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The role of cerebellar nuclear GABAergic neurotransmission in eyeblink motor control

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The role of cerebellar nuclear GABAergic neurotransmission in eyeblink motor control

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LIST OF ABBREVIATIONS

aCSF – artificial cerebral spinal fluid

BC – brachium conjectivum

CR – conditioned response

CS – conditioned stimulus

DGG – γ-D-glutamylglycine

EMG – electromyography

GZ – gabazine

IN – interposed nucleus

IO – inferior olive

IR – infrared

LED – light emitting diode

MCP – middle cerebellar peduncle

PBS – phosphate-buffered saline

PC – Purkinje cell

PM – premotoneurons

PTX – picrotoxin

SLR – short-latency response

UR – unconditioned reflex

US – unconditioned stimulus

CHAPTER 1: INTRODUCTION

1.1 Dissertation Organization

This dissertation investigates the role of the intermediate cerebellum in eyeblink classical conditioning. It is written in an alternative thesis format, containing a general introduction, 4 research papers, general conclusions, and acknowledgements.

Chapter 1 presents a general introduction to eyeblink classical conditioning, the research hypotheses, background, and a literature review. It describes in detail the paradigm for training, the circuits subserving eyeblink conditioning, the controversy surrounding the function of deep cerebellar nuclei, the effect of blocking the cerebellar CS input, and the functional role of tonic interactions in eyeblink conditioning circuits.

Chapters 2-5 are organized in journal paper format for 4 manuscripts, 2 of which are submitted to Brain Research and 2 that have already been published in which I am a co-author:

Ch. 2: S.B. Ryan, K.L. Detweiler, K.H. Holland, M.A. Hord, and V. Bracha (2006). A long range, wide field-of-view infrared eyeblink detector. *Journal of Neuroscience Methods*, 152: 74-82.

Ch. 3: K. Parker, S. Zbarska, A. Carrel, and V. Bracha. Blocking $GABA_A$ receptors in interposed nuclei: effects on conditioned and unconditioned eyeblinks. Submitted to *Brain Research*.

Ch 4: K. Parker and V. Bracha. Inactivating the middle cerebellar peduncle abolishes the expression of short latency conditioned eyeblinks. Submitted to *Brain Research.*

Ch. 5: V. Bracha, S. Zbarska, K. Parker, A. Carrel, G. Zenitsky, and J. R. Bloedel (2009). The cerebellum and eye-blink conditioning: Learning versus network performance hypotheses. *Neuroscience,* (Epub ahead of print).

Chapter 6 contains general conclusions and recommendations for future directions with this research. References are at the end of each chapter.

1.2 Introduction

Classical conditioning is an intensely studied form of associative and motor learning known to be critically dependent on the intermediate cerebellum. Both the cerebellar cortex and cerebellar nuclei, comprising the intermediate cerebellum, are required for generating conditioned eyeblink responses (CRs). However, their precise roles in CR learning and expression are highly debated. Investigations of this issue thus far have provided contradictory results leading to two competing hypotheses explaining cerebellar cortical and nuclear involvement in motor learning. These hypotheses are based on behavioral results of disconnecting the cerebellar cortex from the rest of the circuit. The first is the cerebellar cortical learning hypothesis which postulates that while the cerebellar cortex stores and generates CR motor commands, the role of the cerebellar nuclei is simply to transmit these commands to the rest of the motor systems. On the other hand, the cerebellar cortical timing hypothesis proposes that CRs are stored in cerebellar interposed nuclei and that the cerebellar cortex only modulates the appropriate timing of these interposed nuclear-generated motor commands.

