Abstract

This study examines the response of Weak and Transient burst deep cerebellar nuclear (DCN) neurons when they are stimulated by physiological patterns of Purkinje cell inhibitory input *in vitro*. We also report the DCN neurons response to regular patterns of Purkinje cell firing defined by a CV2 analysis, as well as complex spike discharge. Currently the functional significance, and input parameters that generate rebound bursts in the DCN are unknown. Here we report that there are subtle differences in the Weak and Transient cell burst response suggesting the presence of differential coding by DCN neurons. Reverse correlation between DCN cell bursts and Purkinje cell input revealed an elevation-pause pattern of Purkinje cell firing triggers a rebound burst, while CV2-defined patterns and complex spikes failed to reliably trigger rebound responses. This work identifies a framework for future studies to investigate the relationship between Purkinje cell input and DCN output.
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<th>Definition</th>
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<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AHP</td>
<td>After hyperpolarizing potential</td>
</tr>
<tr>
<td>CF</td>
<td>Climbing fiber</td>
</tr>
<tr>
<td>CPCCOEt</td>
<td>mGluR blocker</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CV2</td>
<td>Coefficient of variation on a spike-to-spike basis</td>
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<tr>
<td>DCN</td>
<td>Deep cerebellar nuclei</td>
</tr>
<tr>
<td>DIC-IR</td>
<td>Differential interference contrast with infrared light transmission</td>
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<tr>
<td>DL-AP5</td>
<td>DL-2-amino-5-posphonopentanoic acid</td>
</tr>
<tr>
<td>DNQX</td>
<td>6,7-dinitroquinoxolinedione</td>
</tr>
<tr>
<td>$E_{Cl}$</td>
<td>Equilibrium potential for chloride</td>
</tr>
<tr>
<td>$E_K$</td>
<td>Equilibrium potential for potassium</td>
</tr>
<tr>
<td>$E_{Na}$</td>
<td>Equilibrium potential for sodium</td>
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<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
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<tr>
<td>FSL</td>
<td>First spike latency</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>HCN</td>
<td>Hyperpolarization-activated cyclic nucleotide gated</td>
</tr>
<tr>
<td>HVA</td>
<td>High voltage activated</td>
</tr>
<tr>
<td>$I_H$</td>
<td>HCN current</td>
</tr>
<tr>
<td>$I_{NaP}$</td>
<td>Persistent sodium current</td>
</tr>
<tr>
<td>$I_T$</td>
<td>T-type calcium current</td>
</tr>
<tr>
<td>IPSC</td>
<td>Inhibitory postsynaptic current</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>ISI</td>
<td>Interspike interval</td>
</tr>
<tr>
<td>JNJ-16259685</td>
<td>3,4-Dihydro-2H-pyran[2,3b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methane</td>
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<tr>
<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>LVA</td>
<td>Low voltage activated</td>
</tr>
<tr>
<td>MF</td>
<td>Mossy fiber</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MPEP</td>
<td>2-Methyl-6-(phenylethynyl)pyridine</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cell</td>
</tr>
<tr>
<td>PC-DCN</td>
<td>Purkinje cell-deep cerebellar nuclei neuron</td>
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<tr>
<td>PF</td>
<td>Parallel fiber</td>
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<tr>
<td>PSP</td>
<td>Post synaptic potential</td>
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<tr>
<td>TB</td>
<td>Transient burst</td>
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<tr>
<td>TTL</td>
<td>Transistor-transistor logic</td>
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<tr>
<td>WB</td>
<td>Weak burst</td>
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CHAPTER 1: INTRODUCTION

1.1 Information Transfer

Information in the brain is transferred through neurons by electrical and chemical signals. The primary unit of the electrical signal generated for communication is a transient electrical spike known as an action potential. Action potentials are generated by the movement of ions through channels in the cell's membrane that permit ion flow in relation to specific electrochemical gradients. There are hundreds of different ion channels found in the brain. They are generally grouped by their selectivity to different ions (Na⁺, K⁺, Ca²⁺, and Cl⁻), their methods of activation or gating (ligand-gated, voltage-gated, etc.), whether they pass ions through the membrane (ionotropic receptors), or initiate secondary messenger cascades within the cytosol (metabotropic receptors) (Bean, 2007). The specific complement of ion channels and their net influence on the membrane potential will affect spike output in a neuron. Action potentials in turn cause release of neurotransmitters at the synapse which bind to postsynaptic receptors that create a postsynaptic potential (PSP) on the neuron. PSPs can be excitatory or inhibitory (EPSPs or IPSPs) depending on which neurotransmitter is released from the presynaptic neuron and which receptor the neurotransmitter acts upon. Generally, multiple PSPs are required to trigger an action potential, such that patterns of presynaptic spike input do not directly translate to postsynaptic output. Yet the precise frequency or pattern of spikes generated in response to presynaptic input reflects the outcome of synaptic integration and thus the basic transfer of information in the brain, a process defined as neural coding (Borst and Theunissen, 1999; deCharms and Zador, 2000). Neural coding can take on many forms, but the main elements of a neural code are found in the pattern, frequency, and timing of spike discharge. Neural coding has been historically classified into two broad subcategories: rate coding (dealing with the frequency of output spikes correlated to input intensity) and temporal coding (dealing with spike timing relative to a specific input). This study focuses on the basis for neural coding in the primary output path of the cerebellum.
1.2 Cerebellar Anatomy and Network

The cerebellum is a complex structure that rests caudal to the brain and above the pontine region. It is well known for its role in motor control, specifically in the coordination of voluntary motor movement, balance, and eye movement. Along with motor control, the cerebellum has been shown to play a role in motor learning by exhibiting various forms of synaptic plasticity (Marr, 1969; Albus, 1971; Eccles, 1973). More recently, an appreciation has been gained as to additional roles for cerebellum in cognition and emotion (Schmahmann and Caplan, 2006; Fatemi et al., 2012; Koziol et al., 2012; Stoodley, 2012).

The cerebellum is viewed as a processing unit that compares sensory input to cortical motor commands on a moment-to-moment basis, serving essentially as a comparator of intended versus actual movements. The structure and arrangement of the cerebellum can be described based on gross anatomy, highly organized circuitry, and function. Anatomically the cerebellum can be divided into three lobes: the anterior lobe, posterior lobe, and the flocculonodular lobe. Two large transverse folds, the primary fissure and posterolateral fissure separate the anterior and posterior lobes. The cerebellum can also be divided into three parts based on functional criteria: the Vestibulocerebellum, which regulates balance and eye movements, the Spinocerebellum that has a role in controlling body and limb movements, and the Cerebrocerebellum, which is involved in planning movements and evaluating sensory information (Apps and Garwicz, 2005).

The internal structure and circuitry of the cerebellum has been well described (Ito, 1984). There are five primary neuron types within a highly layered cerebellar cortex. The surface layer is called the molecular layer and contains Purkinje cell dendrites and granule cell axons referred to as parallel fibers (PF). The molecular layer also contains basket and stellate cells, which are small inhibitory interneurons that receive excitation from PFs and provide feed forward GABAergic inhibition to Purkinje cells. The middle layer of cerebellar cortex is the Purkinje cell layer that contains only the cell bodies of Purkinje cells. The most ventral layer is the granule cell layer containing granule cells as well as inhibitory Golgi cell interneurons that are in receipt of afferent mossy fibers (MF). The output projection from cerebellar cortex is unique in the brain in reflecting only an inhibitory input from Purkinje cells to neurons of the deep cerebellar nuclei (DCN).
1.2.1 The Deep Cerebellar Nuclei

The DCN are located in the white matter at the base of the cerebellum. These cells are important in generating the final output of the cerebellum after sensory-motor information is processed in the cerebellar cortex. Three bilateral nuclei (medial, interpositus, and lateral) receive input from specific areas of the cerebellar cortex with the exception of the vestibulocerebellum. Projections from the cerebellar cortex are generally topographic, with the Lateral nuclei receiving most connections from the lateral cortical hemispheres, the Interpositus nuclei receiving inputs from the paravermis region, and the Medial nuclei receiving input from the vermis (Ito, 1984). It has been suggested that there are three different types of neurons in the DCN (Chan-Palay, 1977; Ito, 1984): large diameter presumed glutamatergic projection neurons, small diameter GABAergic projection neurons, and local interneurons. Glutamatergic DCN neurons provide significant cerebellar output to the thalamus and red nucleus as well as a minor projection path back to the cerebellar cortex as MFs (Chan-Palay, 1977; Ito, 1984; Sultan et al., 2003; Uusisaari et al., 2007). Small diameter GABAergic DCN neurons project to the inferior olive, which is the source of the climbing fibers (CF), creating an inhibitory olivo-cortico-nuclear feedback loop (Jacobson et al., 2009). It has also been shown that some of the projection neurons in the DCN are glycinergic, with similar firing properties to neighbouring glutamatergic neurons (Bagnall et al., 2009). Given that DCN neurons represent the final output of the cerebellum they are a key step in the transfer of information after signal processing in the cerebellar cortex.

There are two types of afferent inputs to the cerebellum: MFs that arise from spinocerebellar, vestibular, reticular, and pontine nuclei and CFs that originate in the inferior olive. MFs provide excitatory inputs to both DCN neurons and granule cells in the cerebellar cortex (Sugihara et al., 1996; Shinoda et al., 2000). The axons of granule cells give rise to PFs to provide excitatory input to a large number of Purkinje cells, which can create synchronous firing in Purkinje cells. Stellate and basket cell interneurons provide feed forward inhibition to Purkinje cells. Unlike MF inputs, CF inputs only synapse onto DCN neurons and Purkinje cells. Each Purkinje cell receives one CF input, and approximately 200,000 PF inputs (Barlow et al., 2002). Each CF wraps around the proximal dendrites of a single Purkinje cell to form several hundred synaptic inputs. Because of this, a single CF input provides a massive depolarization that creates
a prolonged, large amplitude complex spike of ~50 ms duration followed by a high frequency burst of smaller amplitude action potentials in Purkinje cells. This pattern of output contrasts sharply with that of simple spikes (single Na\(^{+}\) dependent action potentials) generated by PF input (Eccles et al., 1966).

A key question in neural coding is the relative role of complex versus simple spike discharge of Purkinje cells and the nature of input signals they serve to encode. This includes not only the generation of spikes in Purkinje cells, but also the pattern of pauses in firing that follow complex spikes, given that this will provide a window of time in which inhibitory input to DCN cells is relieved, facilitating spike output from DCN neurons.

The nature of synaptic input and output outlined above defines a central dichotomy of synaptic processing in the cerebellum. Purkinje neurons in the cortex receive mainly excitatory input but project only GABAergic output to DCN neurons that must convert the signal to the only excitatory output provided to other brain regions. Both of these neurons need to correctly process their synaptic input to generate the appropriate DCN spike train. Despite this, the neural code used by DCN neurons to translate the inhibitory input of Purkinje cells remains unknown.

1.3 Physiology of DCN Neurons

DCN neurons typically fire spontaneously at 10-20 Hz (Thach, 1968; LeDoux et al., 1998). Purkinje cells provide a large inhibitory input, which decreases DCN cell spike output and thus presumably limits information transfer. However, DCN cells possess the ion mechanisms necessary to generate a rebound depolarization after a period of inhibition that drastically increases their firing frequency (Jahnsen and Llinas, 1984a; Huguenard and Prince, 1992; Aizenman and Linden, 1999; Tadayonnejad et al., 2009b; Pedroarena, 2010; Sangrey and Jaeger, 2010). Rebound depolarizations have also been shown to provide a computational advantage by increasing the rate and duration of spike output, as well as controlling first spike latency (FSL) and spike precision (Kepecs and Lisman, 2003; Heil, 2004; Person and Perkel, 2005; Sangrey and Jaeger, 2010; Engbers et al., 2011).
Figure 1.1 DCN processing of cerebellar cortical output.

A, Coronal tissue section indicating the position of the three deep nuclei at the base of cerebellum. B, Schematic diagram of the GABAergic projection by Purkinje cells onto large diameter projection neurons of the DCN, which in turn generate the excitatory output from cerebellum. C, Recordings from DCN cells indicating two distinct phenotypes of rebound burst discharge in response to membrane hyperpolarizations in the form of high frequency Transient Burst or Weak Burst responses.
1.3.1 Rebound Bursting

Multiple in vitro and in vivo studies have provided evidence that the threshold for a rebound burst can be reached with inhibitory synaptic inputs (Gardette et al., 1985; Llinas and Muhlethaler, 1988; Aizenman et al., 1998; Aizenman and Linden, 1999; Zhang et al., 2004; Tadayonnejad et al., 2009b; Zheng and Raman, 2009b). Other groups have recently questioned the probability of evoking rebound bursts with synaptic inhibitory inputs (Alvina et al., 2008), although close examination revealed a high probability of evoking rebound bursts in vitro when stimulus intensities were appropriately considered (Tadayonnejad et al., 2009b). Previous studies in vitro have also provided evidence that there are at least two phenotypes of rebound bursting in DCN neurons, identified as a Weak Burst or Transient Burst output (Molineux et al., 2006; Molineux et al., 2008a). Following an inhibitory synaptic stimulus train (50-100 Hz), Weak Burst neurons have maximal rebound frequencies of <140 Hz and show a continuous decline in firing frequency over several seconds. Transient Burst neurons instead generate rebound bursts that show a characteristic 2-5 spike burst with a frequency > 140 Hz that is followed by a large AHP and long interspike interval before returning to resting values of spontaneous discharge (Tadayonnejad et al., 2010b).

It is currently unknown what role these different rebound phenotypes play in the transfer of information. However, Weak Burst neurons were found to be GAD-67 negative, which indicates that weak burst neurons correspond to non-GABAergic cell types (Molineux et al., 2006). Transient Burst cells were found to be either GAD-positive or GAD-negative, meaning they correspond to both GABAergic or non-GABAergic cells (Molineux et al., 2006). It has been previously shown that GABAergic neurons project to the inferior olive (Best and Regehr, 2009). Therefore, Transient Burst neurons may contribute to the nucleo-olivary feedback loop deemed important for controlling Purkinje cell activity. An overview of the anatomy and DCN burst phenotypes are shown in Figure 1.1.

1.4 Ionic Factors of DCN Firing

DCN output neurons contain a variety of voltage-gated calcium channels (including high voltage-activated channels (HVAs) and low voltage-activated channels (LVAs)) which mediate a
calcium conductance within specific voltage ranges (Raman et al., 2000; Gauck et al., 2001; Zheng and Raman, 2009b; Person and Raman, 2010). It has been suggested that most HVA calcium channels have no effect on the tonic firing of DCN cells, with the exception being the N-type (Cav2.2) calcium channel. If the N-type channel is blocked using ω-conotoxin GVIA DCN neurons exhibit an increased firing rate and strong tendency to burst erratically upon loss of a calcium-gated potassium current that regulates tonic firing frequency (Alvina and Khodakhah, 2008; Molineux et al., 2008). The rebound response of DCN neurons is generally split into an early phase (~100 ms after the end of a hyperpolarization), and a late phase (up to seconds after release from hyperpolarization). It is expected that a host of HVA calcium channels are activated as part of the late phase of rebound response to contribute in ways not yet fully assessed (Zheng and Raman, 2009b).

