Filling in the Gaps: Cerebellar Granule Cell Layer Oscillations and the Role of Gap Junctions in the Anesthetized Rat

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ABSTRACT

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Local field potential (LFP) oscillations within the granule cell layer (GCL) of the cerebellar cortex have been recorded in the alert, awake rat at frequencies between 5-15 Hz. In rodents and primates, these oscillations occur while the animal is immobile, and terminate with the initiation of movement. The low-frequency oscillations are related to Golgi-granule cell activity; Golgi cells afferent connections show strong divergence and are heavily implicated with the temporal organization of the cerebellar cortex. Gap junctions have been identified within the GCL between Golgi cells, indicating a possible mechanism by which the Golgi cells electrical potential could spread quickly across the GCL, synchronizing the oscillations. Here, the presence of these oscillations under anesthesia was recorded in order to study both the regional specificity and if these oscillations can be pharmacologically modulated. Regional specificity was of oscillations was shown with the recordings in cerebellar cortex across multiple channels. These oscillations are related to Golgi-granule cell activity: a modulatory factor might be gap junction connections between Golgi cells, which are believed to be involved in the synchronization of neuron populations resulting in synchronous rhythms. To investigate the modulatory effect of gap junctions, the oscillatory power of signal in the presence of gap junction blockers carbenoxlone and mefloquine was examined. Drugs were locally introduced into the cerebellar cortex, resulting in a consistent decrease in the power in oscillations between 5-15 Hz. These findings add to the mechanistic description of the genesis of cerebellar GCL oscillations.

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CHAPTER 1

GENERAL INTRODUCTION

In order to appropriately interact with our environment, we require the ability to integrate external influences and coordinate specific responses. Seemingly simple movements, such as pouring a cup of coffee, require the complex integration of various brain structures involved in sensory processing and motor control. The successful planning and execution of movements, whether simple or complex, requires extensive and specific communication and synchronization among visual, vestibular, somatosensory and motor areas. Among this network of structures responsible for motor control, the cerebellum has been proposed as contributing to timing, coordination and balance of movements.

In 1809, Luigi Rolando observed that damage to the cerebellum results in general motor control problems, the first report of functions supported by this structure (Glickstein, Strata, & Voogd, 2009). In subsequent animal experiments throughout the 1800's, it was found that damage to the cerebellum did not result in a complete loss of movement, but rather an overall loss of coordination (For review see Ito, 2002). Damage or dysfunction of the cerebellum does not inhibit movement per se; instead, it results in the specific loss in the fine tuning and regulation of motor control. Further investigation into the role of this brain structure has lead to findings implicating the cerebellum with coordination and timing, as well as demonstrating its role in attention, language and other cognitive functions (Ito, 2006; Wolf, Rapoport, & Schweizer, 2009).

An important tool in studying neural activity in the central nervous system (CNS) is the recording of oscillations, rhythmic fluctuations in electrical potential generated by neural tissue. Recordings of local field potential (LFP) oscillations within the cerebellum have contributed to advances in the understanding of the function and organization of neural activity in this structure. In studying the role of the cerebellar cortex in motor

control, low-frequency oscillations have been recorded in awake, alert animals (Courtemanche, Pellerin, & Lamarre, 2002; Hartmann & Bower, 1998; Pellerin & Lamarre, 1997). How these oscillations are modulated within the cerebellar cortex is not yet understood. The aim of the current study is to alter these oscillations and to elucidate a modulatory mechanism for rhythm generation in the cerebellar cortex. We begin with an overview of the anatomical and circuitry elements of the cerebellar cortex and its signaling inputs and outputs, followed by a review of signal patterns and oscillatory activity in general motor control, and specifically, the activity in the cerebellar cortex. Finally, based on previous findings, we hypothesize a possible mechanism by which these oscillations may be modulated.

1.1 Anatomy & Circuitry Elements

The cerebellum is a very distinct structure in the CNS. Once known as the "lesser brain" (Bower & Parsons, 2003), it appears to be completely separate from the cerebrum, attached only through fibers extending from the brainstem. The cerebellum is a mass of tightly overlapping layers, with an immense number of neurons held inside. This structure is made up of three lobes: anterior, posterior and flocculonodular (Voogd, 2003). The anterior and posterior lobes are separated by the primary fissure. Underneath the posterior lobe is the flocculonodular lobe that rests against the posterior side of the brain stem (Voogd, 2003). The cerebellum receives input for three distinct sources: the spinal cord, the vestibular system and the cerebral cortex. The spinal cord provides information on position of muscles via the ventral spino-cerebellar tract (Saab & Willis, 2003). The vestibular system provides information on equilibrium and the cerebral cortex relays primary motor and sensory input (Saab & Willis, 2003).

The cerebellar cortex is composed of a diverse number of cell types, organized into cellular layers. Like the cerebrum, the cerebellum is made up of white matter and a gray matter outer cortex. The white matter contains deep cerebellar nuclei (DCN) which are the interface for outgoing cerebellar signaling (Saab & Willis, 2003; Voogd, 2003). The outer gray matter integrates these signals across three cellular layers. The most superficial cellular layer is the molecular layer, followed by the Purkinje cell layer and the GCL (Bower, 2002; Llinas, Walton, & Lang, 2004).

Each layer of the gray matter contains different cell types (Fig. 1.1). The somas of granule and Golgi cell bodies, along with Lugaro and unipolar brush cells rest within GCL (not shown in Fig. 1.1) (Geurts, Timmermans, Shigemoto, & De Schutter, 2001; Melik-Musyan & Fanardzhyan, 2004). The Golgi cell is an inhibitory interneuron whose axonal projections mostly remain in the GCL, while granule cell axons extend up to the molecular layer (Llinas et al., 2004). Above the GCL, the Purkinje soma rests within the Purkinje cell layer, its elaborate branching network of dendrites extending up to the molecular layer where it has synaptic connections with many different neurons. The molecular layer contains parallel fibers initially coming from the granule cells, dendrites of the Purkinje cell as well as basket and stellate cells (Eccles, 1967; Kalinichenko & Okhotin, 2005).

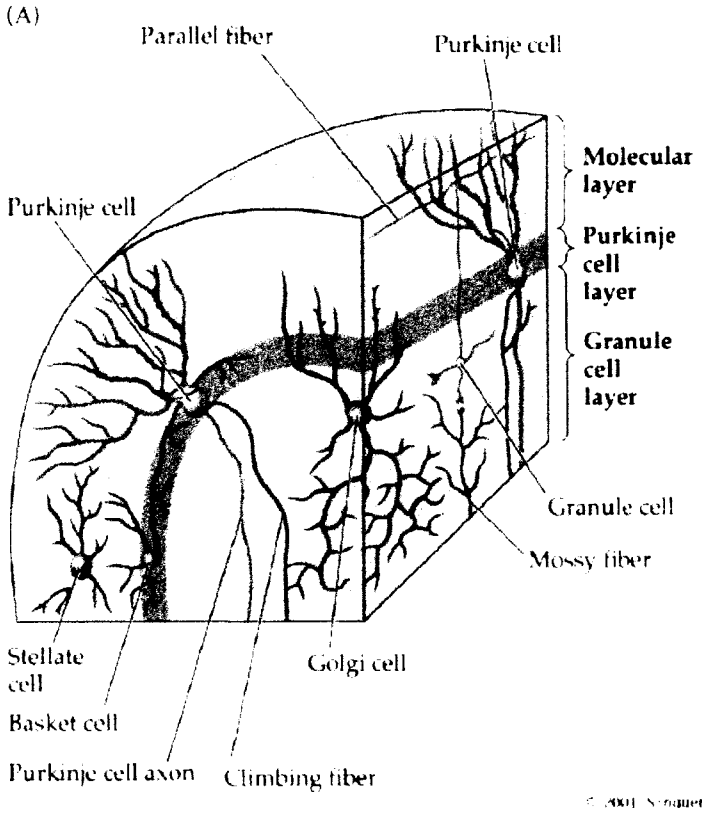

Figure 1.1: Gray matter of the cerebellum showing three distinct layers: Molecular, Purkinje cell and granule cell layer (From Purves et al., 2001).

1.2 Afferent Cerebellar Pathways

The cerebellar cortex receives input through two fiber pathways; climbing fibers and mossy fibers. Both pathways carry information from different brain regions, but share a common endpoint, Purkinje cells. The climbing fiber pathway is a simple, potent, and direct system for the transmission of excitatory inputs. Climbing fiber axonal projections transmit information from the inferior olivary nucleus directly to the Purkinje

cell carrying low-frequency but powerful inputs from the spinal cord, brain stem, cerebellar nuclei and the motor cortex. One climbing fiber synapses with one Purkinje cell at many dendritic points, forming a very strong connection (Llinas et al., 2004).

