. B-E Four examples of the normalized power for the optical (black) and the single-unit firing (red) and the computed r-value. F Histogram of occurrence rate versus r-values for the comparison of the optical and single-unit power for each of the 278 imaging periods (125 s duration) acquired with single-unit recordings of 131 cerebral cortical neurons in 5 $tg/tg$ mice.
Contribution of P/Q-type Ca\(^{2+}\) channels and synaptic transmission to the LFOs

The next question addressed whether a decrease in P/Q-type Ca\(^{2+}\) channel function underlies the high power LFOs observed in the \(tg/tg\) mouse. Therefore, we tested whether acute reduction in P/Q-type Ca\(^{2+}\) current in the WT mouse with 1.5 μM of the specific P/Q-type Ca\(^{2+}\) channel blocker, \(\omega\)-Agatoxin (Teramoto et al., 1993), would recapitulate the LFOs observed in the \(tg/tg\) mouse. Based on previous reports of the in vivo efficacy of 100 nM \(\omega\)-Agatoxin (Hoebeek et al., 2005), 1.5 μM likely blocks the majority of cortical P/Q channels. In WT animals, \(\omega\)-Agatoxin produced a significant increase in the power of LFOs from 17.2 ± 13.3 to 33.1 ± 32.3 \((F_{1,6} = 18.4, p < 0.0001, n = 4, \text{Fig. 6C})\). As shown in example spectral maps (Fig. 6A), there are regions within the cerebral cortex with increased power in the frequency band of interest. However, the power levels did not enter the high power state and remained well within the power levels observed in WT mice. A complimentary question is whether a further reduction in P/Q-type Ca\(^{2+}\) channel function in the \(tg/tg\) mouse results in the transition to a high power state. Because we were specifically interested in the transition from low to a high power state in this experiment, the \(\omega\)-Agatoxin was applied only after a minimum of 6 contiguous imaging periods confirmed the presence of a low power state. Both the example experiment (Fig. 6B) and the population results (Fig. 6D) show that \(\omega\)-Agatoxin does not result in high power LFOs in the \(tg/tg\) mouse. The power of the LFOs (60.7 ± 49.6) in the presence of \(\omega\)-Agatoxin was not significantly different \((F_{1,6} = 0.01, p = 0.92, n = 4; \text{Fig. 6D})\) and nearly identical to baseline power (60.2 ± 46.1). Taken together, these two experiments suggest that a reduction in the P/Q-type Ca\(^{2+}\) current favors the
development of network instabilities in the cerebral cortex as evidenced by the spontaneous high power state in the \( tg/tg \) mouse and by the increased LFO power in WT mice in the presence of \( \omega \)-Agatoxin. However, as \( \omega \)-Agatoxin did not produce a high power state in WT or \( tg/tg \) mice, the mechanisms underlying LFOs must involve changes in the cerebral cortical circuitry beyond an acute reduction in P/Q-type Ca\(^{2+} \) channel function.

The next series of experiments assessed if the instability in the \( tg/tg \) cerebral cortex is unmasked by altering the balance of excitation or inhibition. As noted in the Introduction, abnormalities in both glutamate and GABA neurotransmission are present in the \( tg/tg \) mouse cerebral cortex (Ayata et al., 2000; Tehrani and Barnes, Jr., 1995; Tehrani et al., 1997). Given that the majority of fast excitatory inputs, either via thalamocortical or corticocortical projections, are mediated by post-synaptic ionotropic glutamate receptors (AMPA and NMDA) (Sherman and Guillery, 2011), we bath applied 100 \( \mu \)M DNQX and 200 \( \mu \)M APV to block fast glutamatergic neurotransmission. As for the experiments blocking P/Q-type Ca\(^{2+} \) channels, we applied the antagonists in the low power state. In WT mice, DNQX and APV result in a significant reduction in the low frequency power (example maps, Fig. 7B), decreasing baseline power from 22.0 \( \pm \) 23.0 to 13.0 \( \pm \) 20.3 \( (F_{1,6} = 10.2, p = 0.0023, n = 4; \text{ Fig. 8A}) \). As expected, in the WT cerebral cortex, blocking ionotropic glutamate receptors decreases spontaneous cortical activity.
Figure 22. ω-Agatoxin blockade of P/Q-type Ca²⁺ channels increases LFO power in WT but not in tg/tg mice. A Example maps from a baseline imaging period (top row) in a WT mouse show small regions of high power that expand upon application of 1.5 µM ω-Agatoxin. B Example maps for the same experiment in a tg/tg mouse. ω-Agatoxin application does not alter the power of the spontaneous activity in the tg/tg mouse cerebral cortex. C and D summarize the average power in the baseline period (gray) and upon application of ω-Agatoxin (white) in the WT (C, n = 4) and tg/tg mouse (D, n = 4).
In contrast and unexpectedly, DNQX/APV in the *tg/tg* mouse produces a significant increase in power from 11.2 ± 13.8 to 61.2 ± 117.0 (*F*$_{1,18}$ = 177.4, *p* < 0.0001, *n* = 10; Fig. 7A and 8B) and resulted in a high power state in 3 of 10 *tg/tg* mice. However, on average, blocking ionotropic glutamate receptors does not produce the high power state observed spontaneously in *tg/tg* mice and nor did DNQX/APV application result in the extremely high power values observed spontaneously in *tg/tg* mice (Fig. 2). These findings suggest that reduced post-synaptic, ionotropic glutamatergic activation contributes to the LFOs and increases the likelihood of a high power state.

In the cerebral cortex, excitatory glutamatergic synaptic transmission also involves metabotropic receptors, mainly type 1 and 5 (Ferraguti and Shigemoto, 2006; Alexander and Godwin, 2006). Furthermore, given the slower time of action of these receptors on post-synaptic neurons, one could hypothesize a role in the LFOs. Therefore, we also tested the effect of blocking metabotropic glutamate receptors by applying MPEP (30 µM), a metabotropic glutamate receptor type 5 antagonist, and LY367385 (50 µM), a metabotropic glutamate receptor type 1 antagonist into the optical chamber. This cocktail of MPEP and LY367385 reliably blocks metabotropic glutamate receptor activity in the cerebral cortex of mice (De and Sherman, 2012). The application of MPEP and LY367385 significantly increases power in the LFOs in *tg/tg* mice (39.4 ± 22.6 to 115.2 ± 98.7, *F*$_{1,6}$ = 52.5, *p* < 0.0001, *n* = 4; Fig. 8D), therefore, on average reaching a high power state. Furthermore, MPEP and LY367385 resulted in the high power state in 3 of 4 *tg/tg* mice. In contrast, inhibition of metabotropic glutamate
receptors in WT mice significantly reduces the power of spontaneous cerebral cortical activity from $21.0 \pm 13.5$ to $15.3 \pm 8.5$ ($F_{1,6} = 12.7$, $p = 0.0008$, $n = 4$; Fig. 8C). Similar to blocking ionotropic glutamate receptors, though more effective, reducing metabotropic receptor activation increases the likelihood of entering a high power state.