The main objective of this thesis was a thorough examination of the cerebellar cortical timing hypothesis, which recently became one of the leading concepts in the field

(Mauk, 1997; Medina et al., 2000). The timing hypothesis is based on observations indicating that either permanently lesioning the cerebellar cortex, or blocking its projections to the interposed nuclei, does not eliminate CRs, but instead changes their timing. To examine this concept, we first developed a unique method for precise recording of eyeblink timing and amplitude. In the next step, we determined the cause for conflicting reports of the behavioral effects of temporarily disconnecting the cerebellar cortex. We found that the effects of disconnecting the cerebellar cortex depend on the extent of the disconnection. We established that changes in CR timing are likely associated with an incomplete block of the cerebellar cortical input in interposed nuclei. In the third group of experiments we tested CRs with altered timing for their dependency on the sensory information/motor commands from the cerebellar cortex. These complex experiments required a unique combination of simultaneous drug injections in two different parts of eyeblink conditioning networks – the interposed nuclei and the middle cerebellar peduncle. We found that the generation of normally timed and short-latency CRs (SLRs) depends critically on signals from the cerebellar cortex. Collectively, these findings critically weaken the cerebellar cortical timing hypothesis and they support the notion that plastic changes underlying eyeblink conditioning are distributed across several parts of eyeblink circuits.

1.3 Research Hypotheses

I. Development of a new, precise eyeblink sensor.

This dissertation examined two predictions of the cerebellar cortical timing hypothesis. Such work required accurate measurements of the timing and amplitude of eyeblink responses. Traditionally, eyeblink responses were measured with electromechanical sensors that are not only inadequate for precise eyeblink measurements, but could also potentially stress the experimental subject. The need for a qualitatively better method of eyeblink measurement led to the formulation of the following objective*: develop a reliable and precise remote-sensing device that would permit highly accurate measurements of eyeblink amplitude and timing.* We successfully developed this sensor (Ryan et al., 2006).

II. Testing the cerebellar cortical timing hypothesis: dependence of behavioral effects on the dose of GABA-A blockers.

The cerebellar cortical timing hypothesis assumes that the complete disconnection of the cerebellar cortex from interposed nuclei only shortens the latency of CRs and does not abolish them. This assumption, however, contradicts results from other laboratories and it is also inconsistent with prior results obtained in our laboratory (Bracha et al., 2001; Aksenov et al., 2004). *Contrary to assumptions of the cerebellar cortical timing hypothesis, we hypothesized that the behavioral effects of disconnecting cerebellar cortical projections from the interposed nuclei, via GABAergic block, should depend on the completeness of the disconnection. We predicted that an incomplete block should shorten CR latency and facilitate non-associative components of eyeblinks. We further proposed that a more complete block of cortical inputs with larger doses of a $GABA_A$ blocker will abolish CRs.* These predictions were tested and confirmed in the experiments described in Chapter 3 (Parker et al., 2009).

III. The dependence of short-latency CRs on the direct nuclear and indirect cerebellar cortex-mediated, CS signal inputs.

The cerebellar cortical timing hypothesis predicts that short-latency CRs are driven by CS information reaching cerebellar nuclei via glutamatergic input from collaterals of mossy fibers. In our preliminary studies we found that this prediction is incorrect because fast glutamate blockers injected in cerebellar nuclei did not abolish short-latency CRs. *Based on this finding, we hypothesized that SLRs could be driven by CS information that reaches the cerebellar nuclei indirectly via incompletely blocked cortical projections.* To address this hypothesis we designed experiments combining microinjections of a $GABA_A$ blocker in the interposed nuclei (to evoke short-latency CRs) with microinjections of a sodium channel blocker in the middle cerebellar peduncle (to block all CS information to the cerebellum). These experiments are described in Chapter 4 (Parker and Bracha, 2009).

1.4 Background and Significance

The studies presented in this dissertation employ a simple form of associative learning known as classical conditioning of the rabbit eyeblink response. This model allows for the study of underlying circuits to elucidate the structures involved in this form of learning and memory as well as their neurophysiological mechanisms. Previous studies have shown that the intermediate cerebellum is the essential component of eyeblink conditioning circuits. The intermediate cerebellum consists of the intermediate cerebellar cortex and the cerebellar interposed nuclei. The specific role of these structures in eyeblink conditioning is poorly understood and controversial.