The second afferent pathway of the gray matter is mossy fibers. The mossy fiber pathway is a more intricate pathway, involving a divergence of signals across many interneurons, but whose destination is also the Purkinje cell. Mossy fibers transmit signals from the cerebral cortex, spinal cord, vestibular nerve and reticular formation (Llinas et al., 2004). The fibers enter through the superior, middle and inferior cerebellar peduncles and continue up to the GCL and form connections with granule and Golgi cell. Golgi and granule cells all receive input signals, and through this heavily interconnected network, the neurons integrate the information received (Marr, 1969). The granule cell axons travel up to the Purkinje cell layer and connect to an overlying Purkinje cell, but also forms a t-shaped bifurcation, becoming a parallel fiber, to contact with the dendrites of a row of Purkinje cells, Golgi cells and interneurons in the molecular layer and GCL (Bower, 2002). Each Purkinje cell may receive up to 200 000 parallel fiber synapses with a weaker resulting output (Harvey & Napper, 1991).

1.3 Signal Patterns

The Purkinje cells produce two output signals, simple and complex spikes, both displaying a striking contrast. Complex spikes occur from climbing fiber activation and are characterized by an all-or–nothing system and have very low frequencies (Llinas & Hess, 1976). Due to the climbing fibers' extensive synaptic branching with the Purkinje cell dendrites, this pathway results in a powerful multi-peaked action potential by the Purkinje cell which has a long duration (Granit & Phillips, 1956). Complex spikes are

characterized by a very low-frequency output, with increases and decreases time-locked to limb direction and speed (Ebner, 1998; Ebner, Johnson, Roitman, & Fu, 2002).

The mossy fiber afferent network results in a very different signal output, the smaller simple spikes. These spikes are regulated by parallel fiber activity and characterized by a much shorter action potential with a thinner and smaller shape, compared to complex spikes (Eccles, 1966; Eccles, Llinas, & Sasaki, 1966). The simple spike's activity has been related to movement in terms of limb and eye acceleration and velocity (Ito, 2006). Rather than a direct route of the climbing fiber pathway, the mossy fiber pathway should be examined by the net excitation of the parallel fibers from the GCL, and the modulation of neurons within this layer. The activity of the neurons in the GCL is coordinated with simple spikes, exemplified with the pharmacological stimulation of the small groups of granule cells, resulting in Purkinje cells excitation through their parallel fiber connections (Barbour, 1993). The activation and modulation of the granule and Golgi cells are of great importance to the overall output of the GCL and the Purkinje cell.

1.4 Golgi cells

As one of the few inhibitory interneurons in the GCL, the Golgi cell is heavily involved with temporal regulation of its activity (D'Angelo, 2008; Maex & De Schutter, 1998; Marr, 1969). Granule cells receive input from mossy fibers and interneurons including Golgi cells. Golgi cells exert control by inhibiting granule cells through feedforward and feedback loops and can affect the overall timing of the granule cells spiking (Marr, 1969; Vos, Maex, Volny-Luraghi, & De Schutter, 1999). The Golgi cells divergent output and inherent properties enable it to play a key role in the synchronization of activity within the GCL.

Golgi cell display specific properties that promote rhythmic inhibition of granule cells. Individual Golgi cells possess intrinsic pacemaking and resonance properties, seen *in vitro* with the regular beating of Golgi cells at frequencies within the theta band range (Dieudonne, 1998; Forti, Cesana, Mapelli, & D'Angelo, 2006; Solinas et al., 2007). These finding complement *in vivo* recordings, with unitary activity showing spontaneous rhythmic firing found in both awake and anesthetized animals (Edgley & Lidierth, 1987; Holtzman, Rajapaksa, Mostofi, & Edgley, 2006; Vos et al., 1999) The wide-spread synaptic connectivity of the Golgi cell combined with its cellular properties has allowed the Golgi cell to play a key role in temporal coordination within the GCL which may lead to the generation of synchronous oscillation (Dugue et al., 2009; Forti et al., 2006; Hartmann & Bower, 1998; Maex & De Schutter, 1998; Pellerin & Lamarre, 1997).

1.5 Oscillations

Synchronous oscillations during movement allows for communication between separate motor network areas (Donoghue, Sanes, Hatsopoulos, & Gaal, 1998; Schnitzler & Gross, 2005). Task-related synchronous oscillations have been identified within the primary motor cortex (MI), and between MI and the primary sensory cortex (SI) (Baker, Olivier, & Lemon, 1997) as well as across the paramedian lobe (PM) of the cerebellar cortex of the primate (Courtemanche & Lamarre, 2005). These oscillations are present while animals are alert and immobile, and decrease with the initiation of movement (Baker et al., 1997; Courtemanche & Lamarre, 2005). Of interest in the present study are the low-frequency oscillations in the GCL of the cerebellar cortex.

The LFP oscillations recorded within Crus II and paramedian lobule in GCL are present at frequencies ranging from 7 to 30 Hz, displaying local synchrony within the cerebellar cortex (Courtemanche, Chabaud, & Lamarre, 2009; Hartmann & Bower, 1998;

Pellerin & Lamarre, 1997). The oscillations appear to be generated locally, apparent through multi- and single-unit recordings in the GCL which are phase-locked to oscillations and through individual cellular properties which contribute to the generation of those rhythms (Courtemanche et al., 2002; Dugue et al., 2009; Hartmann & Bower, 1998; Pellerin & Lamarre, 1997).

A potential mechanism for the synchronous activity of Golgi cells are electrical coupling through gap junctions. Gap junctions are transmembrane channels that connect the cytoplasm of adjacent cells together (Belluardo et al., 2000). These channels allow for the flow of K+ ions, small metabolites and intracellular signaling molecules between cells, and can result in an increased synchrony of firing between individual neurons (Bennett & Zukin, 2004). Gap junction subtype connexin36 (Cx36)-forming proteins are expressed in the cerebellar cortex, within the GCL and molecular layer (Condorelli, Belluardo, Trovato-Salinaro, & Mudo, 2000; Ray, Zoidl, Wahle, & Dermietzel, 2006). Across the CNS, Cx36 gap junctions have been linked to the generation of widespread synchronous inhibitory activity across networks (Deans, Gibson, Sellitto, Connors, & Paul, 2001). Synchronized rhythmic output from the inferior olive and in turn Purkinje cell complex spikes have been attributed to Cx36 gap junctions between the inferior olivary neurons (Blenkinsop & Lang, 2006). Gap junctions in the GCL between Golgi cells allows for the rapid spread of depolarization signal to transfer between Golgi cells, promoting the rhythmic inhibition of granule cells (Dugue et al., 2009).

1.6 Goal of Present Study

Combined with previous findings, the present study explored the mechanism underlying GCL oscillations within Crus II and the paramedian lobule in the anesthetized rat. It addresses the localization of low-frequency oscillations as well as the role of Golgi

cell electrical coupling in the generation of low-frequency oscillations recorded within the GCL. Our aim was to alter the oscillations within the GCL by targeting gap junctions between Golgi cells with gap junction blockers carbenoxolone (CBX) and mefloquine. The current study provides further insight into the modulatory factors which affect these local circuits.

CHAPTER 2

GAP JUNCTION BLOCKERS CARBENOXOLONE AND MEFLOQUINE DECREASE LOW-FREQUENCY OSCILLATIONS IN THE GRANULE CELL LAYER OF THE ANESTHETIZED RAT

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(To be submitted)

ABSTRACT

Local field potential (LFP) 5-15 Hz oscillations within the granule cell layer (GCL) of the cerebellar cortex have been recorded in the awake rat. Local circuit interactions involving Golgi cells may play a role in the genesis or resonance of these oscillations and gap junction connections within the GCL may modulate these LFP oscillations. Gap junctions have been identified between Golgi cells, and appear to be involved in synchronizing neural populations in local circuits. Here, we recorded LFP oscillations under urethane anesthesia to determine if the cerebellar cortex slow oscillations display local properties and if they can be manipulated pharmacologically. We found 5-15 Hz oscillations similar to the GCL oscillations observed in the awake rat, confirming that sensory input is not necessary for their generation. The laminar nature of these oscillations was examined with multi-channel recordings, showing oscillations within a limited number of adjacent recording sites. To investigate the role of gap junctions in LFP oscillatory signal, carbenoxolone and mefloquine were introduced through local injections adjacent to the recording site. Both drug manipulations were found to significantly decrease 5-15 Hz oscillatory power over a 30 minute recording session. No significant decrease was found following the injection of the vehicle. Our results indicate that at least part of the circuitry that generates GCL LFP oscillations is localized within the GCL and that electrical coupling through Golgi cells is involved in their manifestation.