**Figure 23.** DNQX/APV application increases the high power domains in the cortex of $tg/tg$ mice but not WT mice. **A** Spectral analysis demonstrates a large increase in high power domains upon application of DNQX and APV (100 and 200 µM, respectively) compared to baseline activity (top row) in the $tg/tg$ mouse. **B** Baseline power (top row) is suppressed by DNQX/APV application (bottom row) in the WT mouse.
In the cerebral cortex of the \textit{tg/tg} mouse, there is a decrease in the efficacy of fast GABAergic inhibition (Tehrani and Barnes, Jr., 1995; Tehrani et al., 1997). Therefore, one possibility is that the increase in LFOs is due to a reduction in cortical inhibition. We tested whether suppression of GABA\textsubscript{A}ergic transmission in the \textit{tg/tg} cortex facilitates a transition to the high power state. Application of 1 \textmu M of the GABA\textsubscript{A} receptor antagonist GABAzine does not change the baseline LFO power (9.6 ± 7.3 to 7.2 ± 5.4, $F_{1,8} = 5.57$; $p = 0.0211$; $n = 5$, data not shown). The results based on blocking glutamatergic and GABAergic receptors show that high power LFOs are not due to an increase in excitability. Instead, the LFOs are facilitated by a reduction in excitatory synaptic transmission.
Figure 24. Blocking ionotropic and metabotropic glutamate receptors facilitates high power LFOs in the tg/tg mouse. A Bath application of DNQX/APV (white) to the cerebral cortex causes a significant decrease in the power compared to the baseline (gray) state in WT mice (n = 4). B Power is significantly increased from baseline (gray) in the presence of DNQX/APV (white) in the tg/tg mouse (n = 10). C Bath application of the metabotropic glutamate receptor type 5 antagonist MPEP (30 µM) and the metabotropic glutamate receptor type 1 antagonist LY367385 (50 µM) results in a significant decrease in power (white) compared to baseline (gray) in WT mice (n = 4). D In contrast, blockade of metabotropic glutamate receptors in tg/tg mice results in a significant elevation of the power (white) compared to baseline (gray, n = 4).

Contribution of NO signaling to cerebral cortical LFO activity

The suppressive effects produced by TTX, the firing discharge of cerebral cortical neurons, and the facilitatory actions of blocking ionotropic and metabotropic glutamate receptors imply a major neural contribution to the LFOs observed in the tg/tg mouse cerebral cortex. However, we also tested blood flow contributions to LFOs, particularly given the high incidence of migraine in patients with CACNA1A mutations (Jen et al.,
2004) and reports of altered nitric oxide signaling in the cerebellum of the *tg/tg* mouse (Frank-Cannon et al., 2007; Rhyu et al., 2003). Two prominent pathways exist in the cerebral cortex to regulate neurovascular coupling: the neuronal nitric oxide synthase (nNOS) pathway that produces nitric oxide (NO) in response to neural activation and the cyclooxygenase (COX) pathway that produces multiple signaling molecules including prostaglandins in response to astrocyte activation (Attwell et al., 2010).

In order to parse their relative contributions to the LFOs, we separately blocked the NOS and prostaglandin pathways, applying the blockers in the low power state. L-NAME (1 mM) was applied to inhibit NOS mediated production of NO (Moore and Handy, 1997). Unexpectedly, blocking NOS results in a dramatic increase in the LFOs in the *tg/tg* mouse (Fig. 9A). The spectral maps from an example experiment show that across most of the cerebral cortex the LFOs exceed the high power threshold and there are large regions of very high power (Fig. 9A) similar to that observed spontaneously (Fig. 1). On average, L-NAME increases power 10 fold in the cerebral cortex of the *tg/tg* mouse (*F*$_{1,10}$ = 41.1, p < 0.0001; n = 6, Fig. 9C). Application of L-NAME produced a high power state in 6 of 6 *tg/tg* mice. In contrast, L-NAME in WT mice resulted in only a modest increase in baseline power (*F*$_{1,6}$ = 4.15, p = 0.0465; n = 4, Fig. 9C) and never resulted in a high power state.

The observation that L-NAME evokes LFOs in the cerebral cortex of the *tg/tg* mouse coupled with the demonstration that nNOS expression is elevated in the
cerebellum of the tg/tg mouse (Frank-Cannon et al., 2007; Rhyu et al., 2003) led us to examine the protein expression levels of nNOS and eNOS in the cerebral cortices of tg/tg and WT mice. Densitometric analysis of nNOS protein levels normalized to the α-Tubulin control shows that protein expression is unchanged in the cerebral cortex of the tg/tg mouse versus WT mice ($t_6 = -1.85$, $p = 0.11$, $n = 4$ each of WT and tg/tg; Fig. 9E). Similar to the nNOS result, Western blot analysis of eNOS levels in the cerebral cortex shows no difference between tg/tg and WT mice ($t_6 = -1.35$, $p = 0.23$, $n = 4$ each of WT and tg/tg; Fig. 9F). We did not examine cerebral cortical expression of inducible nitric oxide synthase because previous studies have demonstrated very low expression levels in the absence of acute pathological insult (Calabrese et al., 2007).

Bath application of indomethacin (100 µM) to inhibit COX mediated production of prostaglandins (Vane, 1971; Faraci, 1992) does not result in the transition from the low to the high power state in the tg/tg cerebral cortex. In fact, the baseline power did not change significantly ($F_{1,6} = 4.4$, $p = 0.0403$; $n = 4$, Fig. 9D). Similarly, indomethacin in WT mice did not result in a high power state, producing a very modest increase in LFO power from $8.9 \pm 12.2$ to $15.0 \pm 11.9$ ($F_{1,6} = 7.3$, $p = 0.0091$; $n = 4$, Fig. 9D).

Together these results suggest that the NO signaling plays a major role in the transition from low to high power state in the tg/tg mouse. In contrast, the prostaglandin pathway controlling cerebral blood flow does not. As both nNOS and eNOS expression is similar between the tg/tg and WT mice, the effect of L-NAME in the tg/tg mouse in
generating abnormal LFOs is not due to the disruption of a high basal level of NO production.
Figure 25. Blocking NO-synthase facilitates LFOs in the cerebral cortex. A Frequency, power, and phase maps showing large regions of high power in a tg/tg mouse cerebral cortex upon bath application of 1 mM L-NAME, a non-specific nitric oxide (NO) synthase antagonist. B Example maps of the spontaneous activity in the same tg/tg mouse prior to NO synthase blockade. C and D Summaries of the average power during the baseline period (gray) in WT and tg/tg mice in the presence (white) of either L-NAME or the cyclooxygenase inhibitor, indomethacin (WT, n = 4 for both drug treatments; tg/tg, indomethacin n = 4, L-NAME n = 6). E Western blot (left) of total cerebral cortical protein probed for neuronal NO-synthase and α-Tubulin loading control in WT (n = 4) and tg/tg (n = 4) mice. Summary (right) of densitometric analysis of neuronal NO-synthase protein expression normalized to α-Tubulin in WT (gray) and tg/tg (white) mice. F Similar to E, Western blot (left) of endothelial NO-synthase protein expression in WT (n = 4) and tg/tg (n = 4) mice with α-Tubulin control. Densitometric analysis (right) of endothelial NO-synthase expression in WT (gray) and tg/tg (white).
Abnormal cerebral cortical responses to sensory input and direct cortical stimulation in \textit{tg/tg} mice