There are at least three key ion channels proposed to be responsible for this early phase of rebound burst in DCN neurons. These are the LVA T-type calcium channels (Cav3 family), the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, and a slow persistent sodium current (I_{NaP}) (Jahnsen and Llinas 1984; McCormick and Pape 1990; Muri and Knopfel 1994; Huguenard 1996; M. L. Molineux et al. 2006; Biel et al. 2009)(Gauck et al., 2001; Sangrey and Jaeger, 2010). T-type channels generate a fast activating and inactivating current (I_{T}) (Perez-Reyes 2003; Iftinca et al. 2006) distinct phenotypes of rebound burst discharge in response to membrane hyperpolarizations in the form of high frequency Transient Burst or Weak Burst responses. HCN channels generate a non-inactivating inward current (I_{H}) that activates directly with increasing levels of hyperpolarization (Biel et al. 2009)(Engbers et al., 2011) and then undergoes a relatively slow deactivation upon depolarization over ~ 500 ms (see(Engbers et al., 2011)). Sodium channels can change gating modes to include the expression of a slow persistent sodium current (I_{NaP}) that is subject to modification by slow shifts in membrane potential (Taddese and Bean, 2002). An alternate form of sodium channel activation that can occur in DCN cells is a resurgent current that follows spike discharge (Afshari et al., 2004). While resurgent sodium current has potential to contribute to rebound bursts, it has received little attention to date. Finally, it is known that calcium influx will subsequently activate calcium-dependent potassium channels that further shape the rebound response (Aizenman and Linden, 1999; Czubayko et al., 2001; Feng and Jaeger, 2008; Molineux et al., 2008a).
1.4.1 Rebound Burst Phenotypes

Therefore it is known that DCN cells generate a rebound burst upon relief of a hyperpolarization at least through direct activation of $I_T$ or $I_{NaP}$ and an inward tail current generated upon deactivation of $I_H$ (Molineux et al., 2006; Molineux et al., 2008a; Sangrey and Jaeger, 2010; Engbers et al., 2011; Steuber et al., 2011). These channels are relatively inactivated (T-type and sodium channels) or deactivated (HCN channels) at resting potential but their properties are such that a membrane hyperpolarization will increase their availability prior to relief of a hyperpolarization. However, given the amount of information available through experimental data and modeling studies, the current study focuses only on the role of $I_T$ and $I_H$. The role of Cav3 calcium channels in generating a rebound response has attracted significant attention. It has been shown that the Transient and Weak burst rebound phenotypes correlate with the expression of a specific Cav3 calcium channel isoform. Transient Burst neurons are correlated with expression of the Cav3.1 isoform, and show a distinct lack of Cav3.3, whereas the Weak Burst phenotype is correlated with Cav3.3 expression. Rebound burst frequency and duration are correlated with the duration and depth of hyperpolarization (Aizenman and Linden, 1999; Czubayko et al., 2001; Pedroarena, 2010; Tadayonnejad et al., 2010b), suggesting that bursting behavior is generated by a voltage-gated current. It was then shown that Transient Burst neurons generate 7X more $I_T$ following a hyperpolarization than Weak Burst neurons, suggesting that Cav3 channels provide a significant amount of current underlying a rebound burst (Molineux et al., 2008a). It was recently shown that physiological levels of membrane hyperpolarization activate only a small proportion of available $I_T$ and $I_H$, but that these are sufficient to contribute to a rebound response. Furthermore, at least 50% of the early phase rebound response is generated by an $I_T$-mediated depolarization. $I_H$ instead provides an increase in spike frequency by reducing the membrane time constant and thus the extent of $I_T$ inactivation following release from a membrane hyperpolarization (Engbers et al. 2011). It was also shown that $I_H$-mediated depolarizations cause an inverse voltage-FSL relationship, such that larger membrane hyperpolarizations are followed by a FSL. $I_H$ was further shown to produce a 35% increase in the precision of the FSL of a rebound (Engbers et al. 2011).

Understanding the physiological role of rebound bursts is restrained by the fact that the majority of previous work used only square wave current injections, and then to membrane
potentials well below the equilibrium potential for chloride ($E_{\text{Cl}}$), the expected maximum hyperpolarization encountered during Purkinje cell input. A recent study (Engbers et al. 2011) focused on providing near physiological patterns of membrane voltage deflections using step and ramp voltage clamp commands at the soma to study the degree of $I_T$ and $I_H$ activation. However, this stimulus also cannot be considered truly physiological given that most Purkinje cell inputs terminate in dendritic regions of DCN cells and give rise to a fluctuating voltage response in relation to ongoing presynaptic input. In order to study how DCN cells and their ion channels respond to physiological patterns of activation one must keep in mind the complexities of a synapse. Synaptic depression proves to play an important role in the Purkinje cell to DCN (PC-DCN) synapse. Depression of evoked IPSCs in vitro includes a fast frequency-dependent component which shows rapid recovery, and a frequency-independent component from which recovery is slow (Telgkamp and Raman, 2002). As stimulation rate increases, each IPSC decays less completely, essentially creating a tonic synaptically mediated hyperpolarization (Telgkamp and Raman, 2002; Person and Raman, 2012). These properties have been suggested to limit the sensitivity of DCN cells to basal inhibition, while allowing responses to changes in Purkinje cell activity (Telgkamp and Raman, 2002). Purkinje cell inhibitory inputs also show multiple forms of synaptic plasticity. It has been shown that PC-DCN synapses exhibit either short term depression or short term facilitation depending on the frequency of input (Pedroarena and Schwarz, 2003). Other studies established that local GABA spillover slows the onset and limits the extent of synaptic depression during high-frequency signaling (Pugh and Raman, 2005). The PC-DCN synapse can also undergo long-term potentiation (LTP) or depression (LTD) depending on the amount of rebound depolarization elicited during the induction protocol. The polarity of change in synaptic strength is then related to rebound bursts (Aizenman et al., 1998).

The generation of a rebound response (as measured by a somatically injected current pulse) can also be shaped by the timing and plasticity of MFs that project collaterals to DCN cells as they course upwards to the granule cell layer. It was found that high frequency burst stimulation of MFs can give rise to LTD of these synapses that is linked to the activation of group I metabotropic glutamate receptors (mGluRs) and protein translation (Zhang and Linden, 2006). The generation of LTP also follows its own set of rules in requiring a specific timing relationship between synaptic stimuli and postinhibitory rebound (Pugh and Raman, 2008). It was then
shown that a prolonged post rebound period of firing is mediated in part by an interplay between L-type calcium channels and mGlur1 and mGlur5 interaction (Zheng and Raman, 2009a). Direct stimulation of Purkinje cell axons then can activate MF collaterals, which in turn could activate mGlur1 and mGlur5 receptors at the PC-DCN synapse. Pharmacology is the general method used to block mGluRs to ensure they are not altering the properties of the synapse of interest (Zheng and Raman, 2010).

1.4.2 Physiological Stimuli

The ability for DCN cells to respond to physiological patterns of Purkinje cell input remains largely unexplored. The exceptions are found in attempts to record from DCN cells in vivo while delivering direct stimuli to the overlying region of Purkinje cells (Hoebeek et al., 2010; Bengtsson et al., 2011). These studies reported that statistically defined rebound bursts could be detected in DCN cells in vivo, but primarily in relation to particularly large hyperpolarizing responses generated following 100 Hz stimulus trains delivered directly to Purkinje cells at the cortical level, or spontaneously during recording (presumably reflecting the activity of complex spikes). Numerous smaller IPSPs potentially reflecting discharge of simple spikes in Purkinje cells were without noticeable influence with regard to rebound burst responses (Hoebeek et al., 2010; Bengtsson et al., 2011). Other studies and models have emphasized the potential importance of the relative synchrony of Purkinje cell firing in relation to sensory input (Shin and De Schutter, 2006; Shin et al., 2007b; Shin et al., 2007a), and even the synchrony of pauses in Purkinje cell firing following complex spike discharge in a population of Purkinje cells (Steuber et al., 2007; De Schutter and Steuber, 2009).

The precise pattern of Purkinje cell input that is important to be decoded by DCN cells is also a subject of debate. Purkinje cells fire very regularly in vitro slice preparations (Hausser and Clark, 1997; Raman and Bean, 1999). Recent work in vivo reported that instead, Purkinje cells in either anesthetized or awake behaving rats or mice could exhibit irregular patterns of firing comprised of both brief elevations and pauses of firing rate (Vos et al., 1999; Goossens et al., 2001; Jorntell et al., 2010). This conclusion is also supported by past and recent evidence that Purkinje cell and cerebellar cortical activity reflects an ongoing interplay with neocortical
regions given the known feedback provided to cerebellum by way of pontine and inferior olivary nuclei (Rowland and Jaeger, 2005, 2008; Ros et al., 2009). Evidence was recently provided using a CV2 analysis that Purkinje cells contain precise regular spiking patterns that last up to hundreds of milliseconds and comprise over half the spikes (Shin et al., 2007b). Interestingly, it was also found that tactile stimulation increased the regularity of spiking (patterns were faster and lasted longer following stimulation). The study concluded that Purkinje cell spike patterns provide a relatively constant synaptic conductance to the DCN neurons. Moreover, these patterns were found in some cases to coincide with similar patterns in nearby Purkinje cells (Shin et al., 2007b). The coincident patterns would then provide what could be seen as synchronized pauses (a period where there is a lack of Purkinje cell-mediated inhibition). Recently there has been a growing interest in synchronized pauses in Purkinje cells, which can be argued as a type of temporal code due to the fact that these pauses provide optimal conditions for rebound bursts (De Schutter and Steuber, 2009). It has also been observed that DCN neurons time-lock their spikes to a synchronous population of Purkinje cells in vivo, which suggests that Purkinje cell synchrony is preferentially relayed to downstream premotor areas (Person and Raman, 2012).

The relative importance of an elevation in firing rate in Purkinje cells as a means of producing a strong hyperpolarization, or a pause in firing that will temporarily relieve inhibition of DCN cells remains a key question in the field (Steuber et al., 2007; Alvina et al., 2008; Jorntell et al., 2010; De Zeeuw et al., 2011).

1.5 Neural Coding

One of the most important questions when considering how information is transferred in neural circuits is how they transform the synaptic inputs to a meaningful output signal, a process called 'neural coding' (Borst and Theunissen, 1999; deCharms and Zador, 2000). This means that the underlying mechanism for neural coding is in the ionic channels’ affect on action potential generation. The literature reveals two general types of neural coding: rate coding and temporal coding. Rate coding was originally defined as reflecting a change in the mean firing rate of a neuron with respect to a given presynaptic input signal (Borst and Theunissen, 1999; Stein et al., 2005). However, there has been some confusion in recent years due to different averaging
procedures, where spikes can be averaged over time, over several trials of an experiment, or over a population of neurons (Rieke et al.). In the case of rate coding, the exact timing of spikes or statistical relation of spikes to one another has no relevance. Time-related parameters instead describe a temporal code. Temporal coding strategies are based on more precise measurements such as FSL, precision, phase, correlations, and synchrony of firing rather than just a mean rate of firing (Gauck and Jaeger, 2000; Butts et al., 2007). FSL is an important temporal coding parameter, and was shown capable of coding a particular stimulus dimension, as seen in the auditory cortex where FSL correlates well with the direction of sound sources (Brugge et al., 1996; Eggermont, 1998). FSL in the case of DCN neurons has been measured from stimulus onset in relation to the first spike of a rebound burst discharge. Note that in the typical slice preparations the FSL is just measured as a the time from the end of the provided stimuli (current injection or synaptic stimulation (Engbers et al., 2011)). Although that definitions works in vitro it is much harder to assess using a continuous stimuli with no defined end.

1.5.1 DCN Neural Coding

It is suggested that burst firing represents a special neural code combining both frequency and rate coding (Gabbiani et al., 1996; Lisman, 1997; Mauk et al., 2000; Sherman, 2001; Kepecs and Lisman, 2003; Oswald et al., 2004; Doiron et al., 2007; Oswald et al., 2007; Heck et al., 2013). DCN cells thus provide the opportunity to explore how Purkinje cell inhibitory input is encoded using either of the two different burst phenotypes recognized to date. Past work has established that DCN cells exhibit rate coding (information that is carried by the firing rate) in terms of a graded rebound firing frequency generated with respect to the magnitude of a preceding hyperpolarization (Jahnsen and Llinas, 1984a; Jahnsen, 1986a; Aizenman and Linden, 1999; Gauck and Jaeger, 2000; Czubayko et al., 2001; Rowland and Jaeger, 2005; Molineux et al., 2008a; Tadayonnejad et al., 2009b; Pedroarena, 2010; Sangrey and Jaeger, 2010; Tadayonnejad et al., 2010b). More recently the role of FSL and spike precision has been recognized as a key factor in rebound bursts in DCN cells, and in relation to more physiological levels of stimulation (Gauck and Jaeger, 2000; Hore et al., 2002; Engbers et al., 2011; Engbers et al., 2013; Heck et al., 2013). Other factors considered important in the nature of Purkinje cell
input is the duration of the pause that follows an increase in firing (i.e. following a complex spike) (Steuber et al., 2007; De Schutter and Steuber, 2009; Person and Raman, 2012) and the synchronicity of Purkinje cell firing (Shin and De Schutter, 2006; Heck et al., 2007; Shin et al., 2007b; De Schutter and Steuber, 2009; Jaeger, 2011; Person and Raman, 2012; Heck et al., 2013).

Therefore it is important to consider both the absolute and relative timing of Purkinje cell spikes and pauses in order to assess the neural coding mechanisms present in the PC-DCN synapse.

The background data existing in the field and briefly summarized above led me to propose the following:

**Hypothesis:** Intrinsic membrane properties underlying rebound bursts allow Transient and Weak burst DCN cells to differentially encode physiological patterns of inhibitory input.

**OBJECTIVES**

1. To determine how Transient and Weak burst DCN neurons respond *in vitro* to physiological stimuli delivered to Purkinje cell axons as stimulus templates derived from recordings obtained *in vivo* during spontaneous and sensory-evoked stimuli to anesthetized rats.

**SIGNIFICANCE**

These experiments will seek to identify the physiological Purkinje cell firing patterns that can reliably trigger rebound bursts in Transient and Weak burst neurons. Although many previous studies have looked at the rebound burst phenotypes, the functional significance and input parameters that generate the rebound response in the DCN are unknown. Given this, my findings will be significant to understanding the parameters of Purkinje cell input that are relevant to the DCN.
CHAPTER TWO: METHODS

2.1 Animal Care

Timed pregnant Sprague-Dawley rat dams were purchased from Charles River Laboratories (Charles River, Quebec, Canada). Animals were maintained and all dissection procedures were completed in accordance with the guidelines established by the Canadian Council on Animal Care. DCN cell recordings were obtained from male rats P12-P17.

2.2 Solutions and Pharmacology

All chemicals were obtained from SIGMA (St. Louis, MO) unless otherwise indicated. Slice preparation and recordings were performed under the circulation of an artificial cerebrospinal fluid (aCSF) composed of (mM): NaCl (125), KCl (3.25), CaCl$_2$ (1.5), MgCl$_2$ (1.5), NaHCO$_3$ (25), and D-glucose (25). The aCSF was continuously bubbled with carbogen (95% O$_2$, 5% CO$_2$) gas. The internal pipette solution for all current-clamp recordings consisted of (mM): K-gluconate (130), EGTA (0.1), HEPES (10), NaCl (7), MgCl$_2$ (0.3), pH 7.3 with KOH. Di-tris-creatine phosphate (5), tris-ATP (2), and Na-GTP (0.5) were added daily from a frozen stock solution. The theoretical reversal potentials created by these solutions at 35 °C were: $E_{Na} = 80$ mV; $E_K = -98$ mV; $E_{Cl} = -75.7$ mV. As established by previous comparisons of DCN cell output first recorded in on-cell mode followed by a whole-cell patch configuration, this internal electrolyte preserves the firing pattern of DCN cells (Tadayonnejad et al., 2009b), greatly reducing any potential influence of whole-cell configuration on the nature of spike output recorded here.