INTRODUCTION

Synchronous oscillations are present across the sensorimotor areas in the mammalian brain, and function in the planning and execution of movement (Baker et al., 1997; Donoghue et al., 1998; Schnitzler & Gross, 2005). The cerebellum is involved in sensorimotor processing and displays low-frequency local field potential (LFP) oscillations that are synchronized to rhythms within the primary somatosensory and motor areas (Courtemanche & Lamarre, 2005; O'Connor, Berg, & Kleinfeld, 2002). The oscillations are present within the granule cell layer (GCL) of awake, alert animals, and have been recorded in rodents (7-8 Hz & 15-16 Hz) and primates (10-25 Hz) (Courtemanche et al., 2002; Hartmann & Bower, 1998; O'Connor et al., 2002; Pellerin & Lamarre, 1997). The oscillations are associated with specific stages of motor control and are present during movement preparation (Courtemanche et al., 2002; Dugue et al., 2009; Hartmann & Bower, 1998; O'Connor et al., 2002). These oscillations appear to be generated within the GCL through local network interactions, as suggested by single and multi-unit recordings in the GCL which are phase-locked to oscillations, and by the individual cellular properties which could contribute to the generation of those rhythms (Courtemanche et al., 2002; Dugue et al., 2009; Hartmann & Bower, 1998; Pellerin & Lamarre, 1997).

Golgi cells are the main inhibitory interneuron within the GCL, and are involved with the coordination of precise temporal patterns within this network (D'Angelo, 2008; Forti et al., 2006; Maex & De Schutter, 1998). Golgi cells display intrinsic pacemaking and resonance properties at frequencies within the theta band range (Forti et al., 2006; Solinas et al., 2007) and activity of Golgi cells may therefore provide wide-spread and rhythmic synchronous inhibition of the granule cells. Rhythmic activity of Golgi cells

could lead to the independent generation of oscillations and promote within-network resonance of oscillations.

Golgi cell synchronized activity can now been attributed to electrical coupling. Gap junctions between Golgi cells allow for the rapid spread of depolarization between Golgi cells, permitting synchronous rhythmic inhibition of granule cells (Dugue et al., 2009). Connexin36 (Cx36)-forming proteins are expressed in the cerebellar cortex, within the GCL and molecular layer (Condorelli et al., 2000; Ray et al., 2006). Across several brain regions, Cx36 gap junctions have been linked to synchronizing inhibitory networks, and are a likely mechanism for network synchrony within the GCL (Deans et al., 2001). Although Golgi cell interactions through electrical coupling have been tested *in vitro* (Dugue et al., 2009), their contribution to population activity *in vivo* has yet to be shown.

In the present study, we show the localized presence of LFP oscillations within the cerebellar cortex at 5-15 Hz during urethane anesthesia. We employed two gap junction blockers, carbenoxolone (CBX) and mefloquine, to examine the influence of electrical coupling on the oscillations within the GCL. Carbenoxolone is a potent non-specific gap junction blocker, while mefloquine specifically blocks Cx36 and Cx50 subtypes (Bocian, Posluszny, Kowalczyk, Golebiewski, & Konopacki, 2009; Cruikshank et al., 2004). Cerebellar oscillations were decreased significantly following the local application of the gap junction blockers, while they were not affected by vehicle administration. The presence of oscillations in the anesthetized animal confirms that they can be generated without patterned sensorimotor inputs associated with awake behavior. In addition, we show here that gap junctions located within the GCL are

involved with the presence of slow LFP oscillations, and removing the electrical coupling between the Golgi cells decreases the oscillatory activity within the network.

EXPERIMENTAL PROCEDURES

Subjects, Drug Treatment and Recordings

Experimental procedures conformed to institutional policies and guidelines of the Concordia University Animal Research Ethics Committee, in accordance with the Canadian Council on Animal Care. Sprague-Dawley male rats (250-500 g, Charles River, St-Constant, Qc) were initially anesthetized with a 5% isoflurane and 95% oxygen mixture during placement of a catheter into the jugular vein. Isoflurane was stopped and urethane (0.8 g/ml) was introduced through the jugular catheter; anesthesia level was maintained constant throughout the experiment via toe pinch reaction. The animals were placed in a stereotaxic apparatus and body temperature was monitored via a rectal thermometer and maintained at 37 °C. The cerebellum was exposed by making a 2-2.5 mm diameter opening on the posterior surface of the skull, allowing direct access to Crus II/paramedian lobules. Two tungsten microelectrodes with an impedance between 1.0-1.2 MΩ (FHC, Bowdoin, ME) were fixed together to form a bipolar electrode in which one tip extended 500 µm in front of the other. The bipolar electrode was inserted into the cerebellar cortex at an angle of 50° from the horizontal plane, parallel to the sagittal plane, and 2-3 mm lateral to the midline. In a subset of experiments, hippocampal activity was also recorded simultaneously with using tungsten micrelectrodes placed at P, -3.8 mm; L, 2.8 mm; V, 2 mm, relative to bregma.

The stereotaxic apparatus was grounded, and the cerebellar signals were referenced to a wire placed along the contralateral cerebellar hemisphere or a stainless

steel screw placed in the skull posterior to lambda. We recorded the LFPs in the cerebellar cortex monopolarly and bipolarly in order to locate the largest oscillations between 5-15 Hz. Microelectrodes were slowly driven through the cerebellar cortex using an oil-hydraulic micromanipulator (Narishige, MO-10, East Meadow, NY). Recordings were analog-filtered (0.1 Hz to 500 Hz passband) and amplified with an A-M Systems Model 1700 amplifier (Everett, WA). Monopolar and differential signals were recorded using the software package Experimenter's Workbench in SciWorks (DataWave Technologies, Longmont, CO). LFP signals were recorded for two-min periods at a sampling rate of 1024 Hz.

Pharmacological treatment during recordings was performed by locally injecting the gap junction blockers CBX (100 µg/µl) (Bocian et al., 2009; Ozden, Sullivan, Lee, & Wang, 2009) or mefloquine (10 µM) (Cruikshank et al., 2004), and compare to the saline vehicle (Sigma-Aldrich, Oakville ON). Drugs were injected adjacent to the recording site through a 16-gauge cannula fixed 500 µm posterior to the microelectrode tips. Drug or saline solutions were injected very slowly to prevent physical disruption of the tissue structure surrounding the injection site using an infusion pump attached to the cannula via polyethylene tubing (CMA 400 syringe pump, CMA/Microdialysis, Solna, Sweden). Injections were delivered at a constant rate of 0.15 µl/min over a 20-min period. LFP signals were recorded in two-min periods before and after drug administration. Following the optimal placement of microelectrodes for recording oscillatory LFPs, a two-min recording period was taken prior to injection. Following this 20-min injection period, LFP signals were monitored for 6 two-min recording periods, acquired at five-min intervals post-injection, for a total post-injection monitoring time of 30 min. Heart rate was monitored throughout all recordings, with two subdermal electrodes attached behind the

forelimbs, making for a transcardial electrocardiogram signal that provided an estimate of heart rate and heart rate frequency.

Data analysis

Rhythmic activity of LFPs was quantified using custom routines written in the MATLAB environment (R2006a, The MathWorks, Natick, MA) using FFT and FILTER functions. The power spectrum from 0 to 512 Hz was calculated using Fast Fourier Transforms (FFT), which quantified spectra within consecutive two-s windows across each two-min recording period. Power between 5 and 15 Hz was integrated for each window providing an ensemble of 59 values for each two-min recording period. To evaluate the effects of the drugs on the 5-15 Hz oscillations, we compared the average integrated power values pre- and post-injection, using repeated measures ANOVAs for each experiment in Statistica (StatSoft, Tulsa, OK). The percentage of integrated values was calculated for each experiment and averaged across experiments with CBX and saline groups were compared using a factorial repeated measure ANOVA.