Given the highly abnormal activity in the cerebral cortex of the \textit{tg/tg} mouse, the next question was if the \textit{tg/tg} mouse cerebral cortex differs from WT mice in sensory evoked cortical activation or the response to direct cortical stimulation. Stimulation of the C2 whisker used a 5 Hz train at random intervals between 6-8 s. The stimulus application strategy was designed to avoid the frequency band of the LFOs. The response amplitude was defined as the power evoked in the stimulus frequency range (0.125-0.167 Hz) (Llano et al., 2009). In both WT and \textit{tg/tg} mice air-puff delivered to C2 evokes a patch of activity in the contralateral barrel cortex (Fig. 10A). The response in \textit{tg/tg} mice is reduced relative to WT ($F_{1,6} = 7.0$, $p = 0.0089$, $n = 4$ each for WT and \textit{tg/tg}; Fig. 10B) and demonstrates a deficit in the processing of sensory inputs into the somatosensory cortex.

The reduction in the response to C2 whisker puff could occur outside of the cerebral cortex. Therefore, we also investigated direct intracortical stimulation in the motor cortex using a brief 5 Hz train at random intervals between 6-8 s. Direct cortical stimulation evokes a response ipsilateral to the stimulating electrode and a transcallosal, contralateral response (Fig. 10C). As for whisker puff, the ipsilateral and contralateral responses are lower in the \textit{tg/tg} compared to the WT mice as shown in the example power maps. The population data confirms the response to direct cortical stimulation in the \textit{tg/tg} mouse is significantly reduced both ipsilaterally ($F_{1,6} = 24.0$, $p < 0.0001$, $n = 4$) and
contralaterally ($F_{1,6} = 8.7, p = 0.0037, n = 4$) compared to WT mice (Fig. 10D).

Therefore, the responses to both sensory input and direct cortical simulation is reduced in the $tg/tg$ mouse cerebral cortex and the later result demonstrates a deficit in information processing that is intrinsic to the cerebral cortex.

**Figure 26.** Cortical responses to direct electrical stimulation and sensory input are reduced in the $tg/tg$ mouse. **A** Example power maps of evoked response to air-puff stimulation of the left, C2 whisker in WT (left) and $tg/tg$ (right) mice. The air-puff stimulus consisted of train of 5 pulses of 9 PSI, 120 ms at 5 Hz delivered at random intervals of 6-8 s. These maps were generated at the frequencies of the stimulation (0.125-0.167 Hz) and not at the LFO frequency. Note a 1X objective was used, resulting in a larger imaged area compared to other figures. **B** Population summary of the power of the cortical response evoked by whisker stimulation in the WT and $tg/tg$ mouse ($n = 4$ each). **C** Similar power maps of the response to direct cortical stimulation of the motor cortex using trains of 5 pulses of 200 µA, 100 µs at 5 Hz in the WT mouse (left) and the $tg/tg$ mouse (right). Stimulation electrode is evident on left. **D** Summary of the power evoked by cortical stimulation in the ipsilateral (-i) cortex (i.e., the side with the stimulating electrode) and the contralateral (-c) response in WT and $tg/tg$ mice ($n = 4$ each).
LFOs are decreased by therapeutic agents used to treat EA2, acetazolamide and 4-aminopyridine.

We reasoned that if the LFOs in the cerebral cortex are important in the neurological abnormalities in patients with CACNA1A mutations, the agents used to treat EA2, acetazolamide (ACTZ) and 4-aminopyridine (4-AP), should reduce the LFO activity in tg/tg mice. As shown in the example experiment, bath application of the carbonic anhydrase inhibitor ACTZ (4 mM) nearly eliminates the LFO activity in a high power state to within the baseline range (Fig. 11A and C). On average, the power of the LFOs is reduced significantly by ACTZ from 218.7 ± 82.0 to 44.0 ± 51.9 ($F_{1,6} = 173.9$, $p < 0.0001$, $n = 4$; Fig. 11C) and converted from the high power state to a low power state in all of the tg/tg mice tested. Similarly, as shown in an example experiment (Fig. 10B) and the average data, bath application of 500 µM 4-AP, a K+ channel antagonist, during the high power state also greatly reduces the power of the LFOs from 377.2 ± 273.0 to 173.7 ± 208.6 ($F_{1,10} = 57.0$, $p < 0.0001$, $n = 6$; Fig. 11D), though not quite as effectively as ACTZ. Therefore, the high power LFOs that characterize the tg/tg mice are markedly decreased by the two common EA2 therapies.
Figure 27. High power LFOs are reduced by therapeutic agents used to treat EA2. A and B Examples of the frequency, power, and phase maps demonstrate the high power LFOs in the tg/tg mouse cerebral cortex in the baseline period (top row) and following (bottom row) application of either ACZT or 4-AP. C and D Summaries of average baseline (gray bars) power and the average power upon drug application (white bars), either ACZT (n = 4) C or D 4-AP (n = 6).
Figure 28. Mechanism of cerebral cortical dysfunction in P/Q channelopathies. The abnormal LFO activity is facilitated by a reduction in excitatory synaptic transmission in the cerebral cortex. The therapeutic agents (ACZT and 4-AP) reduce the LFOs by facilitating excitatory synaptic transmission. The LFOs represent the fundamental instability in the cerebral circuitry that produces episodic symptoms.

Discussion

The major finding in this study is the presence of episodic and highly abnormal LFOs in the cerebral cortex of the $tg/tg$ mouse. The oscillations are at the same frequencies observed in the cerebellar cortex, highlighting that the $tg/tg$ mutation results in similar instabilities in widely different circuits within the CNS (Chen et al., 2009). The transient nature of the LFOs in the $tg/tg$ mouse cerebellar and cerebral cortices
emphasizes the multi-faceted, episodic dysfunction characteristic of many channelopathies. Episodic abnormalities are particularly marked in the P/Q-type Ca\(^{2+}\) channelopathies (Rajakulendran et al., 2012).