As the study focused on the effects of stimulating Purkinje cell inhibitory input, all recordings were undertaken in the presence of bath applied excitatory ionotropic synaptic blockers (DL-2-amino-5-phosphonopentanoic acid (DL-AP5; 25 μM) and 6,7-dinitroquinoxolinedione (DNQX; 10 μM), and the mGluR blockers 2-Methyl-6-(phenylethynyl)pyridine (MPEP; 1 μM), CPCCOEt (10 μM), and (3,4-Dihydro-2H-pyran[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methane (JNJ 16259685; 1.5 μM)).

2.3 Preparation of Tissue

Rats were anaesthetized by inhalation of isoflurane until unresponsive to a tail pinch. The rat
was then decapitated by guillotine and the dorsal aspect of the skull removed. The exposed cerebellum was immediately bathed with an ice-cold stream of aCSF solution from a 50 ml syringe. To obtain slices in the parasagittal plane the cerebellum was immersed in ice-cold aCSF and continuously bubbled in the vibratome chamber with carbogen gas while 240 μm slices were cut. Slices were then transferred to a holding chamber and incubated at 35 °C aCSF for 30-35 min, and allowed to cool to room temperature (~21 °C) in a carbogen-gassed holding chamber.

### 2.4 Electrophysiology

Cerebellar slices were transferred from the holding chamber into the recording chamber of a Zeiss Axioskop FS-2 microscope and maintained at 32-34 °C as a submerged preparation. Each slice was perfused with aCSF at a rate of 2-3 ml/min and held in place using a platinum harp strung with fine nylon wires. DCN neurons were imaged using differential interference contrast optics with infrared light transmission (DIC-IR) detected using an IR-sensitive Newvicon camera (DAGE; Michigan City, IN) mounted in the light path of the microscope. The region containing the DCN was first identified using a low power (4x) objective under visual guidance, and individual cells subsequently identified by a (40x) immersion objective under DIC-IR optics. DCN neurons were distinguished according to somatic size: large (>20 μm) or small (<20 μm) diameter (Czubayko et al., 2001). All experiments carried out here focused on large diameter neurons primarily from Interpositus and Lateral nuclei, with some cells included from medial DCN.

Current-clamp recordings were obtained using an Axon 700B Multiclamp amplifier (Axon Instruments) and digitized using a Digidata 1400A (Axon Instruments). Data was collected with pClamp 10 software with a DC-10 kHz bandpass filter. Pipettes were pulled from thick-walled borosilicate glass and had resistances between 5-10 MΩ. A seal between membrane and electrode was created using negative pressure applied by suction through connected PE tubing. Seal resistance was in excess of 1.0 GΩ before breaking into the cell and gaining whole-cell access. Pipette capacitance was neutralized and series resistance compensated with bridge balance circuitry. Whole-cell recordings were conducted on cells with an access resistance of 8-15 MΩ, with cells rejected for any drift in access resistance of > 20%. Control recordings were made >5 min after break in to allow full stabilization with the internal solution. Control and
baseline recordings were sampled at 40 kHz, while stimulation protocols were sampled at 10 kHz (as was necessary due to the length of the Purkinje cell patterns presented). When sampling at 10 kHz the pClamp-10 software's filter was set to a DC-4 kHz bandpass filter.

2.5 Stimulation Methodology

The first set of experiments involving stimulation of Purkinje cell axons used recordings of Purkinje cell firing patterns obtained in awake mice or anesthetized rats in vivo (Shin et al., 2007b). Purkinje cell spike trains were time-stamped using custom offline software (see (Shin et al., 2007b) and software written in MATLAB R2007B converted the time-samples into TTL pulse trains to use as physiological stimulus patterns to activate Purkinje cell axons in vitro. The TTL pulse trains were then delivered as a digital-to-analog output from with PClamp software and the Digidata to the input of a Digitimer Stimulus Isolation unit. Purkinje cell inhibitory axon input to DCN neurons was then stimulated using 0.1 - 0.2 ms pulses defined by the TTL pulse train to a concentric bipolar electrode (Frederick Haer, CBCMX75(JL2)) positioned on the slice surface just dorsal to the recording site and outside of the DCN nuclei. Stimulus strength from the isolation unit used to define maximal IPSC amplitude was restricted to < ~30 volts, as higher voltage stimuli could create electrolysis at the electrode tip and cause tissue movement during high frequency stimulation. In all cases the stimulating and recording sites were directly monitored by video imaging to confirm the lack of electrolysis or movement during stimulation, with either effect leading to rejection of recorded data. It was found that these effects were problematic when delivering the high frequencies of Purkinje cell input characteristic of mouse recordings. Data presented here thus focus entirely on stimulation frequencies and recordings relevant to rat tissue.

2.6 Cell Control Protocols

Four control protocols were used to determine cell health and proper characteristics to be included in the data set (expanded analysis below, see results):

1) A spontaneous firing pattern in order to check spike height (>65mV), baseline frequency, and the presences of the AHPs.

2) A series of hyperpolarizing steps was used to ensure the neuron was capable of producing
a rebound burst, and also used to categorize cells into the two burst phenotypes (Weak or Transient). A rebound burst was identified according to an increase in spike frequency over the initial 100 ms following a hyperpolarizing stimulus that exceeded 2 times the standard deviation of the mean spontaneous firing frequency over 10 secs at rest in each cell for a membrane potential of ~-60 mV at the AHP trough.

3) A 50 Hz (25 pulse) train of synaptic inputs was delivered in voltage-clamp to test for a synaptic connection between our Purkinje cell and DCN neuron. To normalize our stimulus intensity, we increased the stimulating voltage (up to ~30V) and then reduced our IPSC to 60 % maximal.

4) A 100 Hz (25 pulse) train of synaptic inputs was delivered in current-clamp to insure our stimulus intensity was strong enough to elicit a rebound response. Any cell which did not provide a statistically defined rebound at 60% maximal was rejected.

If the cell passed the rejection criteria above it was presented with our Purkinje cell input patterns. Each cell was given 2 minutes rest in between Purkinje cell input patterns. After the two minute rest we provided the cell with a single sweep 50 Hz (25 pulses) of synaptic stimulation to measure IPSP amplitude. Post-hoc analysis was used to look at the change in IPSP amplitude over time, and if no significant difference was seen the cells stimulation protocols were considered acceptable. Further details and analysis of these control recordings can be seen in Chapter 3.

2.7 Data Analysis

Data was analyzed using Clampfit 10 software and custom MATLAB R2007B scripts. Custom MATLAB scripts were used to align the perioral stimulus times in order to study the DCN frequency response (Fig. 3.2,3.6-9). We also used similar scripts to perform our reverse correlation, in which we locate the first spike of a burst response (time 0, unless otherwise indicated) and look at the Purkinje cell frequency before and after the defined burst rebounds (Fig 3.10-12). Again the same process was used to align the complex spike timings and study the DCN frequency response (Fig 3.15).

Instantaneous frequency plots are constructed by convolving Gaussian kernels with a standard deviation inversely proportional to the neuronal firing frequency (Paulin; Steuber et al.,
Following Steuber et al., we set the Gaussian standard deviation $\sigma_k$ for each spike $k$ to

$$\sigma_k = \min(ISI_{\text{before}}, ISI_{\text{after}}) / \sqrt{2\pi}$$

where $ISI_{\text{before}}$ and $ISI_{\text{after}}$ are the interspike intervals (ISIs) directly before and after the spike. This limits the individual contribution of each spike to the maximum instantaneous rate just before or after it (Steuber et al., 2011). This method was originally developed for spike trains that show large variations in successive ISIs as seen during a rebound response in the DCN. A visual representation of the formula can be seen in **Figure 2.1**.

Coefficient of variation (CV) was used to measure the regularity of our Purkinje cell input patterns, and $CV_2$ was used to measure the short term regularity (Holt et al., 1996; Shin et al., 2007b). CV is the standard deviation of the ISIs divided by the mean ISI in your spike train, while $CV_2$ is the same calculation done on a spike to spike basis, only looking at the previous and next ISI instead of the whole spike train. The number of regular patterns in our Purkinje cell patterns was measured using a threshold value (0.2) for $CV_2$ as done by Shin et al.

$$CV = \frac{\text{Std}(ISI)}{\text{Mean}(ISI)}$$

$$CV_2 = \frac{2|ISI_{n+1} - ISI_n|}{(ISI_{n+1} + ISI_n)}$$

Custom MATLAB scripts were used to assess and analyze the metrics associated with our reverse correlation analysis as well as to plot the SEM in the frequency plots.

A) PC pattern baseline: The average Purkinje cell frequency over the entire 100 second recording.

B) PC preburst duration: The period of time in which the Purkinje cell frequency is greater than the PC pattern baseline.

C) PC preburst frequency: The maximal frequency reached above the PC pattern baseline during the PC preburst duration.

D) PC rate of decline: The slope measured from the time of the PC preburst frequency, until the zero time point (the first spike of the burst).

E) Pause duration: The time in which the Purkinje cell frequency was below 50% of the PC pattern baseline.

See **Figure 3.16** for a graphical representation of the values described above. Statistical analysis
including student t-tests and linear regressions were performed in OriginPro 8 unless otherwise stated. Average values are plotted as mean ± SEM; * p < 0.05, ** p < 0.01, *** p < 0.001, and ns, not significant.

2.8 Purkinje cell Patterns

During post-recording analysis we discovered that our PC Spontaneous stimulus file only contained the simple spikes recorded from the Purkinje cell, as the complex spikes had been filtered out by Shin et al. We believe that this had minimal effects on our initial analyses of the Purkinje cell firing pattern as it corresponds to only 1% of the total spikes (51/4589) in the PC Spontaneous input file. However, to ensure that we do not draw incorrect conclusions as a result of these missing spike times, our linear regression, CV2, and complex spike analysis were only performed on the PC Sensory-evoked stimulus pattern, which contained the full set of both simple and complex spikes.
Figure 2 Gaussian Rate Coding.

A method for plotting the spike rate of a DCN neuron, in which each spike is convolved with a Gaussian kernel. To account for high variability in DCN spikes trains, the standard deviation of the kernel ($\sigma_k$) is set according to:

$$\sigma_k = \min(ISI_{before}, ISI_{after}) / \sqrt{2\pi}.$$  

Where $ISI_{before}$ is the ISI preceding spike k, and $ISI_{after}$ is the ISI directly following spike k. These Gaussian kernels then give a smooth and accurate spike rate code for both DCN and Purkinje cell neurons. Method adopted from (Paulin, 1996; Steuber et al., 2011).
CHAPTER THREE: RESULTS

3.1 Cell Selection

The activity of large diameter DCN neurons was recorded under whole-cell conditions primarily in the Interpositus and Lateral nucleus in transverse tissue slices of rat cerebellum at near physiological temperature (32-34°C). Cells were accepted for recording if they satisfied the minimal requirements of exhibiting spontaneous and tonic discharge of spikes of at least 65 mV peak amplitude and evoked inhibitory responses to stimulation of Purkinje cell axons. Given the continuous manner of tonic firing inherent to DCN cells, resting membrane potential was adjusted by bias current injection and measured at the trough of the AHP as $-61 \pm 1.85$ mV ($n = 17$) (after subtracting junction potential), with a spontaneous firing frequency of $10.72 \pm 3.11$ Hz for Weak burst neurons ($n = 9$) and $8.96 \pm 1.90$ Hz for Transient burst neurons (Fig. 3.1A). Recordings were confined to putative output cells identified by a large soma diameter (15-20 µm) and a set of three spike afterpotentials consisting of a fast AHP (fAHP), a depolarizing afterpotential (DAP), and a slow AHP (sAHP) (Fig. 3.1A) (Uusisaari et al., 2007; Molineux et al., 2008b; Uusisaari and Knopfel, 2011). Cells were further confirmed to be capable of evoking a rebound increase in firing frequency or spike burst following a membrane hyperpolarization invoked by a set of square wave current pulse injections (500 msec) to a maximal membrane potential of ~90 mV.

A rebound burst was identified by an increase in spike frequency over the initial 100 msec following a hyperpolarizing stimulus. The increase in frequency must have exceeded 2 times the standard deviation of the mean spontaneous firing frequency over 10 secs at rest in each cell, for a membrane potential of ~60 mV at the AHP trough. Cells were further identified as exhibiting a Transient or Weak burst phenotype according to the pattern and frequency reached in the initial 100 msec following release from a hyperpolarizing current pulse. As previously described (Molineux et al., 2006; Molineux et al., 2008b; Tadayonnejad et al., 2009a), Transient burst cells characteristically exhibited an initial high frequency burst followed by a gradual return of firing frequency to resting levels over 1-2 secs. In contrast Weak burst cells exhibited a more modest increase in peak rebound frequency without a prominent initial high frequency burst, followed by a gradual return to resting frequency over 1-2 secs (Fig. 3.1B). To
assess the burst response following a physiological level of hyperpolarization, Figure 3.1B compares the bursts triggered by a 500 msec current step that evoked a membrane potential shift down to ~-75 mV (our theoretical Cl⁻ reversal potential). A difference in burst frequency was readily apparent from this level of hyperpolarization, with a mean increase in spike frequency (above baseline levels) in Transient burst neurons of 161.57 ± 28.54 Hz (n = 8) and 15.36 ± 5.53 Hz (n = 9) in Weak burst neurons.

3.2 Purkinje Cell Inhibitory Potentials

Purkinje cell axons were stimulated using a concentric bipolar electrode positioned above the dorsal border of the cerebellar nuclei. Ionic gradients present during whole-cell recordings established a theoretical E_{Cl} of -75.7 mV. The stimulus intensity used for physiological stimulus trains was determined by first testing evoked IPSCs under voltage clamp from a holding potential of -65 mV. The maximal evoked IPSC was first determined (see Methods) and then stimulus intensity adjusted to ~60% of the maximum to ensure a submaximal and consistent initial stimulus between individual cell recordings. At 60% maximal stimulus intensity stimulation of Purkinje cell input evoked an outward current with a peak latency of 6.16 ± 0.64 msec (n = 17) and peak amplitude of 60.69 ± 17.5 pA (n = 17) from a holding potential of -65 mV (Fig. 3.1C). Repetitive stimulation at 50 Hz progressively decreased IPSC amplitude (Fig. 3.1C), as expected for Purkinje cell inputs to DCN cells (Telgkamp and Raman, 2002; Pedroarena and Schwarz, 2003; Telgkamp et al., 2004). Synaptic connectivity and function was judged acceptable if cells responded at this intensity with a detectable IPSP under current clamp conditions, with single pulse stimuli to Purkinje cell axons evoking an average IPSP of -12.66 ± 1.54 mV (n = 17). Repetitive stimulation at 100 Hz for 5 pulses then evoked the expected progressive decrease in IPSP amplitude, with peak IPSP amplitude most often approaching a value around the predicted E_{Cl} (Fig. 3.1D). Excitatory synaptic responses can potentially be evoked by mossy fiber or climbing fiber axons that project collateral inputs to DCN cells as they ascend to make their primary synaptic contact with cells of cerebellar cortex (Gauck and Jaeger, 2003; Pugh and Raman, 2006; Zheng and Raman, 2011). Therefore, any potential co-activation of excitatory inputs was avoided by always recording IPSPs in the presence of blockers of glutamate receptors DL-AP5 (25 μM) and DNQX (10 μM), the Group 1 metabotropic receptor.
blockers JNJ 16259685 (1.5 µM) and CPPCCOEt (10 µM), and the Group 5 metabotropic receptor blocker MPEP (1 µM) (Zhang and Linden, 2006; Knopfel, 2007; Tadayonnejad et al., 2010a; Zheng and Raman, 2011).