Histological identification of recording and injection sites

Histology of the cerebellar cortex was used to confirm the placement of the microelectrodes. Animals were sacrificed and the brains were isolated and stored in formaline (10% solution) until tissue was fixed. The cerebellum was then removed and was put in a 30% formalin-sucrose solution for cryoprotection. The cerebellum was cut in 30-µm sagittal sections and stained with cresyl violet. To assess the dispersion pattern of the injections across the particular cerebellar tissue, control experiment used a dye that was injected at the same rate and volume as the drug injections (Texas Red, 7 mM, sulforhodamine 101 acid chloride, Sigma-Aldrich, Oakville ON).

Multi-Channel Linear Probe Recordings

Linear probe experiments were performed in agreement with French and European guidelines for the experimental use of animals. Recordings were performed on urethane-anesthetized Sprague-Dawley male rats between 300 and 500 g (Charles River Laboratories, France). Animals were initially anesthetized with a ketamine/xylazine mixture and a catheter was inserted into the jugular vein to allow subsequent anesthetization with urethane (0.8 g/ml) as above. Heart rate and oxygen consumption were monitored to adjust anesthesia level, and animal body temperature was maintained at 37 °C with a heating pad.

Electrophysiological recordings were performed with 16 channel linear probes (impedance range 240-290 kΩ) spaced 50 µm apart (model A16, NeuroNexus Tech, Ann Arbor, MI). Signals were acquired using Tucker Davis Technologies System3 (Tucker-Davis, FL, USA) at a sampling rate of 25 kHz, for recording both units and LFPs. The voltage was bandpass filtered between 0.1 Hz to 8 kHz. Signals were grounded to the animal's skin and referenced to a low-impedance wire placed on the surface of the cerebellar cortex adjacent to the recording site. The linear probes were inserted perpendicularly to the surface of the cerebellar cortex at an angle of 40 to 52° from the coronal plane, parallel to the sagittal plane, and adjusted in depth to record cerebellar cortex LFP oscillations of maximal amplitude. For LFP analysis, the multi-site linear probe recordings were down-sampled to 1000 Hz, and were analyzed using custom-programmed routines in the MATLAB environment. The LFP recordings were digitally filtered from 2 – 49 Hz to better visualize the low-frequency oscillations (finite impulse response filter). To identify oscillatory activity that was synchronous across channels,

depth patterns of multi-site recordings were analyzed by generating wave-triggered 16-channel plots centered on a channel of reference displaying large oscillations. A current source density (CSD) analysis (Nicholson & Freeman, 1975) was then conducted to identify the location and amplitude of current sources and sinks associated with low-frequency oscillations between 4 and 12 Hz.

RESULTS

Identification of oscillations during urethane anesthesia in the cerebellar cortex

Oscillations ranging from 5 to 15 Hz were identified in both monopolar and bipolar recordings within the GCL under urethane anesthesia, appearing spontaneously without stimulus (Fig. 2.1). Finding strong low-frequency oscillations was heavily dependent on optimal placement of microelectrodes within the cortex and the amplitude of oscillations was therefore variable between experiments/animals. The monopolar LFP trace shown in Fig. 2.1A displays oscillatory activity (~6 Hz) recorded in the paramedian lobule. The FFT shown in Fig. 2.1B corresponds to the shaded two-s window and shows a clear peak near 6 Hz. Recordings from another animal show oscillations recorded with bipolar recordings (Fig. 2.1C). In this case, and for most of our bipolar recordings, the traces were found to oscillate at lower amplitudes. In this particular example, the peak-to-peak amplitude was around 25 μV (Fig. 2.1C), and displayed a similar peak frequency at ~6 Hz (Fig. 2.1D). Mean frequencies for the FFTs were calculated with the ensemble of two-s windows from each two min pre-injection recording session. The mean peak frequencies observed for experiments ranged from 5.5 to 12 Hz, similar to the frequencies observed within the GCL in awake rats (Hartmann & Bower, 1998; O'Connor et al., 2002).

Localization of oscillations across layers within the cerebellar cortex with multi-site recordings

Strong low-frequency oscillations were identified in two animals using 16-channel linear probes. Recordings reflected either a uniform distribution of LFP oscillations across all recordings depths, or a more localized distribution within deeper recording

sites. Sample traces of ~9 Hz oscillations were recorded across all channels (Fig. 2.2A), with the associated spectrogram show power as a function of recording location and frequencies below 50 Hz, averaged across the entire 11-minute session (Fig. 2.2B). The wave-triggered average (compared to channel #9) for LFPs filtered between 4-12 Hz demonstrates synchronous, phase-locked activity across all channels (Fig. 2.2C top). The corresponding CSD observed between 4 to 12 Hz (compared to channel #9) shows a similar alternating signal pattern across the electrode depth, with no distinct source identified (Fig. 2.2C bottom). Combining these, both the wave-triggered and the CSD analysis confirmed the presence of synchronized rhythmic activity across all recording channels. A second set of recordings in an adjacent track showed a more localized distribution of slow LFP oscillations. In this case, oscillations (~9 Hz) were observed most strongly in deeper channels, but were absent from superficial channels (Fig. 2.2D & E). The wave-triggered analysis confirms localized oscillations between 4-12 Hz, displaying similar valleys and peaks across deeper recording sites, which were not present at superficial distances from 0 to 200 μm (Fig. 2.2F top). The CSD analysis (Fig. 2.2F bottom) indicates sources and sinks across the deeper recording sites (400 to 800 μm), which were not identified at more superficial sites in the cerebellar cortex (compared to channel #15). These recordings therefore confirm that low-frequency oscillations can be localized within specific layers of the cerebellar cortex under urethane anesthesia.

Modulation of oscillations with the gap junction blockers

The mechanisms of LFP oscillation generation were surveyed with the precise local injection of the gap junction blocking drugs CBX and mefloquine. Injection of CBX resulted in a consistent drop of low-frequency (5 to 15 Hz) oscillations (n=4). Fig. 2.3A

shows bipolar recordings of low-frequency oscillations prior to injection. Rhythmic activity with a peak frequency of 10 Hz was recorded prior to CBX treatment (shown in corresponding FFT in Fig. 2.3C) and there was a decrease in oscillatory activity following the 20-min injection period (Fig. 2.3B and D). A repeated measures ANOVA, showed that CBX resulted in a significant reduction in integrated power between 5 and 15 Hz (Fig 2.3E, $p<0.001$).

Pharmacological treatment with gap junction blocker mefloquine reconfirmed CBX findings. Preliminary results have shown a consistent drop of low-frequency (5 to 15 Hz) oscillations following drug administration (n=1). Fig. 2.4A shows bipolar recordings of low-frequency oscillations prior to injection, with the corresponding FFT in Fig. 2.4C. The trace in Fig. 2.4B shows an example of a decrease in oscillatory activity following the 20-min injection period with the corresponding FFT in Fig. 2.4D. Integrated power between 5 and 15 Hz dropped following the administration of mefloquine (Fig. 2.4E, $p<0.001$); injection occurred between recordings 1 and 2 (indicated with an arrow).

Control experiments with saline injections showed no significant decreases in low-frequency (5 to 15 Hz) oscillations over the post-injection recording periods (n=5). Fig. 2.5 shows examples of traces, collected pre- and post-injection of saline (Fig. 2.5A and B respectively) and their corresponding FFTs for the two-s windows showing similar peak frequencies of ~ 6 Hz (Fig. 2.5C and D). Saline injections resulted in no significant changes in integrated power values between 5 and 15 Hz (Fig. 2.5E, $p=.53519$). The observed decrease in signal following local administration of CBX can therefore not be attributed to passage of time or to tissue damage or displacement resulting from local injection. These findings confirm that the gap junction blocker CBX has an affect on the generation/resonance of low-frequency oscillations in the cerebellar cortex.

For an analysis across sessions, the pre- and post-injection integrated power values between 5 and 15 Hz were compared for each recording session. The post-injection 5 to 15 Hz power across the six recording periods was averaged and calculated as a percentage of pre-injection signal. All CBX injection experiments showed a drop in the post-treatment oscillatory power: this drop of power in CBX treatment experiments ranged from 27 to 73% (Fig. 2.6A). Overall, the saline control experiments revealed minimal decreases of 3% and increases of up to 24% (Fig. 2.6B). The group percentage average for CBX and saline was compared using factorial repeated measures ANOVA, showing a significant effect of group, recording period and a significant interaction between the two (Fig. 2.7, $p<0.001$).

To examine the pattern of drug injection, Texas red dye was injected into the cerebellar cortex in a single control experiment. In this experiment, dye was introduced adjacent to the electrodes; bipolar signal shows low-frequency oscillations (Fig 2.8A, corresponding FFT shown in Fig. 2.8C). Dye was injected across the GCL and dispersed partly into the white matter within the paramedian lobule of the cerebellar cortex (Fig. 2.8B).