1.4.1 Classical conditioning of the eyeblink reflex.

Classical conditioning is a training procedure involving the repeated pairing of a neutral stimulus (conditioned stimulus, CS) with an aversive stimulus (unconditioned stimulus, US). The CS is most often a tone with an intensity that does not elicit an eyeblink on its own (**Fig. 1**, CS trace). The US is an airpuff to the eye which elicits a reflex eyeblink response of both the external eyelids and nictitating membrane (**Fig. 1**, US trace). The paradigm most relevant for examining cerebellar involvement in conditioned eyeblinks is delay conditioning. In the delay conditioning paradigm, the US onset is delayed after the CS onset by an inter-stimulus interval, typically 350 ms, and the tone co-terminates with the 100 ms US (**Fig. 1**, CS/US trace).

Rabbits are common subjects in these experiments because of their docile nature (they are easily restrained), and their brains have been well mapped and documented. When properly calibrated, a naïve rabbit will not blink to the tone CS, but it will exhibit an unconditioned reflex blink to the US (**Fig. 1**, naïve rabbit). Over the course of training with paired CS/US presentations, rabbits learn to associate the CS with the succeeding US and they will produce conditioned responses to the CS (CRs, **Fig. 1** CR trace). Training protocols generally involve presenting 100 repeated trials per day of the paired CS and US. Rabbits normally achieve asymptotic performance in 3-5 days.

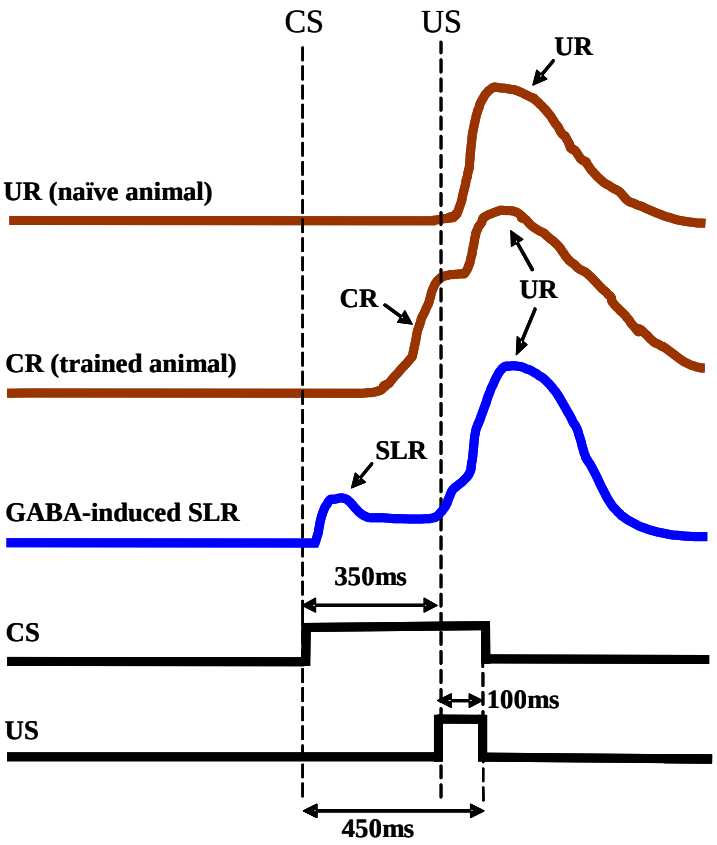

Fig. 1. Schematic diagram of the delay classical conditioning paradigm. CR – conditioned stimulus, CS – conditioned stimulus, SLR – short-latency response, UR – unconditioned response, US – unconditioned stimulus.

Studies of eyeblink conditioning require precise measurements of eyelid movements. Traditionally, eyeblinks in the rabbit were measured using electromechanical methods (Gormezano, 1966; Patterson and Romano, 1987; Bracha et al., 1993). These methods are not optimal because they display inertial artifacts and because their application could stress the experimental subject. A second common approach utilizes photoelectric infrared sensors (Thompson et al., 1994). These sensors however are very sensitive to stray IR light. To improve the precision of eyeblink

recording during conditioning, the first objective of this thesis was to develop a new photoelectric method which would not be sensitive to stray visible and infrared light. This technical objective was successfully achieved and the results are reported in Chapter 3. All experiments reported here utilized this new method.