Cerebral cortical oscillations represent an abnormal excitability state

Several observations demonstrate that the LFOs represent pathological CNS activity. The high power state is almost never observed in WT mice. In contrast, the power levels in the 0.035-0.11 Hz frequency band in \(tg/tg\) mice are over two orders of magnitude greater than the average power in WT mice (Fig. 2B and C). The responses to several pharmacological agents are strikingly aberrant in \(tg/tg\) mice. For example, blocking ionotropic or metabotropic glutamate receptors, as expected, reduces LFO power in WT but increases LFO power in \(tg/tg\) mice. Inhibition of NOS results in high power LFOs in the \(tg/tg\) cerebral cortex but not in WT mice. In WT controls, TTX virtually eliminates LFO power as blocking action potentials greatly suppresses neuronal activity in normal animals, including sensory input (Sachidhanandam et al., 2013) and cortico-cortical connections (Chen et al., 2013; Khalilov et al., 2003). Although TTX greatly reduces the LFOs in \(tg/tg\) mice, the high power state was not eliminated suggesting that local cellular, action potential independent mechanisms can support the oscillations. Similarly, local mechanisms appear capable of generating the LFOs in the cerebellar cortex (Chen et al., 2009). In agreement with previous \textit{in vitro} demonstrations of abnormalities in the somatosensory thalamus and cortex (Sasaki et al., 2006; Ayata et al., 2000), the cortical responses to both somatosensory inputs and to intracortical
stimulation are reduced, suggesting that normal information processing is disrupted. Decreased post-synaptic response to parallel fiber stimulation also occurs in the cerebellar cortex of the *tg/tg* mouse (Chen et al., 2009; Matsushita et al., 2002). Overall, cerebral cortical excitability and synaptic transmission is abnormal in *tg/tg* mice.

*Role of synaptic transmission in the low frequency oscillations*

The high power LFOs in the *tg/tg* mouse are not solely due to an acute decrease in P/Q-type Ca\(^{2+}\) channel function. In WT mice, acutely blocking P/Q-type Ca\(^{2+}\) channels with \(\omega\)-Agatoxin increases the power of the spontaneous cortical activity within the 0.035-0.11 Hz frequency band; however, the power remains well below the high power threshold. Furthermore, \(\omega\)-Agatoxin does not induce high power LFOs in the *tg/tg* mice. The latter finding may reflect that changes in the makeup of voltage-gated Ca\(^{2+}\) channels at axon terminals compensate for the loss of P/Q-type Ca\(^{2+}\) channels (Zhou et al., 2003; Qian and Noebels, 2000; Pardo et al., 2006; Kaja et al., 2007b; Campbell and Hess, 1999). At some central and peripheral synapses the compensation is nearly complete (Qian and Noebels, 2000; Pardo et al., 2006; Kaja et al., 2007b) and additional antagonism of P/Q-type Ca\(^{2+}\) channels may not increase the baseline deficit. The lack of high power LFOs in the presence of \(\omega\)-Agatoxin in WT animals argues that other developmental/compensatory mechanisms are operative in the *tg/tg* mouse and these changes bias the cerebral cortex to generate high power LFOs. In support of the concept that developmental/compensatory changes are important in the pathological CNS activity, the episodic dystonia does not appear before ~21 days of age in the *tg/tg* mouse (Green
and Sidman, 1962; Meier and MacPike, 1971; Seyfried and Glaser, 1985). A recent study of an EA2 allelic disorder, spinocerebellar ataxia type 6, demonstrated that the $CACNA1A$ gene encodes a transcription factor crucial for normal neuronal development in addition to its role encoding the P/Q-type Ca$^{2+}$ channel (DU et al., 2013). Therefore, a constellation of developmental and compensatory alterations in the $tg/tg$ mouse contribute to the instabilities in the cerebral cortex that lead to the high power LFOs.

Although there is compensation for the reduction in P/Q-type Ca$^{2+}$ channel function in $tg/tg$ mouse, synaptic transmission is abnormal. In $tg/tg$ mice, reductions in glutamatergic synaptic transmission occurs in the cerebellum (Matsushita et al., 2002; Chen et al., 2009), thalamus (Caddick et al., 1999) and cerebral cortex (Sasaki et al., 2006; Ayata et al., 2000). In support of reduced synaptic transmission in the cerebral cortex, the present results show decreased ipsilateral and contralateral responses to direct cortical stimulation. We reasoned that a reduction in glutamatergic synaptic transmission in $tg/tg$ mice produces instability in the cerebral cortical circuitry and that further compromise of glutamatergic synaptic transmission may increase the instability. In agreement, LFO power and the probability of entering the high power state increases with blocking ionotropic or metabotropic glutamate receptors. Given the importance of inhibitory interneurons in generating cerebral cortical oscillations (Buzsaki and Wang, 2012; Wang, 2010) and as GABAergic transmission is impaired in $tg/tg$ mice (Sasaki et al., 2006; Tehrani and Barnes, Jr., 1995), one could hypothesize that inhibitory networks play a role in the LFOs. Although it is well documented that blocking GABA$_A$ receptors
increases cerebral cortical excitability and produces seizures (Chagnac-Amitai and Connors, 1989; Dichter and Ayala, 1987), blocking GABA<sub>A</sub> receptors did not have a significant effect on the LFOs. These observations suggest normal excitatory synaptic transmission constrains the cerebral cortical network from entering the high power state in <i>tg/tg</i> mice.

*Role of nitric oxide signaling in the low frequency oscillations*

NO is an additional factor involved in the cerebral cortical LFOs as L-NAME mediated inhibition of NOS reliably produces the high power state in the <i>tg/tg</i> mouse. The contribution of NO signaling likely goes beyond modulation of cerebral blood flow, as blocking COX mediated cerebral blood flow regulation does not produce high power LFOs. NO signaling influences multiple pathways resulting in many downstream effects including modulating neurotransmission, transcription, and vascular tone (Calabrese et al., 2007). Despite similar levels of nNOS and eNOS expression in the <i>tg/tg</i> and WT mice, there is a fundamental difference in the cerebral cortical response to NOS blockade between the WT and <i>tg/tg</i> mice. Because a reduction in basal NO production reliably increases LFO activity in <i>tg/tg</i> mice, downstream signaling must serve to dampen the aberrant cerebral cortical activity that occurs during the high power state. Although there is not a consensus among reports, it is notable that NO modulates the activity of voltage-gated Ca<sup>2+</sup> channels, including facilitating channel activity (Ohkuma et al., 1998; Chen et al., 2002; Jian et al., 2007; Chen and Schofield, 1995; Hirooka et al., 2000) (see however Carabelli et al., 2002; D'Asczenzo et al., 2002; Schuchmann et al., 2002; Yoshimura et al.,
In the \textit{tg/tg} mouse, NO may facilitate voltage-gated Ca\textsuperscript{2+} channel conductance and help compensate for the reduced activity of the mutant P/Q-type Ca\textsuperscript{2+} channels and impaired synaptic transmission.