DISCUSSION

The current study identified the localized presence of spontaneous low-frequency oscillations within the Crus II/paramedian lobule under urethane anesthesia (Fig. 2.1). The LFP oscillations were shown to be synchronous across multi-site recordings at frequencies comparable to those recorded in the *in vivo* awake animals, and were localized within the cerebellar cortex (Hartmann & Bower, 1998). Injection of the gap junction blockers CBX and mefloquine was used to target the Golgi cell electrical synapses, and resulted in a significant attenuation of LFPs oscillations. This is consistent with the involvement of electrical synapses between Golgi cells in the generation/resonance of 5-15 Hz oscillatory activity in the cerebellar cortex.

The rhythmic oscillatory activity showed peak frequencies between 6 and 14 Hz, comparable to frequencies recorded in awake rats, and with Golgi cell firing rates recorded *in vitro* at 35-37 °C (Forti et al., 2006; Hartmann & Bower, 1998; O'Connor et al., 2002). The rhythmic nature of the Golgi cell firing has previously been shown *in vivo*, awake and under anesthesia as well as with *in vitro* studies (Dieudonne, 1998; Dugue et al., 2009; Forti et al., 2006; Vos et al., 1999). Until now, the LFP oscillations have solely been identified within the GCL of awake animals. Coordinated movement is associated with mossy fiber input into the GCL that provides sensorimotor afferent modulation, with spiking patterns in Golgi and granule cells that differ depending on the stimulus, action and source (D'Angelo et al., 2009). It is likely that sensorimotor inputs were minimal under urethane anesthesia, as animals received no outside stimulus. The consistency of the oscillations and their relatively narrow frequency range also suggest that intrinsic properties of Golgi cells and their local network serve as a major mechanism that lead to the temporal coordination of LFP oscillations.

Recordings of LFPs reflect the overall synaptic activity in the local neural population, and cerebellar LFPs quite likely represent primarily the synchronous synaptic activity generated by the GCL network. The actions of inhibitory interneurons provide a mechanism whereby populations of neurons may become synchronous. As seen across other neural network including the visual, somatosensory and hippocampus, the presence of electrical coupling between interneurons can increase the synchrony of neuronal inhibition and firing (Bennett & Zukin, 2004). Within the GCL, the presence of Golgi cell electrical coupling through gap junctions has been shown *in vitro*, with subthreshold oscillations presence, with kainate stimulation (Dugue et al., 2009). Considering the divergent connections of Golgi cells and their resulting widespread influence on granule cells, it is likely that they also have a strong influence on the excitability of granule cells, and Golgi networks. Given the LFP oscillations recorded could be generated/reverberated through Golgi-granule cell activity, by blocking the neural communication and desynchronizing the Golgi cell's rhythmic activity, there is a substantial change in oscillatory power (Fig. 2.3 & 2.4).

The gap junction blockers CBX and mefloquine were used to target the connexin channels present in the Golgi-Golgi cell gap junctions. Results of both sets of experiments show that LFP oscillations were attenuated by the gap junction blockers, while control injections of saline had no effect on oscillations. In addition to elimination of electrical coupling through gap junctions, CBX has also been reported to affect α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) receptors and N-methyl-D-asparate (NMDA) receptors (Tovar, Maher, & Westbrook, 2009). As a result, the decrease of population activity reported here due to the introduction of CBX might not be exclusively due to the modulation of gap junctions. We therefore conducted additional experiments with the more specific gap junction antagonist, mefloquine which is a

specific gap junction blocker that affects CX36 and CX50 connexins, but which displays more limited effects on other cell firing properties as compared to CBX (Cruikshank et al., 2004). Results obtained with mefloquine were similar to those obtained with CBX, and support the role of gap junctions in GCL-related LFP oscillations. Mefloquine can cause an increase in spontaneous synaptic activity and increases spiking frequency of hippocampal interneurons (Cruikshank et al., 2004).

In order to add to the current study, and further investigate the Golgi cell involvement in the rhythmogenesis/resonance of low-frequency oscillations in the cerebellar cortex, future studies should involve recording of unitary activity in complement with LFP oscillations, and explore the effect of gap junction blocking drugs on the timing of the firing of individual cells. To provide insight into the effect of local deactivation on the synchronization within the GCL, multiple recording sites could identify any changes in the level and synchrony of oscillation, identifying how the networks are influenced by deactivation across various distances.

In agreement with current data, our results show the presence of spontaneous low-frequency LFP oscillations under anesthesia and the attenuation of oscillations in the presence of gap junction blockers CBX and mefloquine. These findings not only implicate the role of gap junctions in the genesis of low-frequency oscillations, but also provide a novel method by which these oscillations can be explored.

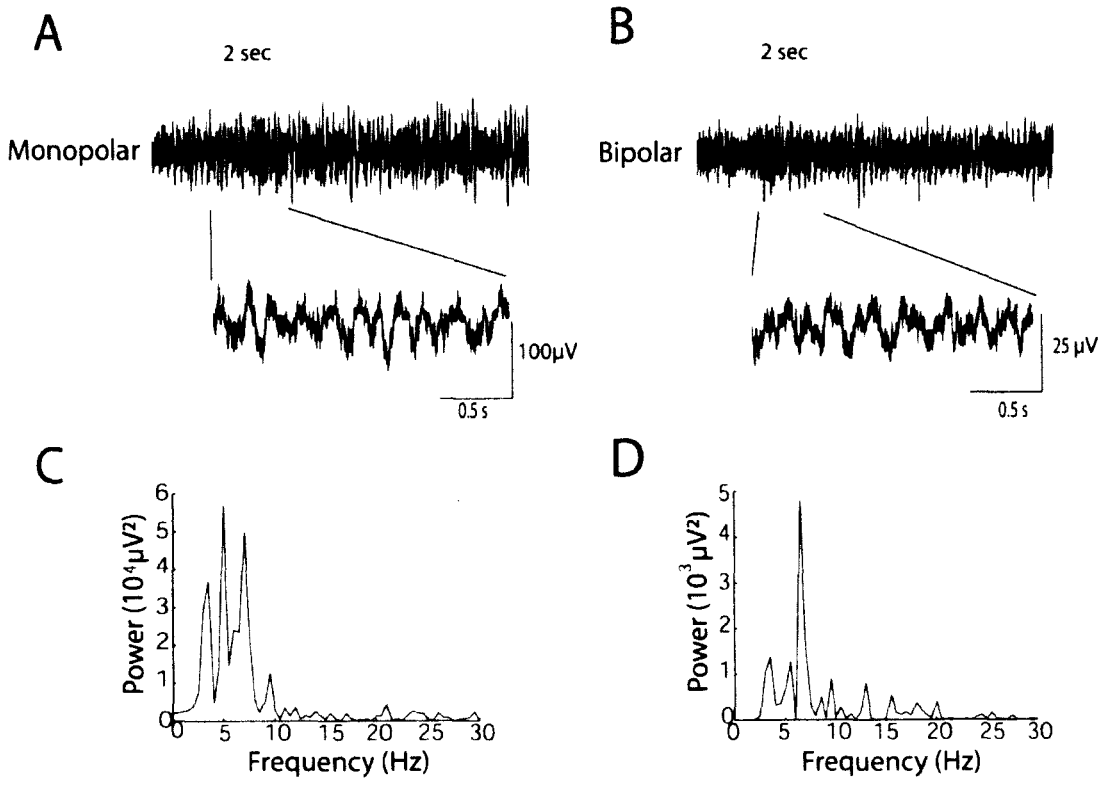

Fig 2.1:

Monopolar and bipolar, LFP oscillations recorded in the anesthetized rat. *A*: Monopolar LFP trace (shaded two second period enlarged below) displaying low-frequency oscillations recorded within the GCL. *B*: Bipolar LFP trace (shaded two second period enlarged below), displaying low-frequency oscillations. *D and E:* Corresponding two second FFT (*A and B respectively*), displaying ~6 Hz oscillations.