3.3 Physiological Stimulation

In order to assess the rebound response capability of DCN neurons, we delivered trains of physiological patterns of stimuli (100 sec) derived from Purkinje cell recordings in anesthetized rats in vivo (Shin et al., 2007b). Purkinje cell firing was originally recorded under resting conditions (PC Spontaneous pattern) or while the animal was receiving a perioral /whisker stimulation at approximately two sec intervals (PC Sensory-evoked pattern) (Shin et al., 2007b). The two Purkinje cell spike patterns are shown as raster plots in Figure 3.2A. Recorded data was parsed into two sec duration records in order to construct average frequency recordings over a time corresponding to 500 msec pre-stimulus time and 1500 msec post-stimulus time. By applying the same time interval parsing to the spontaneous recording of Purkinje cell input we could compare the average for the PC Sensory-evoked pattern to a similar period of the PC Spontaneous pattern (Fig. 3.2A,B).

Raster plots of spontaneous firing in the Purkinje cell recording revealed a fluctuating spike output reflecting periods of relative silence interspersed with segments of firing (Fig. 3.2A). The mean firing frequency over the entire length of 100 sec of spontaneous firing was 46.85 Hz (n = 4537 ISIs) and had a CV = 5.64. A similar analysis conducted over the PC Sensory-evoked stimulus revealed a mean rate of 69.47 Hz (n = 6542 ISIs) and a CV = 2.49. Comparing the effects of perioral stimulation on raster plots, the PC Sensory-evoked pattern exhibited a strong post-stimulus increase in the firing rate for a period of ~200 msec following the sensory stimulus which was not present in the PC Spontaneous pattern over the same relative time intervals (Fig. 3.2A, B). The mean rate of firing over the 500 msec preceding each sensory stimulus (66.13 ± 28.62 Hz, n = 49) was not significantly different from that over the last 500 msec of recording following each stimulus (67.30 ± 29.81 Hz, n = 49, p > 0.05). These initial measurements indicate that firing frequency recovered within 2 sec of the stimulus and these Purkinje cell patterns reflect distinct repetitions of a stimulus that can be averaged without concern for cumulative effects.
Figure 3.1. Control recordings in large diameter DCN neurons.

A, Spontaneous firing in a large diameter DCN neuron at rest set with bias current injection to an AHP trough of ~-60 mV. *Inset* indicates the fAHP, DAP, and sAHP set of afterpotentials characteristic of a large diameter DCN neurons. Bar plots reveal a similar tonic firing frequency in Transient and Weak burst neurons. B, Injecting a 500 ms hyperpolarizing current step triggers a rebound burst. Frequency plots show the key difference in burst frequency between Weak and Transient burst neurons from current pulses that approach ~-75.7 mV, the predicted value of E_{Cl}. Burst frequency is plotted as frequency above baseline firing. C, A 50 Hz train of Purkinje cell synaptic input evokes IPSCs which show characteristic depression over repeated stimuli. The intensity of Purkinje cell axon stimulation was adjusted to ~60% of maximal intensity to provide a comparable submaximal stimulus strength from a holding potential of -65 mV. The mean amplitude of evoked IPSCs is plotted at right. D, Stimulating Purkinje cell synaptic inputs with a 100 Hz (25 pulse) train inhibits DCN neurons and elicits a rebound burst that differs in peak frequency between Transient and Weak burst phenotypes (bar plots). *Horizontal bar* above trace in (D) depicts the duration of a statistically defined rebound increase in spike frequency made up of two consecutive burst ISIs.
Averaging all 49 sensory stimuli over the 100 secs of recording time for both the PC Sensory-evoked and PC Spontaneous patterns was used to assess the nature of the stimulus that could be transmitted if a population of Purkinje cell inputs were activated in a synchronized fashion and converged onto a DCN cell. While the average of the PC Spontaneous stimulus pattern varied between 30-70 Hz over a 2 sec period, no identifiable pattern emerged over the 2 sec segments. However, the PC Sensory-evoked pattern revealed a definite increase in mean frequency, up to ~110 Hz within 200 msec (~40 Hz above baseline), followed by a relative slowing of firing frequency to an absolute value of ~40 Hz (~30 Hz below baseline) between ~500-1300 msec following the stimulus (Fig. 3.2B). It is important to consider that the fidelity of axon transmission in Purkinje cells can become unreliable at high frequencies, with ~50% failure of transmission down the axon for spikes recorded at the soma at frequencies greater than ~257 Hz (as might be reached during a complex spike response) (Monsivais et al., 2005). However, this upper limit is also dependent on the duration of a somatic stimulus discharging a simple spike, with short current pulses evoking an output that can transmit down the axon at frequencies up to ~438±37 Hz (Monsivais et al., 2005). By plotting ISI histograms for the stimulus files we found that 98% of all ISIs in the PC Spontaneous stimulus pattern, and 99.7% of the PC Sensory-evoked pattern, fell below 257 Hz, with all ISIs falling below 438 Hz (Fig. 3.2C). Therefore, both stimulus patterns were considered to deliver rates of Purkinje cell firing relevant to the following frequencies of Purkinje cell axons.

Before delivering either Purkinje cell stimulus pattern, the rebound phenotype of DCN cells was examined using a pair of control tests consisting of hyperpolarizing current steps of 500 msec duration, and a train of stimuli set to 60% maximum IPSC amplitude of 25 pulses, 50 Hz (see methods). A period of at least 2 min was allowed between multiple presentations of the Purkinje cell stimulus patterns, with the 25 pulse stimulus conducted to ensure the continued integrity of the evoked IPSP amplitude and the tonic firing of a given cell at least 1 min before delivery of a Purkinje cell physiological pattern. Repetition of a Purkinje cell stimulus pattern was only conducted if comparison of IPSPs on the first pulse of control IPSP stimulus trains confirmed no significant change in amplitude, and rebound responses were still detected. While long-term plasticity of evoked IPSPs has been reported in the Purkinje-DCN synaptic relay (Morishita and Sastry, 1993; Aizenman et al., 2000; Pedroarena and Schwarz, 2003), we found
Figure 3.2. Sensory-evoked and spontaneous Purkinje cell spike patterns recorded in vivo.

A-C, Records of Purkinje cell firing to perioral sensory-evoked stimuli delivered every 2 secs (left panels) and during spontaneous activity (right panels) in two separate recordings from anesthetized rats in vivo (Shin et al., 2007b) and used as stimulus templates in vitro. In (A) and (C) the 100 sec of continuous recordings are parsed into 2 sec segments corresponding to a 500 ms baseline spontaneous rate of discharge prior to a stimulus, and 1500 ms following a stimulus (total of 49 / recording). The same time intervals are used to parse data in records of sensory-evoked or spontaneous firing of Purkinje cells. A, Raster plots of the timing of spike discharge in relation to each of the 49 separate sensory stimuli, as indicated by each new line entry. Red lines at 500 ms indicate the timing of individual perioral stimuli and the equivalent time in the spontaneous recordings for comparison. B, Mean frequency plots of the two records of Purkinje cell firing shown in (A) averaged over all 49 sensory stimuli, with spontaneous firing rate subdivided over the same intervals. The number of 2 sec recording segments used to calculate mean values are indicated in brackets with grey shaded areas reflecting the SEM. C, ISI frequency histograms for the records shown in (A) and (B), with the X-axis converted to illustrate instantaneous event frequencies. Red dashed lines indicate the reported somatic spike frequency at which axonal condition becomes less reliable (Monsivais et al., 2005).
no significant long-term change in IPSPs evoked by the stimulus patterns presented here. Data were only accepted if no movement of the electrode was detected during the stimulus train, spike amplitude and spontaneous activity continued as in control conditions, and the evoked IPSP amplitude and rebound burst capability were retained following presentation of Purkinje cell physiological stimulus patterns. The data presented were derived from 8 Transient burst and 9 Weak burst neurons that met these criteria.

3.4 Purkinje Cell Physiological Spike Input Patterns Evoke Complex Responses in the DCN

Delivering the Purkinje cell physiological spike patterns evoked a series of IPSPs that resulted in a complex modulation of the spontaneous firing rate of DCN cells, depending on instantaneous variations in Purkinje cell input frequencies. Any DCN cell ISIs that matched the criteria of exceeding 2X the standard deviation of the rate of spontaneous firing under control conditions were then identified. We note that these conditions are substantially different in terms of overall activity compared to the physiological stimulus input files, but choosing a segment of background firing during the stimulus patterns was not possible due to extensive variability in the evoked response depending on variations in the presynaptic interpulse interval. Using the spontaneous rate of firing as the baseline point of comparison is thus expected, if anything, to overestimate the number of rebound responses identified.

Examples of DCN cell responses to the PC Spontaneous input pattern is shown in Figure 3.3. Close inspection of the recordings revealed some expected patterns of IPSP responses based on knowledge obtained from previous work characterizing DCN cell firing in response to fixed frequencies of input. The peak amplitude of the IPSP could approach $E_{Cl}$ but was also affected by the timing of a stimulus in relation to the background swings in membrane potential between the trough of an AHP or spike discharge, a result likely reflecting the level of background membrane resistance and the driving force to $E_{Cl}$ (Fig. 3.3A,B). As previously established (Telgkamp and Raman, 2002; Telgkamp et al., 2004), longer durations or higher frequencies of input stimuli were associated with a frequency-dependent depression of IPSP amplitude during the train (cf. Fig. 3.3A and B). In some cases the depression in amplitude could lead to a depolarizing shift in membrane potential (Pedroarena, 2010), although the extent to which this
reflected a process independent from the underlying conductances driving a DCN cell to discharge another spike could not be determined. IPSP amplitude could also exhibit some degree of recovery within tens of msec during a relative slowing in the instantaneous frequency of the input stimulus (Fig. 3.3B) (Telgkamp and Raman, 2002; Telgkamp et al., 2004). The amplitude of IPSPs and the peak membrane potential reached during the physiological stimulus train was thus extremely variable and difficult to interpret based on visual inspection alone. However, the ability to detect each of the processes indicated above, and even individual IPSPs with normal amplitude near the end of 100 sec of physiological stimulus input indicated that IPSPs were not undergoing progressive failure or rundown over the course of stimuli presented here.

Similarly, the response of DCN cells in terms of spike output was difficult to interpret by visual inspection given the degree of interstimulus frequency variability and in response to even brief changes in the Purkinje cell input stimulus frequency. While all trains of Purkinje cell stimuli hyperpolarized the DCN cell to alter spike discharge by at least lengthening the ISI (Fig. 3.3B, C), in some cases DCN cells would respond to Purkinje cell stimuli by essentially resuming a degree of tonic firing (Fig. 3.3D). This activity would support the conclusions of previous work that the net effect of multiple Purkinje cell inhibitory inputs can be to adjust the overall membrane potential and thus firing rate of DCN cells (Gauck and Jaeger, 2000). Moreover, Purkinje cell input that was similar to or even higher in frequency and number of stimuli than the control stimuli shown to be able to produce a rebound response could fail to evoke a rebound burst (cf Figs. 3.1D and Fig. 3.3C). This failure to evoke a rebound burst was evident even for segments of the record corresponding to Purkinje cell perioral stimuli (Fig. 3.3E). The variability in rebound burst capability during physiological stimulus patterns was also apparent for DCN cells defined as exhibiting either a Transient or Weak burst phenotype.

When a rebound burst was generated, the ISIs falling within the statistically defined range for a burst were often delayed by 1-3 spikes following a brief high frequency Purkinje cell input train (Fig. 3.3B). In this regard, the pattern of many rebound responses evoked by the physiological input stimuli deviated from the Transient or Weak burst phenotypes reliably evoked using an injected current pulse or a fixed frequency inhibitory synaptic input, as traditionally used \textit{in vitro}. Thus, instead of a rapid transition to a burst response of high frequency following a hyperpolarizing stimulus, many rebound responses consisted of a gradual
Figure 3.3 Physiological patterns of Purkinje cell spike firing evoke complex inhibitory responses and spike output in DCN neurons.

A, Expanded view of a Transient DCN neuron response to compare variations in IPSP amplitudes during stimulation of Purkinje cell axonal input using the Spontaneous stimulus template. B, Example recording from a Transient burst DCN neuron showing how similar input frequencies as used in control protocols (~100 Hz) can elicit a rebound increase in spike frequency that satisfy the criteria of burst ISIs (*blue horizontal line*), but with a delay from the end of the Purkinje cell input stimulus train. A progressive depression of IPSPs and rapid recovery are evident in the recordings. C, Example recording from a Transient burst DCN neuron receiving a series of PC Spontaneous input frequencies that are higher than those in (B) but that primarily lengthen the DCN cell ISI without promoting rebound responses. D, Example recording from a Transient burst DCN neuron highlighting how a wide range of Purkinje cell input frequencies and patterns fail to elicit a readily detectable rebound response, with continued discharge of apparent tonic activity. E, Example recording of a Transient burst DCN cell response at the time of a perioral sensory stimulus (*red bar*), and the lack of a burst response to this sensory-relevant stimulus.
transition to a series of ISIs that shortened to within an interval that fell within the burst criteria. In fact, this pattern has been recognized in the past (Pedroarena and Schwarz, 2003; Hoebeek et al., 2010; Pedroarena, 2010) but was not fully considered in the original definition of Transient or Weak burst phenotypes that relied on the average firing frequency over the initial 100 msec following a hyperpolarizing stimulus (Molineux et al., 2006; Molineux et al., 2008b).

3.5 Burst Response Following Purkinje Cell Input

Figure 3.4 illustrates the responses of Transient burst cells to either the PC Sensory-evoked stimulus pattern (Fig. 3.4A) or the PC Spontaneous discharge stimulus pattern (Fig. 3.4B) continuously presented over 100 sec. The occurrence of ISIs in DCN cells that fell within the statistical definition of a rebound burst were classified according to the number of sequential ISIs satisfying this criteria (ie 2 spike burst vs 6 spike burst). The data reveal that in some cells the PC Sensory-evoked stimulus triggered relatively few burst ISIs despite presenting 49 sensory stimulus-relevant Purkinje cell responses at approximately 2 sec intervals (Fig. 3.4A). By comparison, the variable frequency and pattern of the PC Spontaneous input file could evoke in the same cell a complex series of DCN cell spike responses and a high number of rebound burst ISIs throughout the stimulus input train (Fig. 3.4B). We note that the PC Sensory-evoked stimulus input was far more regular in its pattern than the PC Spontaneous input, evoking a lower frequency of discharge in DCN cells over the course of the stimulus than the PC Spontaneous input. The degree of inhibition was also stronger over the initial ~20 sec of recording in the PC Sensory-evoked stimulus, followed by a gradual restoration of firing and increasing number of burst ISIs over the final 30 sec (Fig. 3.4A). The gradual increase in rate of firing near the end of the PC Sensory-evoked stimulus was not due to a loss of evoked IPSPs, in that full amplitude IPSPs were often evoked following even brief pauses in Purkinje cell input near the end of the stimulus train. In addition, retesting the evoked IPSP a minute following the physiological input pattern confirmed no long-term change in the amplitude of the IPSP compared to control conditions (data not shown). However, a key difference between the start and end of the PC Sensory-evoked stimulus was a greater number of pauses in Purkinje cell firing over the final 30 sec of the train; a time period noticeably associated with an increased rebound burst output in the DCN cell. Consistent with this, the same Transient burst cell
responded to a more varied stimulus pattern in the PC Spontaneous stimulus input by discharging a large number of spikes and rebound bursts throughout the 100 sec recording (Fig. 3.4B).