1.4.2 Circuits essential for classical conditioning of the eyeblink response.

Previous efforts from many laboratories have studied and successfully identified circuits that are essential for acquisition and retention of eyeblink CRs in rabbits (Bracha and Bloedel, 1996; Christian and Thompson, 2003; Mauk and Donegan, 1997; Steinmetz, 2000; De Zeeuw and Yeo, 2005). Elucidating the role of individual structures in the circuit is performed using several different methods such as pharmacological inactivation, electrophysiological recording, and lesioning or stimulating specific areas. Studies employing these techniques suggest that the intermediate cerebellum, with the help of underlying brainstem structures, is essential for associative learning and memory (Thompson, 1986; Bracha et al., 2008). Cerebellar components of eyeblink conditioning circuits are outlined in **Fig. 2**. Most current hypotheses assume that the cerebellum receives converging information about the CS and US and that it uses this information to generate CR motor commands (Thompson, 1986; Ohyama et al., 2002). Information encoding the CS has been shown to enter the cerebellum predominantly through the pontine nuclei via mossy fibers making up the middle cerebellar peduncle (**Fig. 2**, CS pathway). However, the US is thought to be supplied to the cerebellum via the inferior olive (**Fig. 2**, US pathway). Lesions and temporary inactivation of the intermediate cerebellum, the pontine nuclei, and the inferior olive have produced CR deficits,

indicating that these structures play a fundamental role in motor learning (Bloedel and Bracha, 1995; Bracha et al., 1996; Christian and Thompson, 2003). Electrophysiological recordings of neurons in interposed nuclei showed modulation and tonic firing patterns that correlated with either the CS, US, or the CR (Berthier and Moore, 1990; Aksenov et al., 2004; Delgado-Garcia and Gruart, 2005). This finding further strengthened notions that the cerebellum processes sensory information related to training and that it might participate in generating CRs. While there is agreement about cerebellar involvement in eyeblink conditioning, the specific roles of the cerebellar cortex and interposed nuclei remain obscure.

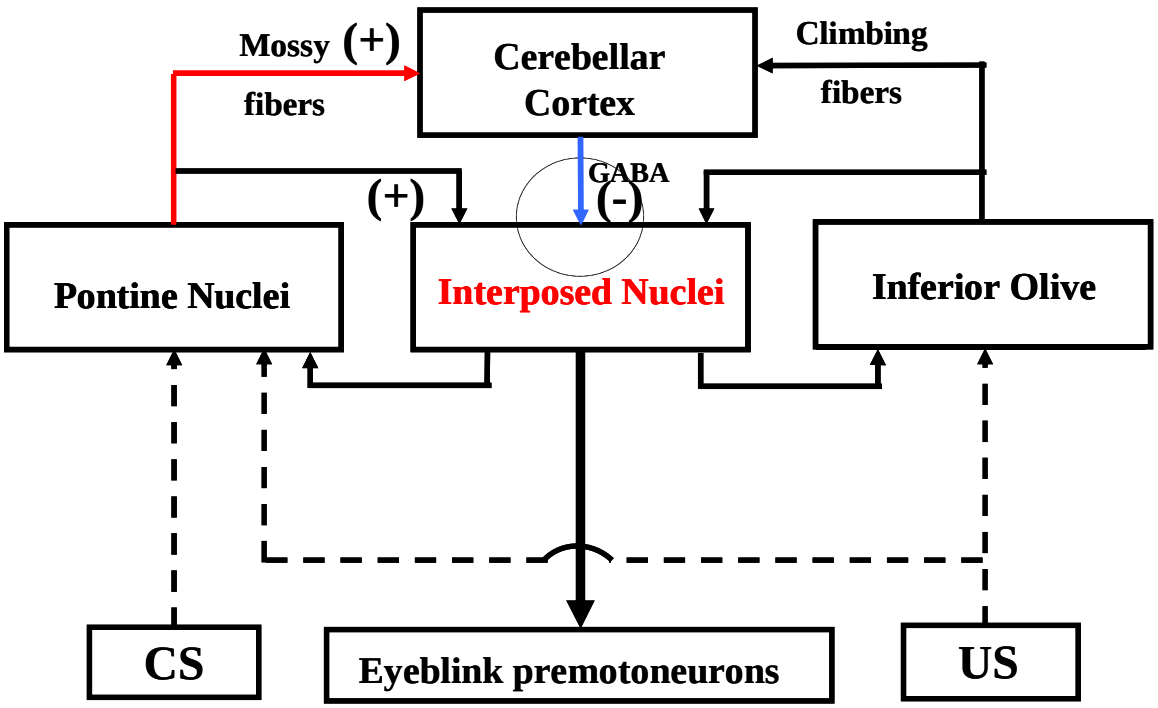

Fig. 2. Schematic diagram of the cerebellar portion of circuits controlling classical conditioning of eyeblink responses. The intermediate cerebellum processes CS and US information and controls the activity of eyeblink