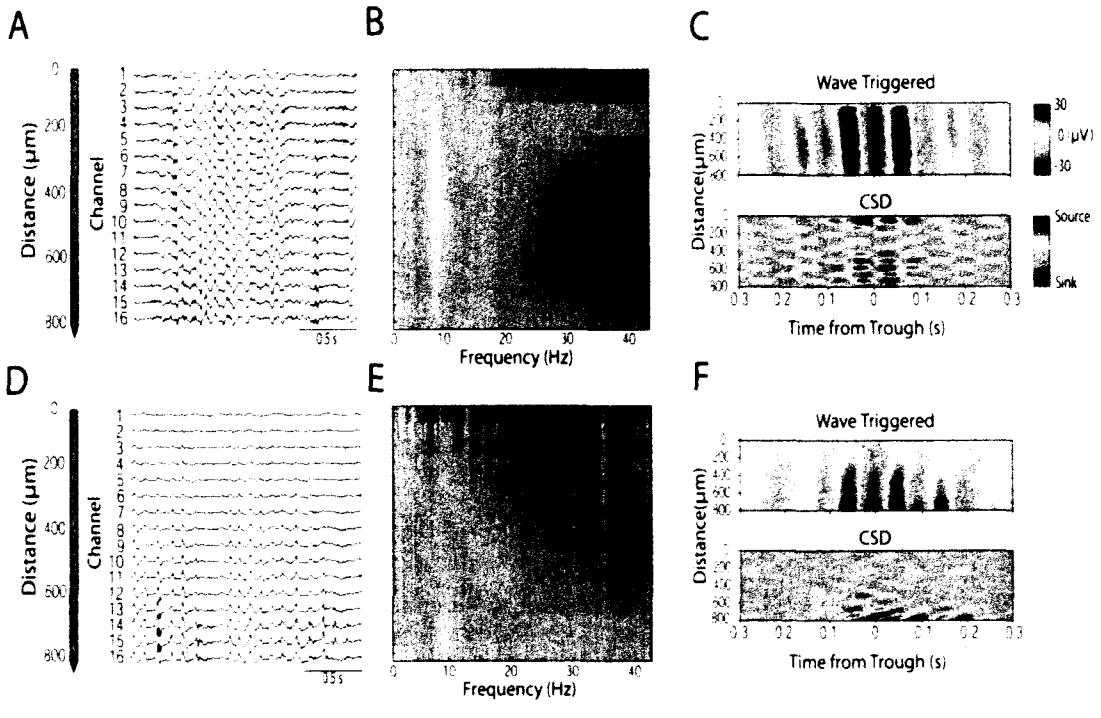
A
B
C
Distance (μm)
Channel
0.5 s
Frequency (Hz)
Wave Triggered
CSD
Distance(μm)
30
0 (μV)
-30
Source
Sink
Time from Trough (s)
D
E
F

Fig. 2.2:

Localization of cerebellar oscillations under urethane anesthesia. *A and D*: Simultaneous trace across 16 channels, microelectrodes spaced 50 µm apart. *B and E:* Spectrogram of recording session corresponding to fig. *A and D respectively*, displaying low-frequency activity at ~9 Hz. *C and F:* Wave-triggered analysis calculated for activity between 4 and 12 Hz. Current-source density (CSD) of data recorded before 4 and 12 Hz, displaying sources and sink patterns across the electrode. *Fig. D-F* were recorded during the same experiment in an electrode track adjacent oscillations analyzed in *fig. A-C*. *Fig C:* The alternating pattern of signal was present across all channels (compared to channel # 9). *Fig F:* The alternating signal pattern was only present across deeper channel (compared to channel #15).

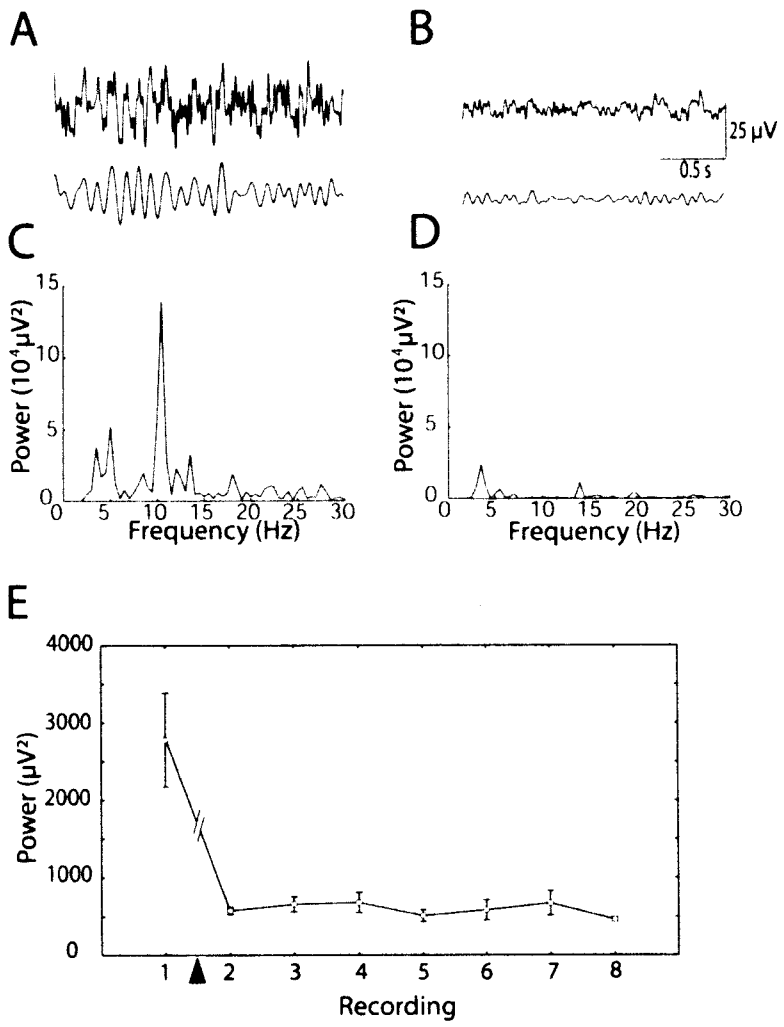

Fig. 2.3:

Effect of a CBX injection on LFP oscillations. *A* and *B*: LFP traces from the cerebellar cortex pre- and post-injection of carbenoxolone (100 µg/µl), showing a decrease in activity between 5-15 Hz (top traces filtered 2-49 Hz, bottom traces filtered 5-15 Hz). *C* and *D*: Corresponding power spectral analysis for the traces shown in *A & B* respectively. *E:* Pre and post measures of 5-15 Hz integrated power with CBX treatment, injection occurred between recordings 1 and 2 indicated by the arrow. After the injection, activity was monitored with two minute recording session, taken every 5 minutes. There was a significant effect of CBX on 5-15 Hz signal.

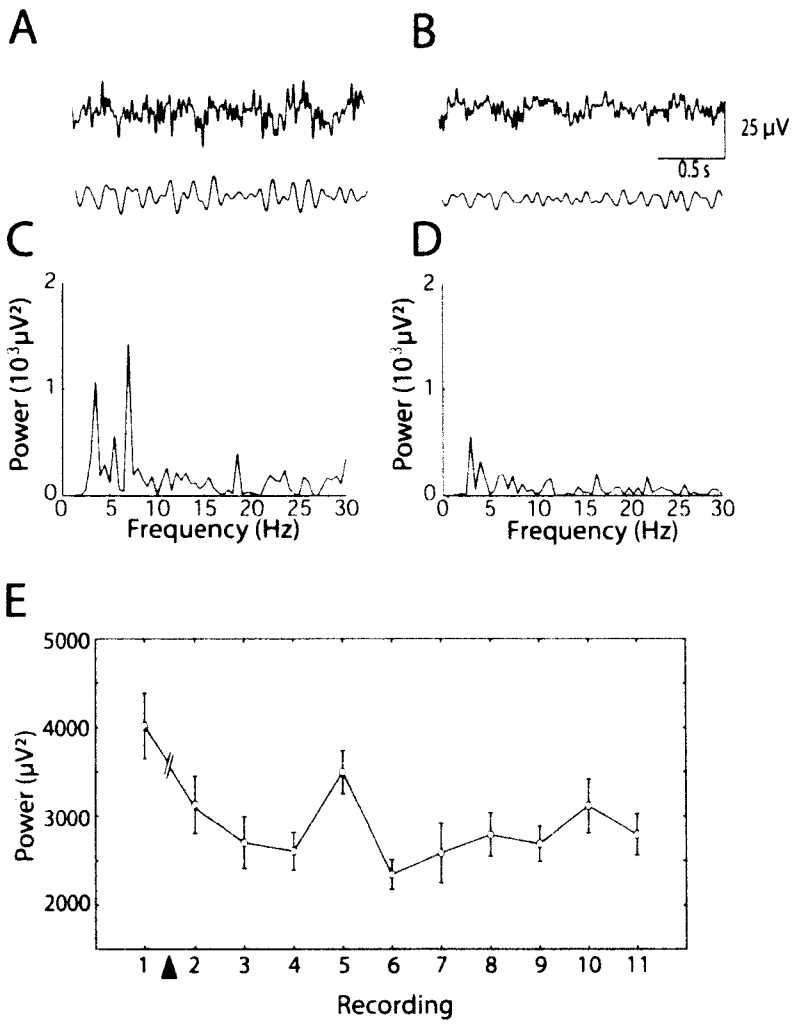

Fig. 2.4:

Effect of mefloquine injection on LFP oscillations. *A* and *B*: LFP traces from the cerebellar cortex pre- and post-injection of mefloquine, showing a decrease in activity between 5-15 Hz (top traces filtered 2-49 Hz, bottom traces filtered 5-15 Hz). *C* and *D*: Corresponding power spectral analysis for the traces. *E:* Pre and post measures of 5-15 Hz integrated power with mefloquine treatment, injection occurred between recordings 1 and 2 indicated by the arrow. After the injection, activity was monitored with two minute recording session, taken every 5 minutes. There was a significant effect of mefloquine on 5-15 Hz signal.

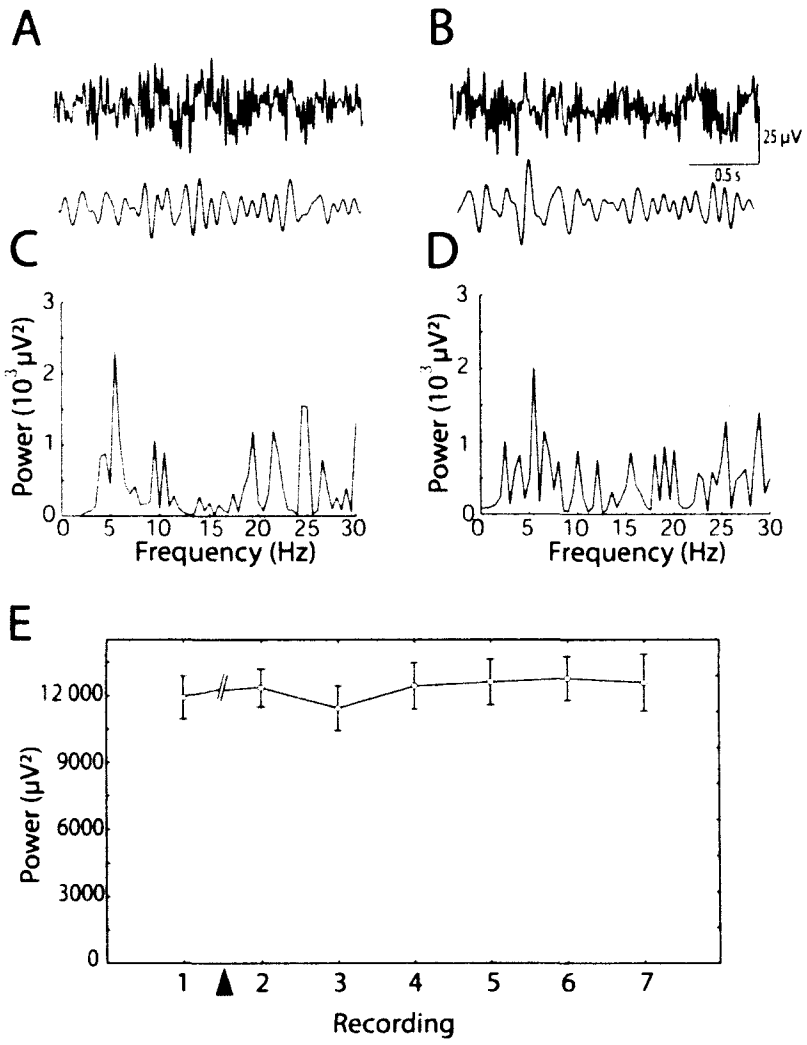

Fig. 2.5:

Effect of a saline injection on LFP oscillations. *A* and *B*: LFP traces from the cerebellar cortex pre- and post-injection of saline, showing no decrease in activity between 5-15 Hz (top traces filtered 2-49 Hz, bottom traces filtered 5-15 Hz). *C* and *D*: Corresponding power spectral analysis for the traces shown in A and B respectively. *E:* Pre and post measures of 5-15 Hz integrated power with saline treatment, injection occurred between recordings 1 and 2 indicated by the arrow. Injection of saline did not affect 5-15 Hz signal.

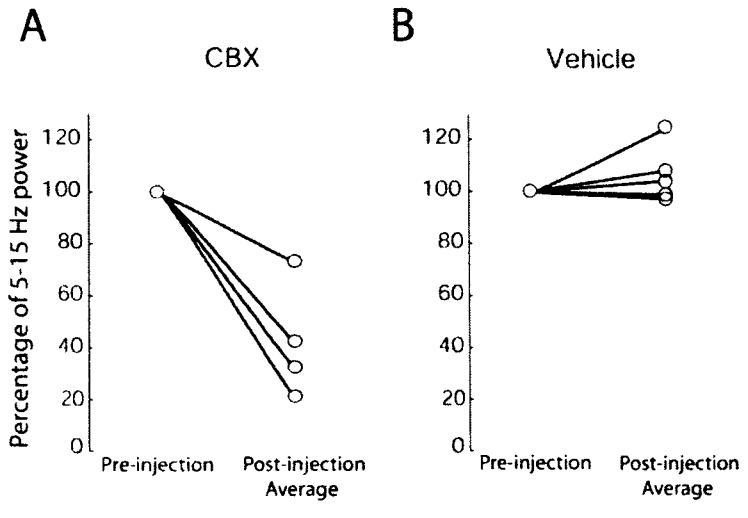

Fig. 2.6:

Average percent change of integrated values between 5 and 15 Hz. Post-injection 5 to 15 Hz power was averaged and calculated as a percentage of pre-injection signal. Each circle in the post-injection average represents a separate experiment. *A*: Percent change during CBX experiments. *B*: Percent change during saline control experiments.

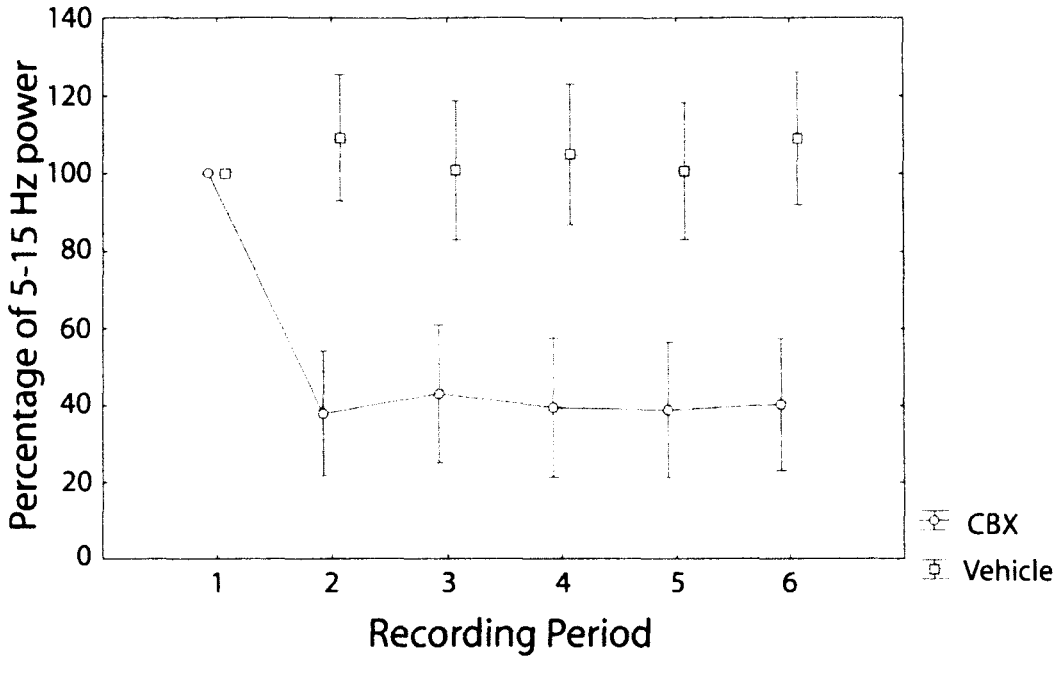

Fig. 2.7:

Average group percentage across recording period. Post-injection was calculated as a percentage of pre-injection signals. Percentage was averaged across recording session within group (CBX or vehicle) and compared to recording period.