\textit{Role for low frequency oscillations in episodic cortical dysfunction}

Many studies in the \textit{tg/tg} mouse have focused on the motor phenotypes, mild ataxia and episodic dystonia, that have been attributed to cerebellar dysfunction (Walter et al., 2006; Raike et al., 2013; Neychev et al., 2008; Campbell et al., 1999). In the human \textit{CACNA1A} diseases, cerebral cortical symptoms are common including migraine and epilepsy as well as sensory motor and cognitive impairments (Jen et al., 2001; Jouveneau et al., 2001; Van and Szliwowski, 1996; Baloh et al., 1997; Rajakulendran et al., 2012; Nachbauer et al., 2014). These diverse findings are thought to reflect abnormalities in either neurovascular coupling (migraine) or abnormal synaptic transmission (epilepsy). The LFOs in \textit{tg/tg} mice provide a possible mechanism for transient cerebral cortical dysfunction in EA2. Therefore, the reduction of LFOs by ACZT and 4-AP argue for a connection between the oscillations in \textit{tg/tg} mice and the human P/Q-type Ca\textsuperscript{2+} channelopathies. In EA2 patients, ACTZ reduces the frequency and severity of ataxic bouts (Griggs et al., 1978) and ameliorates non-cerebellar features including migraine attacks and abnormal EEG activity (Neufeld et al., 1996; Zasorin et al., 1983). Similar to ACTZ, 4-AP effectively decreases attack frequency and severity, interictal ataxia, and migraine occurrence in EA2 patients (Lohle et al., 2008; Strupp et al., 2004; Jung et al., 2010).
In conclusion, abnormal synaptic transmission is central to the episodic LFOs in the cerebral cortex of the \textit{tg/tg} mouse. This conclusion is based on the observations that decreased glutamatergic synaptic transmission and NO synthesis facilitates high power LFOs. The conclusion is reinforced by the corollary findings that LFOs are reduced by 4-AP, an agent that enhances intracellular Ca\textsuperscript{2+} influx and neurotransmitter release (Smith et al., 2000), as well as by ACTZ a drug with multiple effects including the enhancement of NO production (Aamand et al., 2009). In addition to glutamatergic synaptic transmission, the role of NO signaling in the LFOs suggests a deficit in neurovascular coupling. The highly abnormal excitability state represented by the cerebral cortical LFOs is a novel finding and offers a potential mechanism for the non-cerebellar dysfunction in P/Q-type Ca\textsuperscript{2+} channelopathies (Fig. 28).

The findings also raise numerous questions about the LFOs including how they are triggered, propagated within the cerebral cortex and the functions disrupted.
CHAPTER V: FINAL DISCUSSION AND FUTURE EXPERIMENTS

Functional implications of regional differences in PF mediated PC activation

As outlined in the Introduction, the role of PFs in the computations performed by the cerebellar cortex is controversial. Chapter 2 demonstrates that previous apparently contradictory results on whether PFs activate patches or beams of PCs may be due to regional differences in PC phenotype. Therefore, the transfer of information between the mossy fiber-GC inputs to PCs is not uniform. The new findings in Chapter 2 provide evidence of a more complex role for PFs in cerebellar computations and function. An important question arises from these findings: what role do regional differences in the pattern of PF mediated PC activation play in the cerebellar control of movement?

Based on the findings in Chapter 2 and other recent work (Ebner et al., 2012; Apps and Hawkes, 2009), the regional differences in the pattern of GC-PF-PC activation could act to coordinate the movements of different body parts across parasagittal zones of PCs. This view would be an update of an established hypothesis that representations of the body are separately encoded in each of the DCN and that the DCN operate as parallel loops, each controlling different aspects of movement (Thach et al., 1992). The PFs serve to link PCs and synchronize the actions of different body regions encoded within DCN producing coordinated movement. The hypothesis predicts that the spatial pattern of co-activated PCs would depend on the number of joints and/or synergistic muscle
groups co-activated to perform a particular movement. Tasks necessitating coordination across multiple body regions (e.g., multi-joint movements) and, therefore, coordination across the cerebellar nuclei would require the beam-like activation of PCs.

Alterations in PF-mediated PC activation disrupt normal motor control

Mutant mice provide evidence for the fundamental importance of differences in parasagittal zones to the computations performed by the cerebellum and subsequent motor output. Departures from the normal parasagittal organization of the cerebellar circuitry exhibit impaired motor control. Examples of this are the mutant $tg/tg$ mouse and a mouse model of the human disorder spinocerebellar ataxia type 5 (SCA5). Both mice exhibit abnormalities in the cerebellar circuitry resulting in altered patterns of PC activation (Armbrust et al., 2014). Both mouse lines also exhibit little evidence of neurodegenerative PC loss until an advanced age, therefore, reducing a major cofound to understanding how abnormalities in the cerebellar circuitry produce impaired motor control (Levitt, 1988; Isaacs and Abbott, 1995; Sawada et al., 2009; Armbrust et al., 2014).

As described in Chapter 3, the circuitry of the $tg/tg$ mouse exhibits fundamental departures from the cerebellar circuitry of WT mice. The responses to PF and GC stimulation are impaired. Specifically, both the amplitude and the mediolateral extent of PC activation evoked by either direct electrical stimulation of PFs or the pharmacological activation of GCs are attenuated compared to WT mice. Similarly, the sensory evoked
PC patch response is nearly absent in the $tg/tg$ mouse. The altered responsiveness of PCs to GC and PF input likely contributes to the ataxia exhibited by the $tg/tg$ mouse and supports the hypothesis that PFs are integral to normal cerebellar motor control.

Similar to the $tg/tg$ mouse, a recently described mouse model of the human disorder SCA5 also exhibits altered PF to PC excitability (Armbrust et al., 2014). SCA5 is a neurodegenerative disease characterized by limb and gait ataxia (Ranum et al., 1994; Burk et al., 2004) and is caused by mutations in $SPTBN2$ (Ikeda et al., 2006) the gene encoding the $\beta$-III spectrin protein. $\beta$-III spectrin is highly expressed in PCs and plays a role in anchoring and restricting the movement of EAAT4 and mGluR1$\alpha$ in the PC plasma membrane. The anchoring function is disrupted in known mutant forms of $\beta$-III spectrin (Jackson et al., 2001; Ikeda et al., 2006; Armbrust et al., 2014). As described above, both EAAT4 and mGluR1$\alpha$ are non-uniformly expressed at higher levels in parasagittal zones of zebrin II+ PCs.

As in the human disorder, the SCA5 mice exhibit progressive cerebellar degeneration and impaired motor coordination. Flavoprotein autofluorescence and Ca$^{2+}$ optical imaging in SCA5 mice demonstrated multiple abnormalities in the cerebellar circuitry. The mGluR1 component of the postsynaptic PC response is impaired. Similarly, mGluR1 mediated postsynaptic long-term potentiation to PF input is reduced in SCA5 mice. In addition, the mGluR1-dependent long-latency patch response to high frequency PF stimulation is
reduced in SCA5 mice and the long-latency patches do not exhibit long-term potentiation.

The altered sub-cellular localization of mGluR1 was found to underlie several of the cerebellar circuit abnormalities in the SCA5 mouse. Similarly, the sub-cellular mislocalization of EAAT4 may also contribute to cerebellar dysfunction. Though not investigated, one could hypothesize that the abnormal localization of mGluR1 and EAAT4 extends to the parasagittal expression of these proteins, further contributing to impaired motor coordination in the SCA5 mouse. The hypothesis would be consistent with the finding that intrathecal infusion of an antisense oligonucleotide to EAAT4 (but not the glial glutamate transporters EAAT1, EAAT2, or EAAT3) is sufficient to produce cerebellar ataxia (Maragakis et al., 1997; Raiteri et al., 2002).