Despite the clear difference in response of a given cell to either Purkinje cell input pattern shown in Figure 3.4, the final results across 8 Transient burst cells and 9 Weak burst cells was highly variable. Thus, we found no statistically significant difference between the mean values for the total number of evoked DCN cell bursts, number of burst ISIs, or the percentage of burst ISIs in each record when comparing the response between PC Sensory-evoked and PC Spontaneous input patterns in either the Transient burst or Weak burst cells (Fig. 3.5A-C). This degree of variability was detected regardless of maintaining all cells near a membrane potential corresponding to a -61 mV AHP trough. The single significant difference detected between the recordings was in the intraburst frequency between Transient Burst and Weak Burst cells for both the PC Sensory-evoked stimulus and PC Spontaneous stimulus (Fig 3.5D). These results would be consistent with the expectation for a higher burst capability in the Transient Burst phenotype (Molineux et al., 2006; Molineux et al., 2008b; Tadayonnejad et al., 2009a). In addition, the maximum peak intraburst frequency was highest in Transient Burst cells responding to the PC Sensory-evoked stimulus (Fig 3.5D). It is important to note that the higher intraburst frequency of DCN cells was calculated for all bursts irrespective of any temporal association with the sensory-evoked stimuli in the stimulus train. These data suggest that there was very little difference between the ability of Transient Burst and Weak Burst cells to respond (on average) to either physiological input train in terms of rebound burst ISI production. This was particularly surprising in the case of the PC Sensory-evoked stimulus file in which the average sensory-evoked Purkinje cell firing rate reached a maximum peak frequency (110 Hz) even higher than the control stimuli of 100 Hz (25 pulses) used to verify that each cell could respond with a rebound burst to Purkinje cell inhibitory synaptic input (Fig. 3.1D).

We considered that the DCN cell response to the PC Sensory-evoked stimulus may be difficult to detect at the single cell level, but perhaps be better represented as an average value approximating a population event. We thus examined each of the PC Sensory-evoked stimuli over a total of 1600 msec per stimulus, including 100 msec preceding each stimulus (Fig. 3.6A,B). Average values for both Purkinje cell firing frequency and the resulting DCN cell firing frequency were then calculated to maximize the opportunity to detect any stimulus-evoked
Figure 3.4 The DCN cell response to physiological Purkinje cell patterns used as stimulation patterns *in vitro*.

A, B, A representative Transient burst DCN cell response to the PC Sensory-evoked (A) and the PC Spontaneous (B) input patterns that evoke a series of IPSPs and very different burst responsiveness. ISIs that are statistically defined as falling inside a defined burst criteria are marked above each trace (*Bursts*), while the associated Purkinje cell input pattern is shown below. *Black diamonds* above the Purkinje cell inputs represent the timing of each sensory deflection originally delivered *in vivo*. The number of ISIs statistically defined as a rebound burst composed of several intraburst intervals (i.e. multi-spike burst) are colour coded according to the legend below each recording.
Figure 3.5  DCN cell burst ISIs evoked by PC Spontaneous or PC Sensory-evoked stimuli. Mean bar plots comparing the burst response of Transient or Weak burst DCN neurons when stimulated using either the PC Sensory-evoked or PC Spontaneous stimulus input pattern. Shown are comparisons between the mean values of the indicated parameters of DCN cell bursts of between 2-11 spikes over the 100 sec of all recordings in response to either input. A-C, No statistical differences were detected between Transient and Weak burst neurons in terms of the number of bursts detected (A), the number of burst ISIs (B), or the percentage of ISIs classified as bursts within all records (C). D, Comparison between the intraburst frequencies detected in Transient vs Weak burst neurons throughout the 100 sec of DCN recordings for either Purkinje cell input pattern. Mean intraburst frequency is measured as the frequency above baseline (Hz) and represented as mean ± SEM. Sample values shown in brackets in (A-C) reflect total animals used, and those in (D) the total number of bursts in all recordings. Students paired t-test (A, B, C) n.s., not significant; *** p < 0.001, two-sample t-test (D).
change in DCN cell burst production. Mean values were calculated from 49 individual sensory input stimuli within the PC Sensory-evoked stimulus file, for a total of 392 stimuli delivered to 8 Transient burst cells and 441 stimuli delivered to 9 Weak burst cells. This analysis revealed that despite an approximately 40 Hz mean increase in PC Sensory-evoked input per sensory stimulus and a subsequent decrease in firing of 15-20 Hz, neither the Transient nor Weak burst cells showed on average any noticeable change in firing rate after averaging across all stimuli (Fig. 3.6C). A similar analysis for extracted segments of Purkinje cell firing from the PC Spontaneous input file corresponding to the same times as perioral stimuli in the PC Sensory-evoked file revealed no significant pattern in the average Purkinje cell firing rate (Fig. 3.7A,B), nor any identifiable change in the firing frequency of either Transient or Weak burst cells (Fig. 3.7C).

While our control recordings verified that IPSPs were stable between successive presentations of either PC Sensory-evoked or PC Spontaneous files, we considered the possibility that a lack of apparent DCN cell response to the mean Purkinje cell sensory stimulus input could reflect a progressive reduction of IPSP amplitude over time due to such factors as a frequency-dependent depression, a depolarizing shift in $E_{Cl}$, or even habituation to the ~2 sec interval of sensory input. To examine this, we repeated the process of extracting segments of Purkinje cell firing and sensory stimuli to compare the average DCN cell firing frequency over each of four quarters (25 sec segments) of the entire 100 sec stimuli (Fig. 3.8). With this analysis each average for DCN cell firing (1600 msec total record length) was constructed from 12 sensory stimuli in the first three segments and 13 sensory stimuli in the last segment of the stimulus file, generating average recordings constructed from 96-117 records across 8 Transient burst cells or 9 Weak burst cells (Fig. 3.8A,B). These records again revealed no sign of rebound frequency increases in the mean DCN cell firing rate for either Transient or Weak burst cells in any of the quarter sections, or any clear change in the pattern of DCN cell firing between each successive quarter. In fact, the average DCN cell firing frequency changed by no more than ~4 Hz from the baseline values within each quarter segment of the recordings, with no clear indication of a period of inhibition in terms of a decrease in DCN cell firing frequency. A similar result was found for PC Spontaneous files segregated into four quarters and the average DCN cell firing frequency calculated ($n = 96$-117) in relation to times equivalent to
Figure 3.6 DCN neurons do not show a reliable burst response to Purkinje cell sensory-evoked stimuli.

A, A representative and expanded segment of a recording from a Transient burst DCN neuron, with the associated Purkinje cell firing rate in the PC Sensory-evoked stimulus pattern below. *Dashed red lines* indicate the timing of two perioral stimulations. The record in (A) suggests that a DCN cell is largely inhibited by Purkinje cell input unless a sufficient pause in firing permits a return of DCN cell membrane potential to evoke spike discharge. B, C, The mean Purkinje cell firing rate of the PC Sensory-evoked stimulus (B) for all 49 perioral stimuli (*red dashed line*) positioned with respect to the mean DCN cell frequency response over the same time intervals (C). Data are averaged over all sensory stimuli from 8 Transient burst neurons (*left*, 392 sensory stimuli) and 9 Weak burst neurons (*right*, 441 sensory stimuli), with records illustrating mean values 100 ms preceding and 1500 ms following the stimulus. Note that DCN cell frequency is shown on an expanded scale compared to Purkinje cell input frequency.
Figure 3.7  DCN neurons do not show a reliable burst response to the mean Purkinje cell spontaneous input pattern.

A, A representative expanded segment of a Transient burst neuron recording, with the associated Purkinje cell pattern and firing rate below. Red dashed lines indicate the stimulus timings taken from the PC Sensory-evoked record used to compare the Purkinje cell input patterns. B, The mean PC Spontaneous firing rate averaged for 49 2 sec segments of a 100 sec stimulus, as used to assess PC Sensory-evoked stimuli (see Fig. 3.6). Shown is the mean firing rate 100 ms preceding and 1500 ms following the comparable time of sensory input delivered for PC Sensory-evoked stimulus trains in vivo (dashed red line). C, The mean DCN cell frequency response to the PC Spontaneous input pattern shown in (B) averaged over 8 Transient burst neurons (left, 392 stimulus segments) and 9 Weak burst neurons (right, 441 stimulus segments). Note that DCN cell frequency is shown on an expanded scale compared to Purkinje cell input frequency and spikes are truncated in (A). Sample values are shown in brackets, with SEM of the mean values in (B) shown in Figure 3.2B.
those of sensory stimuli in the PC Sensory-evoked file (Fig. 3.9A,B).

Therefore, although the spike frequency response of Purkinje cells to perioral stimuli \textit{in vivo} reached a peak of \(~110\) Hz over \(~400\) msec (a value similar to the control stimuli delivered \textit{in vitro} at 100 Hz, 300 msec that showed a detectable rebound frequency increase), we could find no reliable evidence of a sensory-related rebound response in DCN cells \textit{in vitro} (cf Figs. 3.1D and 3.6B). This was true for comparisons of evoked firing rates within individual cells or across multiple cells, even when sensory stimulus responses were averaged to maximize the potential to locate a measurable response. Note that this result was obtained even when using the baseline firing rate of DCN cells associated with tonic firing under quiescent conditions as the reference point for statistical comparisons of rebound firing frequency, which should have enhanced our potential to locate burst ISIs in these recordings. It is unclear at this time why physiological input patterns containing sensory stimulus-evoked increases in Purkinje cell firing of the magnitude shown here were unable to produce rebound increases in DCN cell firing (considered in Discussion). However, these data highlighted the need to reassess burst firing in DCN cells with respect to the actual pattern in Purkinje cell firing associated with the burst ISIs that were identified.

3.6 Rebound Burst Reverse Correlation

For this purpose we employed reverse correlation between identified DCN cell bursts and Purkinje cell firing frequency irrespective of the actual Purkinje cell perioral stimulus times. The time of the first spike of a defined DCN burst, not each individual burst ISI, was used as the timepoint to align the races for reverse correlation. These timepoints were used to extract segments of Purkinje cell firing 1 sec before and 1 sec after the identified burst ISI to calculate average plots of firing frequency in Purkinje and DCN cells. The sample numbers for each average record varied between 56-231 depending on the number of bursts identified in DCN cell records across all cells (8 Transient, 9 Weak burst cells) and for either PC Sensory-evoked or PC Spontaneous stimulus inputs (Fig. 3.10). The averaged records of firing frequency for Purkinje cells in relation to the average DCN cell frequency for all identified bursts of at least 3 spikes are shown superimposed in Figure 3.10. Two-spike bursts were excluded from the average due to
Figure 3.8 DCN burst output does not vary between each quarter of the PC Sensory-evoked input pattern.

A, B, Mean frequency plots of the DCN response in each successive quarter of the 100 sec PC Sensory-evoked stimulus input for (A) Transient burst and (B) Weak burst neurons. The 100 sec recordings are divided into four 25 sec segments and the mean values calculated for each sensory stimulus applied in vivo. Records illustrate 100 ms preceding and 1500 ms following the stimulus (red dashed line). The first three quarters each contain 12 sensory stimuli, and the fourth quarter contains 13 sensory stimuli, averaged across all cells in each burst phenotype. Sample values are shown in brackets.
Figure 3.9 DCN burst output does not vary between each quarter of the PC Spontaneous input pattern.

A, B, Mean frequency plots of the DCN response during a PC Spontaneous input pattern for (A) Transient burst and (B) Weak burst neurons segregated into successive quarters of the 100 sec stimulus input. Mean firing rate is shown 100 ms preceding and 1500 ms following the time of a sensory stimuli (red dashed line) in the PC Sensory-evoked stimulus record. The numbers of 2 sec segments averaged in each quarter section of the recording are shown in brackets.
the sheer number of them completely altering the average plots. The reason for this difference between two-spike bursts and other bursts is currently unknown (see Discussion). These plots now identified a pattern in the Purkinje cell firing rate in relation to DCN cell bursts that was common to both PC Sensory-evoked and PC Spontaneous input data. In each case, Purkinje cell firing exhibited an increase in frequency preceding a DCN cell burst followed by a relatively rapid decrease in firing rate for up to ~500 msec before returning to baseline values (Fig. 3.10). In each case the mean peak frequency of Purkinje cell firing was in the range of 80-90 Hz. Associated with this pattern was a corresponding initial average decrease in DCN cell firing, and a subsequent rebound increase in firing frequency during the subsequent reduction in Purkinje cell firing rate. The magnitude of the rebound frequency increase in DCN cells was most prominent for Transient burst cells, increasing on average from ~10 Hz baseline firing rate to ~25 Hz, with a more modest increase of ~10 Hz to ~13 Hz in Weak burst cells (Fig. 3.10A,B). Note that these differences in peak rebound frequencies are consistent with the higher intraburst frequencies detected earlier between Transient and Weak burst cells through statistical analyses (Fig. 3.5D).

All aspects of the sequence of Purkinje cell and associated DCN cell firing rate identified through reverse correlation were more prominent for the PC Spontaneous input file compared to the PC Sensory-evoked input file. Thus, while the firing rate of Purkinje cells in the reverse correlated PC Sensory-evoked stimulus input increased from baseline on average by ~30 Hz preceding the DCN Transient cell burst, the Purkinje cell firing rate in the PC Spontaneous-evoked stimulus input increased on average by ~50 Hz (Fig. 3.10). These differences were also associated with larger corresponding decreases in DCN cell firing rate. Thus, there was a minimal detectable decrease in the mean DCN cell firing rate in relation to the PC Sensory-evoked input in Transient burst cells compared to ~4 Hz decrease for the PC Spontaneous input (Fig. 3.10A). The relative duration of the increase in PC Preburst cell firing rate was also more prominent for the PC Spontaneous input file, with Purkinje cell firing rate increasing above baseline levels for ~800 msec compared to ~200 msec for the PC Sensory-evoked input. The subsequent decrease in Purkinje cell firing rate in the PC Spontaneous stimulus input approached the zero baseline for ~300 msec, essentially reflecting a full pause in Purkinje cell firing. A pattern in DCN cell rebound firing was also more clearly evident for the PC Spontaneous input.
file, with an initial rapid and transient elevation in firing for < 100 msec followed by a lower rate of elevated firing for ~600 msec (Fig. 3.10A,B). Notably, this pattern for DCN cell firing closely reflects that described in previous in vitro reports on rebound responses in individual DCN cells, with an initial transient increase and longer duration, lower frequency component (Molineux et al., 2006; Molineux et al., 2008b; Zheng and Raman, 2009b; Engbers et al., 2011; Zheng and Raman, 2011). Unexpectedly, the average rate of Purkinje cell firing identified through reverse correlation in the PC Spontaneous input file exhibited a further late increase after the pause for at least 500 msec, a result of unknown significance as it was not as prominent in the PC Sensory-evoked stimulus input and occurred following the time frame of a DCN cell rebound increase in firing. As indicated, the Purkinje cell firing rate for the PC Sensory-evoked stimulus followed the same general pattern, although with a less prominent magnitude and rate of change of firing frequency (Fig. 3.10A,B). Yet despite these differences in relation to the stimulus input file used, the relative change in Purkinje cell firing was surprisingly similar for records reverse correlated from Transient and Weak burst neuron burst ISIs (Fig. 3.10C ). In the case of the PC Spontaneous input the two records were virtually superimposeable.