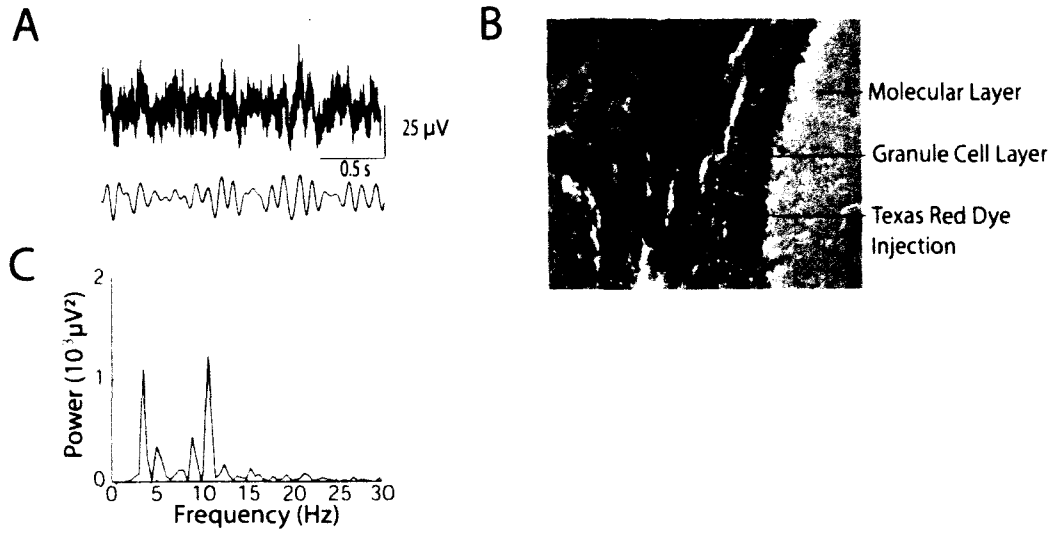

Fig. 2.8:

Dispersion pattern of local injection: *A:* LFP traces from the cerebellar cortex (bottom traces filtered 5-15 Hz). C: Corresponding power spectral analysis for the LFP trace in *A*. *B:* Example dispersion pattern, showing Texas red dye injection within the GCL and white matter.

CHAPTER 3

GENERAL DISCUSSION

To further characterize GCL oscillations, our finding of low-frequency oscillations without active stimulus supports the theory of an intrinsic oscillator within the GCL. To examine the mechanism involved in rhythmogenesis, we altered the local circuits by impeding intercellular communication through gap junction blockage, resulting in decreased low-frequency GCL oscillations. Drug treatments targeted the specific gap junction found within the GCL between Golgi cells. Our findings suggest a modulatory role of gap junctions in the underlying oscillatory activity in the GCL.

Although synchronous oscillations have been identified within the GCL, multi-channel synchronous recordings had yet to be explored. Our recordings show both localized bipolar oscillations and uniform oscillations across multiple channels. We did find a distribution which seemed layer specific. However, in multi-channel linear recording shown here, activity between 4 and 12 Hz displayed similar phases, ie: no phase reversal was observed, and no isolated source of current was identified. Unlike theta oscillations in the hippocampus, in which dipoles have been well characterized (Buzsaki, 2002), dipoles within the cerebellar cortex at low-frequencies have not been identified. Golgi and granule cell unitary firing has been recorded in synchrony with low-frequency oscillations, and fire during the up-phase of oscillations (Dugue et al., 2009). In our recordings, the CSD analysis does show a slight phase shift (fig 2.2F bottom). To further explore this issue, the unitary activity will be analyzed for any phasic relationships to the low-frequency oscillations.

Within Crus II and paramedian lobules, the GCL receives mossy fiber inputs from the pontine nuclei of the brain stem via cortico-ponto-cerebellar pathways that carry signals from cortical sensorimotor areas (Enger & Brodal, 1985). The Golgi cell act to regulate network activity, receiving excitatory inputs and in turn activating the inhibitory

Golgi cell network. Our findings suggest gap junctions are involved in the modulation of low-frequency oscillations.

Other components of the GCL may contribute to the temporal control of the GCL network and the low-frequency oscillations which develop. The Golgi cell is one of the two inhibitory interneurons within the layer which may modulate GCL network activity. The second inhibitory interneuron within the cerebellar is the Lugaro cell. Lugaro cells have axonal projections which may help synchronize the Golgi cell and contribute to the generation of low-frequency oscillations within the GCL (Dieudonne, 2001). Serotonin specifically modulates Lugaro cells (Dieudonne & Dumoulin, 2000), therefore future *in vivo* experimental manipulations could include the modulation of Lugaro cells through injection of either serotonin agonist or antagonists to investigate their role in the modulation of these oscillations.

Other drug manipulations which could alter the presence of low-frequency oscillations within the GCL include decreasing neurotransmission in the GCL network. Glutamate is released from mossy fiber and granule cells, while GABA is released from both Lugaro and Golgi cells. The local application of either a GABA or glutamate antagonist would likely decease synaptic communication between the Golgi and granule cells. By affecting the synaptic communication within the Golgi-granule cell network this may lead to an overall decrease of low-frequency oscillatory activity.

The Role of oscillations in the GCL

Synchronous oscillations have been identified within the GCL in awake animals. The oscillations are strongest during movement preparation but have been identified when animals were at rest (Courtemanche et al., 2009). The current study combined with underlying synchronous oscillations while animals at rest points towards an intrinsic

mechanism for rhythm generation. Network oscillations represent precise neural interactions and are one mechanism for long-range neural communication. The function of local oscillations during phases in which animals are either at rest or under anesthesia may have a purpose. During rest or anesthesia, oscillations may function as to keep local circuits in an "idling mode", waiting for outside inputs. This idling role of oscillations, maintaining the cellular network in active state, may explain the functional significance of these oscillations.

Experimental Considerations from the Present Study

As the present study utilized a new technique to study cerebellar low-frequency oscillations, we found that body temperature and level of anesthesia plays a key role in the level of activity recorded. Urethane level played a significant role in oscillatory power, in a single experiment we found a significant decrease was observed during the 30 minute post-injection period. This was immediately following administration of an additional dose of urethane anesthesia. This is in contrast to all other control experiments in which no decrease in signal was observed. Interestingly, in this recording session monopolar signal decreased while differential signal was not altered. This may be due to an overall global decrease in activity following the administration of urethane, while local networks were not affected. In a number of experiments, heartbeat was found to contaminate neural signal. All recordings were compared to heartbeat signal and any contaminated experiments were discarded.

General Applications of the Current Research

By studying the intrinsic mechanisms which govern the GCL oscillation, we may have some insight into what factors regulate the output of the cerebellar cortex. The Golgi cell receives a convergence of information from spino-cerebellar and cerebro-

cerebellar pathways via mossy fibers. By deciphering the basic properties which modulate activity of in GCL, we have a better understanding of what directs the parallel fiber output, simple spike activity and DCN activity.

The GCL local network activity is related to several pathologies including cerebellar ataxia and autism. Cerebellar ataxia is characterized by motor control issues including tremors and decreased motor control coordination (Schmahmann, 2004). The motor timing dysfunctions are due to abnormal firing of the DCN located within the white matter (D'Angelo & De Zeeuw, 2009). In patients with autism, there is an altered activity of several brain regions which includes abnormal sensory processing (Lane, Young, Baker, & Angley). Neuroanatomical studies of patient with autism have revealed an abnormal structure of the cerebellum, including decreased number and sizes of Purkinje cells and DCN (Palmen, van Engeland, Hof, & Schmitz, 2004). In relation to the current study, by deciphering the mechanisms that modulate the overall activity of the GCL and the resulting inputs received by DCN, we may have insight into what inputs are dysfunctional in these disease states.

Our results show the presence of spontaneous oscillations, supporting current findings of intrinsic rhythmic properties within the GCL. Our experiment presents a new technique, which allows for further examination of the modulatory factors affecting the low-frequency oscillations, targeting the gap junctions within the GCL. Our results show a decrease in low-frequency LFP oscillation within the GCL as a result of the local injection of gap junction blockers CBX and mefloquine. These findings indicate that electrical coupling between the local network plays a role in the generation of low-frequency oscillations within the GCL.

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