In conclusion, the tg/tg and SCA5 mouse are two examples of how alterations in the spatial pattern and strength of PF mediated PC activation cerebellar cortex in the absence of significant PC loss may disrupt normal cerebellar function.
Future experiments to determine the mechanism of reduced PF mediated PC response in the tg/tg mouse cerebellar cortex

The findings in Chapter 3 demonstrate that the response to PF or GC stimulation is reduced, both the amplitude and mediolateral extent. The implication is that the excitability of GCs, PFs and/or PCs is impaired in the tg/tg mouse. However, paired-pulse facilitation, a measure of presynaptic release, does not differ between tg/tg and WT mice (Matsushita et al., 2002; Zhou et al., 2003) suggesting the impaired response is not due to altered synaptic release. If glutamate release from PFs is not responsible for the decreased responsiveness, there are several other mechanisms that could account for this phenomenon.

First, PF and/or GC excitability could be reduced. To test GC and PF excitability, in vivo two-photon Ca$^{2+}$ imaging could be utilized. The strategy is to use an adeno-associated viral vector to sparsely express a Ca$^{2+}$ indicator, GCaMP, in the cerebellar GCs of tg/tg and WT mice (Jackman et al., 2014; Akerboom et al., 2012; Tian et al., 2012). Restricting the Ca$^{2+}$ indicator to a sparse population of granule cells affords the ability to specifically identify granule cells based on their distinctive morphology and avoid contamination of the Ca$^{2+}$ signals from other cell types (e.g., PCs) that may also be transduced by the virus. Two photon imaging is critical to achieve single cell resolution, allowing the identification of PF axons and GC somata in vivo. The remaining approach is similar to that outlined in Chapter 3. An electrical stimulus is applied to the ipsilateral C3 vibrissae and the responses of the PFs and GC somata compared between WT and tg/tg mice. This
approach tests if there is a decrease in the excitability of the GCs and/or the PFs in the \(tg/tg\) mouse. If a deficit is found, 4-AP application is tested to determine if the reduced GC response in the \(tg/tg\) is rescued. It would also be interesting to test if the other major therapy in EA2, ACTZ rescues the reduced responsiveness (Griggs et al., 1978).

Another possible mechanism for the attenuated PF-PC response in the \(tg/tg\) mouse is that AMPA receptors on PCs could be reduced. In another \(Cacna1a\) mutant, the \(rocker\) mouse that similarly exhibits reduced PF-PC transmission in the absence of impaired paired-pulse facilitation, AMPA receptors on PCs are diminished (Kodama et al., 2006). In the \(tg/tg\) mouse, whether AMPA receptors on PCs are reduced or have decreased responsiveness has not been examined, to our knowledge. Therefore, an important next step would be to measure the AMPA receptor number and density on PCs in the \(tg/tg\) mouse. One approach would be to employ a similar freeze-fracture technique as was used to examine the \(rocker\) mouse PCs (Kodama et al., 2006; Tanaka et al., 2005).

The third possible mechanism is a reduction in PC excitability due to reduced P/Q-type \(Ca^{2+}\) currents. This mechanism is highly likely given that P/Q-type \(Ca^{2+}\) channels account for 70-80\% of voltage-gated P/Q-type \(Ca^{2+}\) currents on WT PCs (Mintz et al., 1992; Llinas et al., 1992). Also, compensatory enhancement of L-type \(Ca^{2+}\) channel expression occurs in \(tg/tg\) PCs (Campbell and Hess, 1999). Diminished and/or changes in the voltage-gated \(Ca^{2+}\) channels on PCs in \(tg/tg\) mice could affect the integration of PF inputs. Furthermore, alterations in PC dendritic morphology have been described in the \(tg/tg\) mouse, including ectopic dendritic spines and altered dendritic geometry (Rhyu et
al., 1999), which could decrease somatic responses by altering current spread down the dendritic processes (Roth and Hausser, 2001; Vetter et al., 2001).

To test if $tg/tg$ mouse PCs exhibit abnormal excitability, whole cell voltage and current clamp recordings of PCs in cerebellar slices could be performed (Ovsepian and Friel, 2008). The whole cell recording configuration allows the comparison of depolarization induced PC responses between $tg/tg$ and WT mice. Measures of PC electroresponsiveness such as action potential threshold as well as depolarizing current evoked initial firing rate and train duration could be obtained. Furthermore, alterations of any of the measures of PC electroresponsiveness could be further investigated to determine the specific channels that contribute to any PC abnormalities in the $tg/tg$ mouse.

**Future experiments to test functional consequences of parasagittal differences in cerebellar excitability**

The observation that a beam of PCs is evoked in Crus I while patches are evoked in Crus II to sensory stimuli prompts the question: what is the functional role of parasagittal differences in the pattern of PC activity evoked by mossy fiber-GC input? To answer this important question, the patterns of PC responses to peripheral sensory input throughout the cerebellum should be investigated. Based on the finding that expression levels of EAAT4 influence the spatial extent of PC activation to GC-PF input, the known expression profile of EAAT4 provides a basis for predicting the responses in different
folia. For example, similar to Crus I other folia including lobules VIb, VII, IX, and X as well as the flocculonodular lobule uniformly (or nearly uniformly) express EAAT4 (Gincel et al., 2007; Apps and Hawkes, 2009; Hawkes and Herrup, 1995). The remaining folia exhibit parasagittal bands of high and low EAAT4 expression similar to Crus II. While extrapolation of the response patterns found in Crus I and Crus II to folia with similar EAAT4 expression profiles is tempting, it is possible that factors in addition to EAAT4 influence the geometry of the PC response to mossy fiber-GC-PF input. Furthermore, the other factors may be non-uniformly distributed in the cerebellar cortex in a pattern that is out of register with EAAT4 expression.

The systematic mapping of peripherally evoked PC responses in as many folia as possible would provide further validation of the findings in Chapter 2. Additionally, the mapping results would demonstrate whether the differential contribution of PFs to the responses in Crus I and Crus II is generalizable to the cerebellar cortex or if further regional variations exist. The experimental approach would be a modified version of the Ca$^{2+}$ imaging utilized in Chapter 2 as it affords the spatial and temporal sensitivity to define the response patterns evoked by peripheral input to the cerebellar cortex (Cramer et al., 2013; Gao et al., 2006). Because the strategy is to examine as much of the cerebellar cortex as possible, bulk loading of Ca$^{2+}$ indicator is impractical. A mouse line expressing a genetically encoded calcium indicator, such as GCaMP, specifically in PCs is needed to visualize superficial folia across the cerebellar cortex (Zariwala et al., 2012). Ca$^{2+}$ optical imaging in vivo is used to monitor the response in the cerebellar cortex to stimuli applied
to various peripheral locations. The pattern of the evoked PC responses could then be used to construct a response map of the cerebellar cortex to sensory inputs. While sensory mapping of the cerebellar cortex has been performed previously in rodents (Shambes et al., 1978b; Shambes et al., 1978a; Bosman et al., 2010; Bower and Kassel, 1990), these studies examined a limited number of folia and the electrophysiological techniques used are not optimal for large scale spatial mapping.