Together these data revealed that DCN cells can respond to physiological patterns of Purkinje cell input with a sequential period of inhibition followed by a rebound increase in firing, as suggested by previous work using stereotyped constant frequency patterns to stimulate Purkinje cell axons in vitro (i.e. 100 Hz, 10 pulses). The pattern of Purkinje cell firing associated with a DCN cell burst ISI was also an increase in the rate of firing, as one would predict. However, reverse correlation revealed that a rebound response in DCN cells was also associated temporally with a subsequent decrease in Purkinje cell firing frequency, emphasizing the need to encounter a temporary relief from inhibition to express rebound burst capabilities. Indeed, the importance of a relative decrease or pause in Purkinje cell firing has been shown in in vivo analyses (Shin and De Schutter, 2006), dynamic clamp studies in vitro (Gauck and Jaeger, 2000), and modeling studies (Jaeger, 2007; Steuber et al., 2007; Steuber and Jaeger, 2013). It was interesting that the pattern of average Purkinje cell firing identified by reverse correlation in relation to a DCN cell burst ISI was more pronounced for the PC Spontaneous input than for the PC Sensory-evoked input. At this time, we assume that this signifies a lower sensitivity of DCN cells to spontaneous input from Purkinje cells, such that larger relative shifts in Purkinje cell
firing are required to evoke a rebound burst. This interpretation might also be supported by the similarity of the Purkinje cell firing required to elicit a rebound burst ISI in both Transient and Weak burst neurons (Fig. 3.10C). Nevertheless, the qualitative aspects of Purkinje cell firing associated with generation of rebound burst ISIs in DCN cells was equivalent for either stimulus input pattern.

Previous work *in vitro* established that the intensity of a rebound burst in DCN cells can be finely graded with respect to the magnitude and duration of a preceding hyperpolarization (Aizenman and Linden, 1999; Tadayonnejad et al., 2009a; Pedroarena, 2010; Engbers et al., 2011). Given that burst ISIs detected in DCN cells during physiological input patterns were grouped into clusters of between 2-9 spikes, it suggested a graded response in relation to some aspect(s) of the input stimuli. To determine the relationship between Purkinje cell firing rate and the number of burst ISIs generated in DCN cells, we reverse-correlated the Purkinje cell firing rate in the stimulus input files in relation to DCN cell bursts comprised of 2-9 spikes (1-8 ISIs) (Figs. 3.11-3.12). By far the most common burst ISI pattern identified in DCN cells was that of two-spikes (single ISI) (n = 4006). Reverse correlation showed that this minimal burst response was still associated with a sequential elevation and decline in the rate of Purkinje cell firing for both the PC Sensory-evoked input (Fig. 3.11A) and PC Spontaneous input (Fig. 3.12A). In the case of the PC Sensory-evoked input, Purkinje cell firing only increased on average ~5 Hz from baseline over ~200 msec time, followed by a gradual decline in firing rate back to baseline over a similar timeframe (Fig. 3.11A). The reverse-correlated PC firing frequency in response to the PC Spontaneous input was more exaggerated, with a net elevation in mean firing frequency of ~60 Hz over 500 msec preceding the burst, and a subsequent decline in frequency ~20 Hz below baseline levels for most of 1 sec time (Fig. 3.12A). For DCN bursts with an increasing number of ISIs the pattern of associated Purkinje cell firing rate increased in relative magnitude, although this reached an apparent maximum for cases (in a very general sense) when Purkinje cell firing exhibited a net increase of ~60 Hz above baseline values when DCN burst ISIs were composed of >4-5 spikes (cf. Figs. 3.11 and 3.12). A key difference noted was a shorter duration of the increase in Purkinje cell firing (PC Preburst) of 100-200 msec for the PC Sensory-evoked input file compared to that of the PC Spontaneous input file (500-1000 msec). The duration of the period of PC Preburst prior to a rapid decline or pause in firing also changed in a graded manner,
A. Transient Burst Neurons

B. Weak Burst Neurons

C. PC Sensory-evoked input

   Transient burst

   Weak burst

PC Spontaneous input
Figure 3.10  Reverse correlation reveals the PC firing pattern that evokes DCN cell burst ISIs.

A, B, Superimposed average frequency plots of the Purkinje cell firing rate (red traces) and the associated DCN neuron response (blue traces) for PC sensory-evoked or spontaneous stimuli in Transient burst (A) or Weak burst neurons (B). All data were extracted according to reverse correlation of Purkinje cell firing rates in relation to the timing of the first spike of each statistically defined burst comprised of 3-8 ISIs in DCN cells irrespective of the time of sensory stimuli delivered in vivo. Frequencies were calculated from ISIs using Gaussian smoothing (Fig. 2.1)(Paulin, 1996; Steuber et al., 2011) and shown for data recorded 1 sec before and after the timing of the first spike of a defined DCN cell burst. The data is averaged over all bursts collected from 8 cells for Transient burst neurons (56 bursts in the sensory-evoked input and 159 bursts in spontaneous) and 9 cells for Weak burst neurons (66 bursts following the PC Sensory-evoked input and 231 bursts following PC Spontaneous input). Traces represent mean values (black lines) with SEM indicated by the shaded red area (Purkinje cell firing) or shaded blue area (DCN firing). The occurrence of burst ISIs in DCN cell records is associated with a distinct pattern of Purkinje cell firing rate comprised of a sequential increase in firing followed by a pause in firing, which is reflected in the DCN mean firing rate as a sequence of a slowing in firing rate followed by a rebound increase in firing for up to a sec. C, Superimposed records of the mean Purkinje cell firing rates identified in (A, B) for Transient and Weak burst cells in response to the PC Sensory-evoked or PC Spontaneous input file.
with longer durations of PC Preburst firing as the number of burst ISIs increased in DCN cells, a pattern again most readily detected on visual inspection for the PC Spontaneous input files (cf. Figs. 3.11, 3.12). The largest apparent change as the intensity of a DCN burst increased was an increase in the magnitude and duration of the decrease in Purkinje cell firing rate (PC Pause). Thus, as the number of burst ISIs in DCN cells increased, the relative decrease and duration of the PC Pause increased progressively for bursts comprised of >4-5 spikes for either the PC Sensory-evoked input (Fig. 3.11B-D) or PC Spontaneous input (Fig. 3.12B-D). For the strongest DCN cell burst responses, the decrease in Purkinje cell firing during the PC Pause was represented by a complete cessation of firing for up to ~500 msec from the timepoint at which firing frequency began to decrease (Fig. 3.11D, 3.12D).

Quantifying several different aspects of the reverse-correlated Purkinje cell firing rate to the number of DCN cell burst ISIs confirmed these qualitative assessments of Purkinje cell firing. Figure 3.13A provides a schematic example of a Purkinje cell sequence of elevation in firing followed by a pause in firing in relation to DCN cell burst ISIs and the different parameters measured across all records for 8 Transient burst and 9 Weak burst neurons and for the PC Sensory-evoked input. We focused on the PC Sensory-evoked record as the DCN cell response appeared to be more sensitive to subtle changes in Purkinje cell firing parameters, and the fact that this record contained all of the complex spike timings. The degree of correlation between each Purkinje cell firing parameter identified in Figure 3.13A was then assessed in relation to the number of successive DCN cell burst ISIs recorded, and the linear fit associated with each data set shown in Figure 3.13B (see also Table 1). The first parameter considered was the duration of the PC Pause, which showed a strong linear fit (adj. $R^2 = 0.38$, $F_{1,1} = 1070$, $p<0.05$) with the length of the DCN burst, as initially assessed by visual inspection of recordings (above). The second parameter considered was the maximal frequency attained in the PC Preburst component (Fig. 3.13A, B), which reached a mean value of ~70-90 Hz for the PC Sensory-evoked stimulus pattern shortly before the DCN cell burst. As suspected by the similar apparent peak change in PC Preburst frequencies (Figs. 3.11, 3.12), no clear relationship emerged in the correlation plots (Fig. 3.13B). In a similar fashion, none of the other Purkinje cell firing parameters examined here showed any clear relationship to the number of DCN cell burst ISIs. Thus, no significant relationship became apparent for either the duration of the PC Preburst or
Transient burst

2-3 Spike Bursts

PC Frequency (Hz)

(n = 1721)

Weak burst

PC Frequency (Hz)

(n = 1813)

B 4-5 Spike Bursts

PC Frequency (Hz)

(n = 34)

(n = 34)

C 6-7 Spike Bursts

PC Frequency (Hz)

(n = 8)

(n = 25)

D 8-9 Spike Bursts

PC Frequency (Hz)

(n = 14)

(n = 7)
**Figure 3.11** Reverse correlation with the PC Sensory-evoked input pattern reveals a graded Purkinje cell response in relation to the intensity of DCN rebound firing.

A-D, Shown are average plots of Purkinje cell firing frequency within segments of the PC Sensory-evoked input stimulus identified through reverse correlation in relation to the first DCN cell spike (Time 0) associated with a statistically defined burst ISI. All records were obtained in response to the PC Sensory-evoked stimulus file and shown for Transient burst (A) and Weak burst (B) neurons, with Purkinje cell firing extracted 1 sec before and after the first defined DCN cell spike of a burst. The average Purkinje cell firing frequency is shown for all cases in DCN cell records generating bursts of 2-3 spikes (A), 4-5 spikes (B), 6-7 spikes (C), or 8-9 spikes (D). Shaded components of the Purkinje cell firing frequency represent S.E.M. The number of DCN cell segments used to create a given Purkinje cell firing plot are shown in brackets.
Figure 3.12  Reverse correlation with the PC Spontaneous input pattern reveals a graded Purkinje cell response in relation to the intensity of DCN rebound firing.  

A-D, Shown are average plots of firing frequency from segments of the PC Spontaneous input stimulus identified through reverse correlation in relation to the first DCN cell spike (Time 0) associated with a statistically defined burst ISI. All records were obtained in response to the PC Spontaneous stimulus file and shown for Transient burst (A) and Weak burst (B) neurons, with Purkinje cell firing extracted 1 sec before and after the first defined DCN cell spike of a burst. The average Purkinje cell firing frequency is shown for all cases in DCN cell records generating bursts of 2-3 spikes (A), 4-5 spikes (B), 6-7 spikes (C), or 8-9 spikes (D). Shaded components of the Purkinje cell firing frequency represent S.E.M. The number of DCN cell segments used to create a given Purkinje cell firing plot are shown in brackets.
the PC Rate of frequency decline (Hz/msec) measured from the peak PC Preburst frequency to the lowest frequency during the PC Pause and the number of DCN cell burst ISIs (see Fig. 3.13A, B). The corresponding adjusted R² values are presented in Table 3.1 for each parameter in our Weak and Transient burst neuron recordings.

The lack of correlation between PC Preburst frequency and the number of DCN cell burst ISIs was surprising given that extensive work in previous in vitro studies have reported a strong linear relationship between different forms of stimulus intensity (i.e. level of current injection, number or frequency of inhibitory synaptic stimuli) and DCN burst output (Zhang et al., 1993; Jaeger et al., 1997; Aizenman and Linden, 1999; Czubayko et al., 2001; Rowland and Jaeger, 2005; Molineux et al., 2006; Uusisaari et al., 2007; Molineux et al., 2008b; Tadayonnejad et al., 2009a; Pedroarena, 2010; Engbers et al., 2011; Zheng and Raman, 2011). A key difference, was our use of a physiological input spike train for the first time. We note, however, that the poor correlation detected in many of our parameters may also reflect the high preponderance of burst ISIs associated with only two-spike bursts, which are expected to alter corresponding linear fits to the data set.

Together these data reveal a general Purkinje cell input stimulus that is needed to elicit a DCN rebound response. It consists of a rapid elevation of Purkinje cell firing to ~30-60 Hz above baseline followed by a rapid drop in frequency or pause in firing for at least ~50 msec and up to ~700 msec. We note that these values are only cited with respect to the PC Sensory-evoked stimulus file we have tested in vitro, but provide a benchmark to compare against other stated values of Purkinje cell firing in relation to DCN cell output. The data are also valuable in confirming that one of the most optimal parameters of Purkinje cell output to trigger DCN burst ISIs is the relative pause in the duration of Purkinje cell firing.

3.7 Regular Purkinje Cell Patterns detected through CV2 Analysis

Of interest is the fact that the stimulus files tested here are the same as those used to identify putative patterns of Purkinje cell firing in vivo through a CV2 analysis (Shin et al., 2007b). CV2 analysis is used to perform a spike-by-spike comparison and measures the standard deviation of successive ISIs divided by their mean and multiplied by √2 (Holt et al., 1996).
Figure 3.13 The intensity of a DCN rebound burst is strongly correlated to the duration of a pause in Purkinje cell firing

A, Representative trace of the average Purkinje cell pattern of firing identified through reverse correlation as evoking a 8-9 spike burst in Transient burst neurons in response to the PC Sensory-evoked stimulus pattern. Time 0 reflects the timing of the first spike of a statistically defined DCN burst ISI. Labels identify the parameters measured to compare against the number of spikes evoked per burst in DCN cells. Purkinje cell frequencies are measured with respect to the mean Purkinje cell firing rate over the entire 100 sec input (PC Pattern Baseline). B, Scatter plots showing the value of each of the indicated parameters for the average firing rate of Purkinje cells (as in A) in relation to the number of ISIs present in each evoked DCN cell burst as a measure of burst duration. Records include all bursts present across 8 Transient burst neurons when stimulated with the PC Sensory-evoked stimulus template. The only Purkinje cell firing parameter exhibiting a linear correlation to burst duration is PC Pause duration (top left plot). See also Table 1 for correlation metrics.
Table 3.1. Adjusted R-Squared Values for Purkinje cell reverse correlation metrics.

Shown are the adjusted R-squared values of correlation coefficients between the number of DCN cell burst ISIs and mean Purkinje cell firing metrics identified through reverse correlation from the first ISI of DCN cell bursts.