A multi-folia map of peripherally evoked PC activation patterns could serve as a guide for investigating the series of questions. First, are different patterns of PC activation (i.e., beams and patches) produced in the cerebellar cortex of awake mice during motor behavior? Similar to previous awake imaging studies (Najafi et al., 2014; Chen et al., 2009), the mouse line expressing GCaMP in PCs would undergo a survival surgery to secure a head-post to the cranium and prepare the bone for transcranial optical imaging of the cerebellar cortex. The animal with its head fixed is secured over a running wheel (Najafi et al., 2014) and the motor behavior monitored. This configuration allows the synchronized monitoring of the limbs, trunk, and tail kinematics simultaneous with the optical imaging to determine if patches and/or beams of PC activation occur during locomotion.

An association between movements and patterns of PC activation (i.e., beams and patches) would provide the framework to address the next question: what role does the pattern of PC activity play in motor control? An optogenetic approach is well suited to
testing this question as it affords the spatiotemporal control as well as the cell type specificity necessary to target a subpopulation of PCs (Witter et al., 2013; Fenno et al., 2011; Chaumont et al., 2013; Nguyen-Vu et al., 2013). The experimental design is similar to the awake mapping investigation described above, though the animals in this experiment express both the light gated, third generation inhibitory chloride pump halorhodopsin (eNpHR3.0) and GCaMP in PCs. The dual PC specific expression of eNpHR3.0 and GCaMP3 allows the simultaneous monitoring and manipulation of PC activity.

The strategy is to use the $\text{Ca}^{2+}$ optical imaging to define regions of the cerebellar cortex that exhibit locomotion (or another motor behavior) evoked PC beams and regions that exhibit PC patches. After a baseline period, photostimulation is delivered to inhibit a subpopulation of PCs within the patch or beam. The hypothesis predicts that partial disruption of the synchronous activation of PCs will impair the coordination of the corresponding movement. The changes observed would provide insight into the distinct roles of beam versus patch activation. For example, does inhibition of patch regions effect more distinct aspects of locomotion (i.e., single joints) and does inhibition of beams more global aspects (i.e., multi-joint coordination)?

The next experiment would address the corollary question of the effects of activating a patch or beam of PCs on motor control? Instead of inhibiting a subpopulation of PCs, this experiment excites PCs activated during normal motor control using the excitatory,
light-gated cation channel, channelrhodopsin-2 (ChR2) in PCs. Using the same behavioral evaluation proposed for optogenetic inhibition of PCs, this experiment will assess how beam versus patch activation of PCs effects locomotion. These experiments are crucial to determine the functional role of different patterns of PC activation in motor behavior and, therefore the role PFs serve in the cerebellar cortex.

In conclusion, expanded mapping of the patterns of PC activation in response to sensory inputs followed by the mapping of patterns of PC activation in awake mice is an important extension of the initial findings discussed in Chapter 2. Furthermore, the manipulation of patterns of synchronous PC activity in awake mice would provide insight into the functional role of PC beams and patches and, therefore the role of PFs in motor control.

Cerebral cortical LFOs and phenotypic abnormalities in the \textit{tg/tg} mouse

The results of Chapter 4 raise an important question: what are the functional consequences of the high power, cerebral cortical LFOs in the \textit{tg/tg} mouse? The complex phenotype of the \textit{tg/tg} mouse consisting of distinct episodic abnormalities, paroxysmal dystonia and absence seizures, requires additional experiments to link the cerebral LFOs to a phenotypic trait. However, two pieces of evidence suggest that the cerebral LFOs contribute to attacks of episodic dystonia. First, the attacks evolve in a highly reproducible and stereotypical progression reminiscent of a “Jacksonian march” (Jackson,
1870; Charcot J.M. and Pitres A., 1877), implying the systematic spread of abnormal neural activity across a region of the brain with a highly organized somatotopy, such as the motor cortex. The cerebellum is not known to contain a similar somatotopic organization (Shambes et al., 1978b; Shambes et al., 1978a; Kassel et al., 1984; Bower and Woolston, 1983; Rowland and Jaeger, 2005; Allen et al., 1977; Allen et al., 1978). Second, a Cacna1a mouse line that expresses one mutant P/Q-type Ca\(^{2+}\) channel \(tg\) allele and one null allele throughout the CNS (Tottering\(^{haplo}\) mice) exhibits heightened sensitivity to attack triggers compared to the Tottering\(^{PC-haplo}\) mouse that expresses one \(tg\) allele and one null allele in PCs but is otherwise heterozygous at the P/Q-type Ca\(^{2+}\) channel gene locus (Raike et al., 2013). The difference in trigger sensitivity between these two mouse lines suggests that structures outside the cerebellum contribute to the motor attack.

Alternatively, if the cerebral cortical LFOs do not contribute to the episodic dystonia, the LFOs could relate to other episodic abnormalities known to occur in P/Q-type Ca\(^{2+}\) channelopathies. In the human CACNA1A diseases, cerebral cortical symptoms such as migraine and epilepsy as well as sensory motor and cognitive impairments are common (Jen et al., 2001; Jouveneau et al., 2001; Van and Szliwowski, 1996; Baloh et al., 1997; Rajakulendran et al., 2012; Nachbauer et al., 2014). These diverse findings are thought to reflect abnormalities in either neurovascular coupling (migraine) or abnormal synaptic transmission (epilepsy). The LFOs in \(tg/tg\) mice provide a possible mechanism for transient cerebral cortical dysfunction in EA2. Therefore, the reduction of LFOs by ACZT and 4-AP argue for a connection between the oscillations in \(tg/tg\) mice and the
human P/Q-type Ca$^{2+}$ channelopathies. In EA2 patients, ACTZ reduces the frequency and severity of ataxic bouts (Griggs et al., 1978) and ameliorates non-cerebellar features including migraine attacks and abnormal EEG activity (Neufeld et al., 1996;Zasorin et al., 1983). Similar to ACTZ, 4-AP effectively decreases attack frequency and severity, interictal ataxia, and migraine occurrence in EA2 patients (Lohle et al., 2008;Strupp et al., 2004;Jung et al., 2010). The efficacy in reducing cerebral LFOs by the agents commonly used to treat EA2 suggests that the LFOs represent a mechanism that contributes to EA2 pathogenesis.

Cerebellar and cerebral LFOs in the tg/tg mouse

As discussed, some features of the episodic dystonia exhibited by the tg/tg mouse are inconsistent with abnormal motor activity deriving solely from the cerebellum. Despite this, there is substantial evidence supporting the necessary contribution of the cerebellum to episodes of dystonia (Campbell et al., 1999;Neychev et al., 2008;Raike et al., 2013;Walter et al., 2006;Chen et al., 2009). A new model is required that incorporates the roles of both the cerebellum and the cerebral cortex in the episodic dystonia.