<table>
<thead>
<tr>
<th>Firing parameter</th>
<th>Rebound burst phenotype</th>
<th>Transient</th>
<th>Weak</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PC Sensory-Evoked</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC Preburst Frequency</td>
<td>0.002</td>
<td>-0.0002</td>
<td></td>
</tr>
<tr>
<td>PC Rate of Frequency Decline</td>
<td>-0.0004</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Duration PC Preburst</td>
<td>0.05</td>
<td>0.033</td>
<td></td>
</tr>
<tr>
<td>PC Pause Duration</td>
<td>0.38</td>
<td>0.40</td>
<td></td>
</tr>
</tbody>
</table>
In this way, one can define successive ISIs that are either of similar or shorter duration than the previous ISI, identifying sequential decreases in ISI that signify a pattern (i.e. burst) in firing rate. A CV2 analysis of Purkinje cell firing in vivo was important in providing the first statistical evidence that these cells can generate an output more structured than a simple tonic discharge long proposed by other groups (Tarnecki and Konorski, 1970; Armstrong et al., 1973). The analysis of Shin et al. (Shin et al., 2007b) using the same type of stimulus files presented here reported definable patterns in Purkinje cell output even during spontaneous firing in anesthetized rats in vivo, with the number of defined patterns increasing sharply during perioral / whisker stimulation. Their defined patterns varied greatly in the number of spikes per pattern, with 72% of patterns being comprised of only 2-3 spikes but some were much longer, lasting on average 45 ± 3.5 msec. Their data further suggest that the most common pattern encountered contained a prominent range of spike frequencies in the order of ~65-200 Hz (see Fig. 4B of (Shin et al., 2007b)). This is important, as the reverse correlation analysis we conducted instead defines a duration of the PC Preburst frequency increase associated with burst ISIs in the order of hundreds of msec and a mean peak frequency increase of ~100 Hz (Fig. 3.13B). We thus compared the occurrence of CV2-defined patterns of Purkinje cell firing (reflecting a transient elevation in firing frequency) to the PC Preburst frequency increase defined by reverse correlation to DCN cell burst ISIs. To focus our comparisons on the most effective PC stimulus-DCN cell phenotype pair, we analyzed the PC Sensory-evoked file and Transient burst cells.

A CV2 analysis of the PC Sensory-evoked input file (binned at 0.02) revealed a positively skewed distribution comprised of 6,541 values with a mode of 0.02-0.04 (Fig. 3.14A). The work of Shin et al. (Shin et al., 2007b) established that CV2 patterns below a threshold value of 0.2 correspond to a defined pattern of Purkinje cell firing. We found that in the PC Sensory-evoked stimulus input file, 3,664 values fell below the 0.2 threshold (56% of total values) that defined 643 patterns. A comparison of the duration of CV2-defined patterns revealed a positively skewed distribution, revealing that 78% were less than 50 msec in duration (Fig. 3.14B). This relationship differed considerably from the duration of PC Preburst frequency increases, which exhibited a normal distribution ($r^2 = 0.95, F_{1,8}=389.54, p<0.05$) with the highest number of events of ~650 msec duration, and only 0.4% shorter than 50 msec (Fig. 3.14B).
To determine if CV2 patterns might be associated with DCN cell bursts, we reverse correlated the first ISI of DCN cell bursts to the Purkinje cell input train to identify any CV2 patterns that fell within the preceding 500 msec. A window of 500 msec was chosen in order detect any association of CV2 patterns with DCN cell bursts in which burst ISIs occurred with a delayed onset following a period of high frequency Purkinje cell firing (i.e. Fig. 3.3B). This analysis identified 556 CV2 patterns that occurred prior to a DCN cell burst and 87 CV2 patterns that did not (Fig. 3.14C). A plot of spike frequency and duration of CV2 patterns revealed a general tendency for patterns to exhibit higher frequency spike discharge as the CV2 pattern duration decreased (Fig. 3.14C). Parsing these CV2 patterns into those that were or were not associated with DCN rebound bursts revealed no obvious difference in the plot of CV2 spike frequency vs duration (Fig. 3.14C). Further analysis indicated that of the CV2 patterns that were associated with bursts, the average number of spikes in the pattern was 4.93 ± 0.18, and the average pattern duration was 35.31 ± 1.35 msec (n = 556). Of the CV2-defined patterns that were not associated with bursts, the average number of spikes in the pattern was 5.17 ± 0.46 spikes, and the average pattern duration was 34.58 ± 3.28 msec (n = 87). Therefore, the occurrence of a burst was not associated specifically with either the mean number of spikes/CV2 pattern (p = 0.61) or the mean CV2 pattern duration (p = 0.84). Finally, we assessed whether particular CV2-defined patterns were more effective at evoking bursts by comparing the ability for specific CV2 patterns within the stimulus input to evoke bursts across 8 Transient burst cells. We found that a large number of CV2-defined patterns (377) were associated with a burst ISI in 1/8 cells, and 161 specific patterns with bursts recorded in 2/8 cells (Fig. 3.14D). However, only 1 of 643 CV2 patterns was associated with a burst ISI in more than 4/8 cells (50% probability). Therefore, we interpret these data to indicate that the initial high apparent association between the occurrence of a burst ISI and a preceding CV2 pattern does not reflect a causal relationship, with a surprisingly low probability for any given CV2 pattern to evoke bursts when presented to 8 different cells.

3.8 Climbing Fiber Evoked Pauses in Purkinje Cell Firing

The idea that pauses in Purkinje cell firing are a key signal in the PC-DCN synaptic relay has been previously suggested, especially given that climbing fiber-evoked complex spikes are
Figure 3.14. CV2 defined patterns in Purkinje cell firing are not reliably associated with DCN cell burst responses.

A, A histogram showing the CV2 scores for each ISI in the PC Sensory-evoked stimulus input pattern (0.02 bin width). The red dashed line indicates the threshold CV2 below which ISIs are deemed to belong to a pattern (as defined in (Shin et al., 2007a)). This distribution is comprised of 6,541 CV2 values, with 3,664 values (56%) below the 0.2 threshold, defining 643 patterns. B, Plots of the number of defined CV2 patterns in the PC Sensory-evoked input file of a given duration compared to that of PC Preburst frequency increases, as identified in the same Purkinje cell input stimulus file through reverse correlation from burst ISIs in Transient burst DCN cells (as in Fig. 3.13A). Data are binned at 20 ms. Note the longer peak duration of the most common PC Preburst events (corresponding to 2 spike DCN cell bursts) compared to those of the most frequent CV2 patterns. C, Scatter plot of spike frequency vs duration of CV2 patterns sorted for those that were associated with a DCN burst response (red) or no burst response (black). Inset shows the number of patterns that did or did not associate with DCN bursts. No clear differences emerge between CV2 patterns of Purkinje cell firing and DCN cell rebound bursts. D, Plots of the ability for a specific CV2 pattern within the PC Sensory-evoked input file to be associated with burst ISIs occurring within 500 ms following the Purkinje cell firing pattern across all 8 Transient burst cells. While a large number of specific PC firing patterns could be associated with a burst ISI in 1/8 cells (377), only 1 pattern is associated with a burst ISI in more than 4/8 cells (50% probability).
followed by a pause in simple spike activity (Gauck and Jaeger, 2000; Shin and De Schutter, 2006; Jaeger, 2007; Steuber et al., 2007; Steuber et al., 2011). Our PC Sensory-evoked stimulus file contained 62 complex spike firing times. It is important to note that our complex spikes are represented in the stimulus input file by only the first spike in the complex spike burst, and no associated high-frequency spikelets. This is acceptable given that the high frequency spike doublets have been shown to have very low probability of conducting down the Purkinje cell axon (Monsivais et al., 2005). The last spike of the complex spike can be conducted down the axon (Monsivais et al., 2005), and is expected to be present in the overall PC Sensory-evoked file.

To explore the influence of the complex spike on DCN cell firing we examined the average frequency of Purkinje and DCN cells over a period of time of 200 msec before until 500 msec after the first spike time of complex spikes in all 8 Transient burst cell recordings (n = 496 complex spikes) (Fig. 3.15A). Inspection of the average Purkinje cell firing rate in relation to complex spike timings in the PC Sensory-evoked file showed a rapid transient component (corresponding to the first spike time) followed by an increase of ~120 Hz in Purkinje cell firing frequency over a period of ~100 msec. This increase in firing was then followed by a decrease in firing of ~10 Hz that extended out ~200 msec (Fig. 3.15A). Surprisingly, we found little effect on the DCN cell response, with no apparent decrease in DCN cell firing frequency over the period of increased Purkinje cell firing, and only a minimal (~1 Hz) increase in DCN cell firing frequency during the subsequent period of reduced Purkinje cell activity (Fig. 3.15A). We then refined our criteria to identify complex spikes that occurred within a window of 500 ms preceding a DCN cell burst ISI. This analysis indicated that 139 complex spikes (28%) occurred within a 500 msec window of an identified burst ISI. Averaging a similar period of 200 ms before and 500 msec of time around the occurrence of burst-associated complex spikes then returned a similar pattern of Purkinje cell firing, except that an elevation in DCN cell firing (~3 Hz) became more apparent with an onset latency of ~100 msec after the complex spike time (Fig. 3.15B).

These results are surprising given that the mean Purkinje cell firing rate identified from the PC Sensory-evoked file is substantially different from what would be expected from single cell recordings. Therefore we identified the absolute timing differences in the occurrence of
complex spike with respect to identified DCN cell burst ISIs, which returned a positively skewed
distribution with a range of latencies of between 1-485 ms, and a mean of 131 ± 11.5 msec (n =
139). Of these, 75 difference latencies occurred within 100 msec time, a predicted timeframe for
an immediate effect on DCN cell firing, suggesting some relationship. We then examined the
probability that a given complex spike time was associated with DCN cell burst ISIs when
delivered to all 8 Transient burst cell recordings. Here we found that of 61 complex spikes in the
record associated with burst ISIs, 31 (50%) were associated with a burst ISI in 2/8 cells (Fig.
3.15D). However, only 3 of 61 (5%) of complex spikes were associated with a burst ISI in more
than 4/8 cells (50% probability). Therefore, we interpret these data to indicate that the initial
apparent association between the occurrence of a burst ISI and a preceding complex spike does
not reflect a causal relationship, with a surprisingly low probability for any given complex spike
to evoke bursts when presented to 8 different cells.
Figure 3.15. **Purkinje cell complex spikes are not reliably associated with DCN cell burst responses.**

DCN cell burst ISIs recorded in response to the PC Sensory-evoked stimulus file were used to reverse correlate the occurrence of any complex spikes (first spike timing) within 500 ms preceding the burst. **A, B,** Spike-triggered average recordings of Purkinje cell complex spike responses in the PC Sensory-evoked file and the mean DCN spike frequency recorded across 8 Transient burst cells. Shown are recordings for all complex spikes ($n = 62$) and the mean response in 8 DCN cells ($n = 496$) (A), or for only those complex spikes detected within 500 ms of the first spike time of a DCN burst response ($n = 139$) (B). **C,** Plots of the latency difference between DCN cell burst ISIs and complex spikes that occur within the preceding 500 msec. Data are binned at 10 ms. **D,** Plots of the ability for a specific complex spike within the PC Sensory-evoked input file ($n = 61$) to be associated with burst ISIs across all 8 Transient burst cells. While some number of specific complex spikes are associated with a burst ISI in 1/8 cells (12) or 2/8 cells (31), only 3 complex spikes are associated with a burst ISI in more than 4/8 cells (50% probability).
CHAPTER FOUR: DISCUSSION

4.1 Overview

The ionic basis of rebound burst discharge in DCN cells has been extensively studied through recordings in vitro (Tadayonnejad et al., 2010a). Yet the ability for rebound bursts to contribute to sensory processing in vivo has been more difficult to assess. Several questions remain as to the patterns of Purkinje cell output that trigger rebound bursts, the role of bursts in processing inputs as varied as tactile stimulation to postural control, or the role for different DCN burst phenotypes in responding to Purkinje cell input. The primary means for studying rebound bursts in vitro has been by delivering well-established stimuli of high (constant) frequency pulse trains (i.e. 100 Hz, 10 pulses) or current-evoked membrane hyperpolarizations (500 msec) (Molineux et al., 2008a; Tadayonnejad et al., 2009b; Pedroarena, 2010; Sangrey and Jaeger, 2010; Tadayonnejad et al., 2010b; Engbers et al., 2011). The current study instead used physiological spike trains from Purkinje cell recordings in vivo as stimulus templates to activate afferent inhibitory inputs to DCN cells in vitro.

The hypothesis I set out to test was “Intrinsic membrane properties underlying rebound bursts allow Transient and Weak burst DCN cells to differentially encode physiological patterns of inhibitory input.” To test this I used the physiological patterns from in vivo recordings of Purkinje cell spike output in response to either perioral sensory stimulation or background spontaneous firing. Surprisingly, the PC Spontaneous stimulus file was at least as effective in evoking rebound burst ISIs in DCN cells as the PC Sensory-evoked input. In fact, extensive efforts to identify DCN cell burst ISIs discharged in relation to the sensory stimuli in PC Sensory-evoked trials failed even after averaging hundreds of individual sensory evoked DCN cell responses, highlighting the complexity of distinguishing exactly what input parameters DCN cells encode from the Purkinje cell trains. The alternative approach of using reverse correlation was important in providing the first identification of a physiological pattern of Purkinje cell firing capable of activating burst ISIs in DCN cells, at least in vitro without glutamatergic input. Moreover, reverse correlation identified a temporal pattern in Purkinje cell firing that was common between Transient and Weak burst cells and graded in nature in its ability to trigger 2-9 spike bursts in DCN cells. Whether comparing the activity between Transient or Weak burst
cells or the stimulus trains delivered here, the strongest correlation between Purkinje cell firing and DCN burst output was the relative pause in Purkinje cell firing. Therefore, my research took our work down an unexpected path in which we managed to define a key pattern of inhibitory input relevant to DCN cells. Yet, since this result was found in both Transient and Weak burst cells, it suggests that the associated burst responses in DCN cells do not reflect a specific difference in how the intrinsic properties of Transient vs Weak burst cells might act to differentially encode Purkinje cell input. The work has thus increased our knowledge of the physiological patterns relevant to this motor system, and provided several questions for future investigations.

4.2 PC Sensory-Evoked vs PC Spontaneous Input Patterns

The relative lack of influence of a sensory-evoked Purkinje cell stimulus pattern in evoking burst responses in DCN cells was unexpected, particularly when bursts were readily detected in the same cells in response to the PC Spontaneous input pattern. Moreover, the relative lack of rebound bursts in DCN cells to the PC Sensory-evoked stimulus was detected despite the fact that the elevation in Purkinje cell firing frequency in response to a perioral stimulus on average exceeded 100 Hz (50 Hz above baseline) (Fig. 3.6B). This value is easily within the frequency range of the inhibitory synaptic stimulus of 50 or 100 Hz used in each cell to first verify that Purkinje cell input (adjusted to 60% of maximum) could readily evoke a statistically defined rebound burst. Our use of the mean tonic firing frequency at rest in a cell (-60 mV AHP trough) as the base point of comparison to statistically define rebound bursts is further expected to favour our ability to detect burst output. Moreover, we failed to detect rebound bursts associated with the PC Sensory-evoked rebound stimulus even when averaged over 49 stimuli and for all 8-9 cells, together encompassing 392-441 DCN cell responses (Fig. 3.6). The purpose of this process was to simulate convergence of inputs to multiple DCN cells to determine if rebound response might at least be evoked at the level of a population response from the DCN. Yet, even with these advantages the PC Sensory-evoked stimulus input could in some cells be less effective at invoking bursts than the PC Spontaneous input pattern.

These results might be attributed to a set of different factors. For one, the available patterns of activation for both spontaneous and perioral-evoked sensory stimuli were obtained
from anesthetized rats. The extent to which anesthetic will disrupt the natural pattern of Purkinje cell output can only be estimated, but as indicated above, the average change in frequency for the PC Sensory-evoked input was entirely relevant to that normally tested in vitro. The cerebellum is also organized anatomically into topographic projection patterns that are specific between different regions of cerebellar cortex and specific DCN nuclei. Our cells recorded in Interposed or Lateral nuclei should receive Purkinje cell inputs primarily from the mid or lateral cortical regions. While the Purkinje cell firing patterns used for stimulation were recorded in vivo from the Crus I and II regions (more lateral hemispheres) (Vos et al., 1999; Shin et al., 2007b), it is still possible that Purkinje cells from which the stimulus files were obtained would not project topographically onto the same group of DCN cells recorded here. Recent work has also shown an increasingly wide range of cerebellar neuronal activities inherent to different cerebellar lobules (Mugnaini et al., 2011; Kim et al., 2012; De Gruijl et al., 2014; Heath et al., 2014; Zhou et al., 2014). Thus, a second possibility is that neurons in the Interpositus and Lateral nuclei recorded here may not receive axon projections from these Purkinje cells and/or are simply not “tuned” to respond to this pattern of sensory stimulus. A third factor is the fact that our in vitro recordings were conducted entirely in the presence of blockers for ionotropic and metabotropic glutamate receptors. This was necessary to avoid retrogradely stimulating ascending afferents of climbing fibers or mossy fibers and activating glutamate receptors through their recurrent axon collaterals to DCN cells. A role for metabotropic glutamate receptors in shaping rebound burst responses in DCN cells was in fact recently reported, with metabotropic receptor activation potentiating L-type calcium currents that modulated subsequent rebound bursts (Zheng and Raman, 2011).

It is also important to consider that the Purkinje cell firing patterns were taken from animals of 300 to 500 g (Shin et al., 2007b), indicating an age of ~P60. The stage of DCN development for the P12-P17 animals used for our studies may not yet include a response capability to sensory stimuli, although rebound bursts are still present at this age. In this regard, a final possibility is that the perioral/whisker stimulus used to evoke Purkinje cell firing in vivo simply does not reflect a meaningful motor-related response to DCN cells in this nucleus. This might particularly be the case for any stimuli that provided only somato-sensory stimulation to the perioral region but did not actually deflect a whisker (S-L Shin, personal communication). Nonetheless, the patterns used here can at least be considered representative of two very different
forms of output that Purkinje cells can exhibit, with one reflecting a rapid rise and fall in Purkinje cell firing, and the other a non-structured output. In that sense, the two forms of simulation can be considered valuable to examine even for the sake of comparison, particularly given the magnitude of frequency change in the PC Sensory-evoked stimulus.

4.3 Potential Purkinje Cell Patterns Related to Rebound Bursts

Given that we could find no evidence for DCN cell bursts in relation to the perioral/whisker stimuli, we changed our approach to identify the component(s) of Purkinje cell firing that were capable of evoking the large number of burst ISIs present in our recordings. Using reverse correlation, we identified a Purkinje cell input pattern in physiological spike trains that is capable of producing rebound responses in both Transient and Weak burst neurons. This pattern was comprised of an increase in Purkinje cell firing frequency above baseline followed by a distinct reduction or full pause in Purkinje cell firing (Figs. 3.10-13), referred to here as an “elevation-pause” pattern. As this pattern was relevant to both the PC Sensory-evoked and PC Spontaneous stimulus inputs, it indicates that DCN cells of either rebound phenotype express the basic ionic mechanisms required to respond to this combination of Purkinje cell firing. In the case of the PC Spontaneous input file, the pattern of Purkinje cell firing was also almost identical for Transient and Weak burst neurons, while subtle differences were apparent between Transient and Weak burst neurons in response to the PC Sensory-evoked input (Fig. 3.10C). The main difference between the Transient and Weak burst neurons here is the firing frequency before (~40 Hz to ~60 Hz) the relative increase of ~30 Hz, as well as a short pause duration is seen in the Transient burst neurons. The fact that the magnitude of change in Purkinje cell firing necessary to evoke a burst ISI for either burst phenotype was also lower for the PC Sensory-evoked input suggests that DCN cells have to capacity to preferentially respond to sensory-related stimuli. Together these findings would suggest that our hypothesis that Transient and Weak burst cells have a capacity to differentially encode Purkinje cell input may hold merit at least with respect to a sensory relevant stimulus input pattern.
4.3.1 CV2 Patterns

The data obtained with the PC Sensory-evoked pattern indicates that the relevant Purkinje cell firing rate to induce a rebound burst in Transient burst cells corresponded to a period of ~200-500 msec increase in frequency (~30 Hz above baseline, ~90 Hz peak frequency) followed by a reduction or pause in firing of ~200 msec. We note that this represents a pattern distinctly different from those bursts defined by only 1-2 ISIs (cf. 3.11A and B-D) (see Discussion below on conventional stimulus protocols). Previous work on the perioral-evoked increase in Purkinje cell firing that made up the PC Sensory-evoked stimulus used a CV2 analysis to identify the presence of sequential ISIs of increasing frequency in both anesthetized and awake rodents, although their functional relevance was not determined (Shin et al., 2007b). The CV2-defined patterns of firing elevation could conceivably represent the first half of the pattern we extracted through reverse correlation. However, our analyses uncovered several differences in these two patterns of Purkinje cell firing that argue against this. Thus Transient DCN cell bursts of 4 or more spikes exhibited a maximum PC Preburst frequency of ~90-100 Hz (Fig. 3.11), while the peak frequency within the CV2-defined frequency elevations in the same stimulus train ranged from 50-400 Hz (Fig. 3.14C). Similarly, the duration of PC Preburst frequency elevation was distinctly different in exhibiting a mode of 660 msec compared to CV2 analysis in which most patterns fall within 50 msec (Fig. 3.14B). These differences resulted in 556 CV2 patterns falling within the 500 msec window that defined our relevant Purkinje cell frequency increase according to reverse correlation. As a result, the occurrence of a CV2-defined pattern was very poorly correlated to DCN burst ISIs. If, in fact, CV2-defined elevations in firing might be accompanied by a subsequent pause or decrease in frequency that together could induce burst output in DCN cells, it has not been identified.

4.3.2 Complex Spikes

A physiologically relevant pattern of Purkinje cell firing that more accurately reflects our elevation-pause pattern of firing is the climbing fiber-evoked complex spike. Complex spikes are triggered as an all-or-nothing event by climbing fibers and are comprised of a burst of 3-6 spikes on top of a depolarization of ~20 msec (Davie et al., 2008). The complex spike is also followed
by a pause in firing of simple spikes up to 50 msec generated through intrinsic ionic mechanisms (Davie et al., 2008) as well as feedforward inhibition (Mathews et al., 2012). We note that these relative durations for an elevation and pause (20 and 50 ms) are very different from those extracted through reverse correlation for Transient burst cells, which were ~500 and 200 ms (Fig. 3.15). Our data analysis suggested that complex spikes were also not well correlated to the generation of DCN cell burst ISIs, based on a relatively small percentage of complex spikes that occurred within 500 ms preceding burst ISIs (28%). There was also a wide range of complex spike to DCN ISI latencies even when complex spikes fell within this window, and very few (5%) that were reliably associated with a DCN cell burst ISI in 50% of the cells.

One of the limitations in assessing the role of complex spikes is the number of Purkinje cells we can access for stimulation, as well as the ability to control the synchrony of their arrival. This is important as individual climbing fibers branch and project to multiple different lobules in the cerebellum, with activated Purkinje cells converging back onto the same DCN cell population that receive a climbing fiber collateral input (Garwicz et al., 1998). Purkinje cell output reflecting a complex spike response could then be synchronized across a large number of Purkinje cells in the intact system. Nevertheless, the mean Purkinje cell and DCN cell firing rates we calculated here using spike-triggered averages of complex spike times in the PC Sensory-evoked file (essentially 100% synchrony) evoked a mean firing rate in DCN cells that was difficult to interpret (Fig. 3.15A,B). Further analysis of the role for complex spikes will thus require additional experimental means, such as modeling Purkinje cell input with control over the degree of synchronous input.

4.4 Encoded Purkinje cell Parameters

Previous studies established a possible rate code for DCN neurons as there is a graded rebound firing frequency generated with respect to the magnitude of a preceding hyperpolarization (Jahnsen and Llinas, 1984a; Jahnsen, 1986b; Aizenman and Linden, 1999; Gauck and Jaeger, 2000; Rowland and Jaeger, 2005; Molineux et al., 2008a; Tadayonnejad et al., 2009b; Pedroarena, 2010; Sangrey and Jaeger, 2010; Tadayonnejad et al., 2010b; Engbers et al., 2011). The majority of these studies focused on the classic protocols of single frequency synaptic stimulation, or hyperpolarizing current injections without consideration for the complexities of a
physiological input. The potential for rebound bursts of the nature described through *in vitro* recordings to participate in signal processing in DCN cells in vivo has been less certain. Careful examination of DCN cell unit firing recorded *in vivo* during sensory stimulation provide tantalizing clues as to the presence of a rebound burst process but actual recorded evidence at the single cell level has been elusive. Most recently, reports of whole-cell recordings from DCN cells *in vivo* suggest that rebound bursts can be evoked, although these again were triggered using a stereotyped stimulus pattern (100 Hz, 10 pulses) directly to overlying Purkinje cells (Hoebeek et al., 2010). A second study reported little evidence of rebound firing in vivo, except in relation to large inhibitory responses triggered in relation to apparent complex spike discharge (Bengtsson et al., 2011). The current study is important in verifying that DCN cells can respond to physiological trains of Purkinje cell input by generating spike output with a pattern and frequencies within the range considered to reflect rebound spike bursts *in vitro*. We can now provide evidence that DCN cells can generate rebound spike frequencies to a defined pattern of an initial increase and then decrease or pause in Purkinje cell firing. While these spikes are further correlated to specific aspects of the Purkinje cell input, it is much more difficult to interpret these to specific input signals *in vivo*.

Our results indicate a strong correlation between the number of ISIs in a burst (burst duration) and the duration of the pause in Purkinje cell input. While our other test parameters returned no correlation to burst ISIs, it may be that they relate to burst duration in a non-linear manner not assessed here. The relationship between pause duration of Purkinje cell input and DCN cell bursts has some definite precedent in the literature. It has been argued that synchronized pauses of Purkinje cell output could be a temporal code as they provide optimal conditions for a rebound burst (Jaeger, 2007; De Schutter and Steuber, 2009; Steuber et al., 2011). In fact, the timing of pauses in Purkinje cell spike firing has previously been suggested as being more important even than spike timings (Jaeger, 2007). The number of Purkinje cells impinging on each DCN cells (~20) has led to the suggestion that inhibitory input provides a constant baseline of inhibition, such that a pause in firing would provide a chance for the DCN cell's membrane potential to rise, activating the T-type and HCN channels known to induce a rebound burst (Jahnsen and Llinas, 1984b; McCormick and Pape, 1990; Muri and Knopfel, 1994; Huguenard, 1996; Gauck et al., 2001; Molineux et al., 2006; Biel et al., 2009; Sangrey and
Another proposed important factor is the degree of synchronization of a pause in firing, as relief from a single Purkinje cell’s input would presumably be insignificant. In fact, neighboring Purkinje cells have been shown to synchronize their firing patterns (Shin and De Schutter, 2006), and DCN cells can time-lock to a synchronized Purkinje cell input (Person and Raman, 2012). While our adjusting stimulus intensity to 60% of maximum will recruit less than 100% of all available inputs to a given cell, these inputs can assumed to be 100% synchronized as we are directly stimulating the Purkinje cell axons with a single input pattern. Further tests studying how the duration and timing of Purkinje cell pauses can be altered by varying levels of synchrony would help explain this complex relationship.

One explanation for our poor correlations between different parameters of Purkinje cell input identified through reverse correlation to DCN cell burst ISIs could have been our large number of two-spike burst responses. Our results show that two-spike bursts have the greatest variability in their ability to correlate to all four of our tested parameters of Purkinje cell input (Fig. 3.13). We had expected two-spike bursts to be seen primarily in Transient burst cells that exhibit a high-frequency rebound, but these were also routinely recorded in Weak burst cells. Spike doublets were also detected over a wide range, with frequencies as low as 14 Hz when detected just above the statistical criteria to as high as 240 Hz. An important consideration is that at least 50% of the two-spike bursts evoked by the PC Sensory-evoked input had instantaneous frequencies within 10 Hz of the baseline firing rate in 8 Transient burst DCN cells (8.96 ± 5.3 Hz, mean ± S.D.). While this falls within our statistical criteria of 2 X S.D. above baseline firing rate to signify a burst, this many burst ISIs so close to baseline could raise questions as to their significance as a sensory-relevant deviation in firing. Therefore it is possible that the definition of a burst response used in previous in vitro studies might actually be incorporating non-relevant increases in DCN firing frequency as false-positive burst events in the physiological input trains. If we are including non-relevant DCN ISIs into our analysis, it could explain the lack of a clear elevation-pause pattern in the reverse correlated 2-3 spike burst averages (Fig. 3.11A) as compared to bursts with more ISIs (Fig. 3.11B-D). Therefore it may be necessary to revisit the appropriate level of statistical criteria to apply in assessing these responses in future work.
4.4.1 Conventional Stimuli

In the past a convention emerged in the field to use either a short burst of high frequency Purkinje cell synaptic input (i.e. 10 pulses, 100 Hz) or a direct hyperpolarizing current pulse as the trigger to evoke a rebound burst. Note that many of the earlier studies used current injection to hyperpolarize a cell to non-physiological levels of membrane potential (reviewed in (Tadayonnejad et al., 2010a)). The work contained here now suggest that even recent attempts to confine voltage clamp stimuli to within physiological levels (Engbers et al., 2011) likely overestimated the levels of inhibition to apply as a physiological condition. Our work now reveals that the presynaptic input necessary to generate a rebound burst is an "elevation-pause" pattern. In some ways one could view the standard protocol of a fixed short frequency train of Purkinje cell inputs as a form of “elevation-pause” as it provided a period of synaptic stimulation followed by essentially an infinite pause (no further stimulation). However, our recordings show that physiological input has a much more complex relationship with the membrane potential changes invoked in a DCN neuron and its resulting firing pattern than does a fixed frequency of input. One issue which was noted was the rapid change in relative amplitude of IPSPs during a physiological train, as well as the ability of the DCN neuron to maintain a semi-tonic form of firing during Purkinje cell input. We noted that even subtle changes in Purkinje cell firing, or brief pauses (on an order of msec) could alter the amplitude of the IPSPs generated or the length of the DCN neuron’s ISI (Fig 3.3). Previous in vitro studies also stimulated DCN neurons from a relatively quiescent state in which there was no background Purkinje cell input, allowing evoked IPSPs to promote a much larger degree of inhibition than might a similar input if it was delivered in the presence of Purkinje cell activity. By comparison, a physiological spike train will invoke variability in synaptic inhibition through presynaptic transmitter mechanisms and a constantly changingpostsynaptic conductance state that is not represented in earlier studies (Telgkamp and Raman, 2002; Telgkamp et al., 2004).

Our defining the elevation-pause pattern of Purkinje cell input as a burst trigger, and the extent to which pause duration can influence the magnitude of a burst provide a framework for which future in vitro studies could design a more appropriate stimulus pattern to examine rebound bursting. A new stimulation protocol would ideally include Purkinje cell baseline firing, and then a relatively short elevation of Purkinje cell firing (up to ~90 Hz) followed by a pause of
varying duration in Purkinje cell firing. The one aspect that is still missing in this scenario would be control over the degree of synchrony of inputs. But this could be addressed through use of dynamic clamp or computer modeling in order to explore how synchrony can alter the rebound response (De Schutter and Steuber, 2009; De Zeeuw et al., 2011; Engbers et al., 2011; Jaeger, 2011; Lin and Jaeger, 2011; Steuber et al., 2011; Person and Raman, 2012).
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