The cerebellar output via the projections of DCN neurons influences the activity of many central motor areas. For example, many cerebral cortical areas receive cerebellar input via thalamic nuclei including primary motor cortex, premotor cortex, prefrontal cortex, and posterior parietal areas (Allen and Tsukahara, 1974;Evarts and Thach, 1969;Bostan
et al., 2013). Cerebral cortical areas receiving of cerebellar output also project back to the same region in the cerebellum, forming recurrent loops (Strick et al., 2009). The DCN also projects to sensorimotor areas of the basal ganglia (Bostan and Strick, 2010; Bostan et al., 2010; Hoshi et al., 2005) which in turn form looped projections with many cerebral cortical areas (Alexander et al., 1986). Therefore, the recurrent interconnections of the cerebellum-thalamus-basal ganglia-cerebral cortical systems may serve as a conduit by which abnormal cerebellar activity in the \( \text{tg/tg} \) mouse drives pathological activity in other central motor structures and leads to the rhythmic muscle contractions characteristic of episodes of dystonia (Chen et al., 2009; Campbell and Hess, 1999; Scholle et al., 2010; Raike et al., 2013; Fan et al., 2012).

Support for this hypothesis is provided by a recent investigation of the abnormal circuitry underlying the development of Rapid-Onset Dystonia-Parkinsonism (RDP) (Calderon et al., 2011; Fremont and Khodakhah, 2012). The study illustrates how the synergistic interactions of pathological activity in different CNS structures produce motor impairment. The motor phenotype of the pharmacological RDP mouse model exhibits both parkinsonian symptoms attributed to basal ganglia dysfunction and a generalized dystonia attributed to cerebello-basal ganglia dysfunction. The authors tested the motor cortical contribution to the hypothesized cerebello-basal ganglia derived dystonia and found that bilateral TTX application to the motor cortex reduced the frequency and severity of dystonia. Furthermore, the dystonia exhibited by the RDP mouse is ameliorated by eliminating cerebellar output or disrupting cerebellar projections to the
basal ganglia by lesioning the centrolateral nucleus of the thalamus (Calderon et al., 2011). The phenotype of the RDP mouse exhibits some similarities to the \(tg/tg\) mouse. Therefore, it is plausible that the cerebello-thalamo-basal ganglo-cerebral cortical circuitry contributes to the propagation of the abnormal LFOs from the cerebellar to cerebral cortex of the \(tg/tg\) mouse to produce the episodic attacks of dystonia.

_Future experiments to investigate functional consequences of cerebral cortical LFOs and cerebellar-cerebral cortical interaction in episodic dystonia_

A primary question raised by Chapter 4 is if the LFOs in the cerebral cortex of the \(tg/tg\) mouse are involved in the episodic dystonia. To address this question, the experimental design consists of transcranial flavoprotein and two-photon \(Ca^{2+}\) imaging in the awake \(tg/tg\) mouse cerebral cortex in conjunction with simultaneous electromyographic and video behavioral recordings to monitor the dystonic attacks. Both imaging methodologies are needed as flavoprotein allows monitoring the LFOs throughout large regions of the cerebral cortex and the two-photon imaging allows monitoring individual neurons and glia during the attacks.

A head restraint devices is surgically implanted in \(tg/tg\) mice (Chen et al., 2009) and the cerebral cortex prepared for transcranial imaging (Hishida et al., 2011; Yang et al., 2010; Drew et al., 2011; Drew et al., 2010). A small burr hole is placed in the cranium to allow microinjection of drugs and \(Ca^{2+}\) indicator for those animals undergoing two-
photon imaging (Lee et al., 2006; Houweling and Brecht, 2008). In the restrained animals baseline activity is monitored followed by a challenge with caffeine, a reliable attack trigger. The ensuing attack is monitored and the analyses focused on the spectral content of the flavoprotein fluorescence as well as the glial and neuronal Ca\(^{2+}\) response during the baseline, the dystonic attacks and the post attack period in relation to the behavior. In conjunction with serum fluorescence labeling, two-photon measurements of cerebral blood flow dynamics are also acquired and evaluated for the baseline, attack, and post attack periods to determine if alterations in neurovascular coupling occur during attacks (Drew et al., 2010; Drew et al., 2011).

Using the spectral analysis developed in Chapter 4, the first goal is to determine if upon attack initiation the cerebral cortex exhibits LFOs as observed in anesthetized \(tg/tg\) mice. The hypothesis predicts that the LFOs will be highly coupled with the episodic dystonia. In particular, the analyses focus on whether the LFOs occur in the motor areas of the cerebral cortex. The two-photon Ca\(^{2+}\) imaging data is similarly analyzed to determine if the neurons or glia exhibit LFO activity. If both cell types exhibit oscillations, additional analyses are performed to determine which cell type develops oscillations first relative to attack onset, and ultimately whether both cell types are necessary for the episodic dystonia.

The frequency band and the power of the LFOs may deviate from the anesthetized mouse as was observed in a similar previous comparison of the cerebellar LFOs between the
anesthetized and awake state (Chen et al., 2009). The exact timing between the LFOs and the motor attacks will be evaluated. A correlation between the onset of LFOs and dystonia would support the hypothesized link between the abnormal cerebral cortical activity and the dystonic phenotype of the \textit{tg/tg} mouse. Furthermore, the two-photon data would provide insight into possible cellular mechanisms of both the LFOs and the attacks.

If a relationship is found between the cerebral cortical LFOs and the attacks, the next experiments test if the oscillations are necessary for episodes of dystonia. In the first experiment, the agents (4-AP and ACTZ) shown to suppress the cerebral cortical LFOs in the anesthetized mouse and used to treat the human P/Q-type Ca$^{2+}$ channelopathies are infused into the cerebral cortex of the \textit{tg/tg} mice prior to exposing the mice to an attack trigger to determine if suppression of the LFOs and the dystonia occurs concurrently. A reduction in the attack frequency, severity and/or duration with 4-AP or ACTZ in conjunction with the absence of LFOs would demonstrate that the cerebral cortex contributes to the attacks. If the attacks are blocked this would suggest the LFOs are integral to the episodic dystonia. In contrast, if the therapeutic agents suppress the cerebral cortical LFOs without affecting the attacks, it would imply that the cerebral LFOs are not necessary for the dystonic episodes.

The final experiments test how the LFOs in the cerebellar cortex relate to the LFO activity in the cerebral cortex. To test this question, simultaneous optical imaging of the
cerebral cortex and multi-unit extracellular recordings of cerebellar PCs. In this way, the
temporal relationship of the onset of LFOs may be compared between these two
structures, and the initiating structure identified. Based on previous studies
demonstrating that the cerebellum is necessary for episodic attacks (Campbell et al.,
1999; Neychev et al., 2008; Raike et al., 2013; Walter et al., 2006; Chen et al., 2009), the
hypothesis predicts that the cerebellar cortex initiates the abnormal LFOs in the \textit{tg/tg}
mouse CNS. Also, the effect of manipulating the LFOs in the cerebellar cortex has on
the cerebral cortex or vice-versa would be studied. For example, how does reducing
ACZT or 4-AP applied to the cerebral cortex alter the LFOs in the cerebellum?
Conversely, how does blocking LFOs in the cerebellar cortex with L-type Ca\textsuperscript{2+} channel
blockers (Chen et al., 2009) effect the LFOs in the cerebral cortex? This set of
experiments would be highly informative on how the cerebral cortex and cerebellar
cortex interact to generate the LFOs and episodic dystonia.
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