premotoneurons. The cerebellar cortex and cerebellar nuclei are serially connected and both receive the CS and US information from collaterals of mossy and climbing fibers. (-) inhibitory GABAergic projections; (+) excitatory glutamatergic projections.

1.4.3 The function of the cerebellar cortex and IN – controversy surrounding blocking the GABAergic cerebellar cortical input into the IN.

As can be seen in **Fig. 2**, determining individual roles for the cerebellar cortex and nuclei is complex due to the patterns of their connectivity. Both the cortex and nuclei receive information about the CS and US and can therefore participate in learning and in generating the CR motor command. Further complicating matters, the cortex and nuclei are serially connected and therefore, manipulating either of them could affect both of their contributions to CR expression. Researchers have used two methods to address the function of the cerebellar cortex. They either lesioned the cortex or blocked the inhibitory GABA projections from the Purkinje cells in the cerebellar cortex to the interposed nuclei (Ito, 1984), allowing them to assess CR acquisition and retention in the absence of the cerebellar cortex. In an experiment of this type, a deficit observed following the cortical disconnection would point toward the possible role of the cerebellar cortex.

Results of these studies are controversial and questions about the function of the cerebellar cortex remain unsettled. Specifically, two groups of investigators produced dissimilar results from the same manipulation. One group of researchers lesioned or

functionally disconnected the cerebellar cortex from the cerebellar nuclei using GABA antagonists and found that this treatment did not abolish CRs. The effect of this treatment was rather subtle - rabbits expressed CRs with onset latencies shorter than normal (short latency responses - SLRs) (Garcia and Mauk, 1998; Perrett et al., 1993; Perrett and Mauk, 1995; Krupa et al., 1993). This was a critical finding because it suggested that the CR memory trace is not stored in the cortex because CRs were still present after its removal. This implied that if the trace is stored in the cerebellum, it must to be in the IN. Since removal of the cerebellar cortex affected only CR latency, it seemed to suggest that this structure controls the appropriate timing of CRs. These findings led to the formulation of the ***cerebellar cortical timing hypothesis*** which postulates that plasticity underlying eyeblink conditioning occurs in cerebellar interposed nuclei and that the main function of the cerebellar cortex is to control the adaptive timing of CRs (Medina et al., 2001).

The main weakness of the cortical timing hypothesis is that other researchers failed to replicate the central finding – the SLRs - that led to its formulation. Specifically, instead of producing SLRs, several investigators reported lesioning or the pharmacological blocking of projections from the cortex to the nuclei abolishes CRs (Attwell et al., 2002; Hardiman and Yeo, 1992; Yeo and Hardiman, 1992; Attwell et al., 2001; Bracha et al., 2001; Bao et al., 2002; Skelton et al., 1988). These latter findings indicated that the cerebellar cortex could be involved in the storage of CRs because without it rabbits were unable to express the learned behavior. The abolition of CRs following the cerebellar cortical manipulation is consistent with the ***cerebellar cortical learning hypothesis*** that was originally formulated by Albus (1971) and Marr (1969).

The rabbit eyeblink conditioning version of the cortical learning hypothesis postulates that the main function of the cerebellar cortex is to store memory traces of CRs (e.g. Attwell et al., 2002).

The cortical timing and cortical learning hypotheses ascribe different functions to the cerebellar cortex and IN, leading to different answers to the question about the location of plasticity underlying eyeblink conditioning. Further advancement of the field depends critically on resolving this controversy. Previous attempts to reconcile the differences in findings focused on permanent lesion experiments. However, they were inconclusive, because it is difficult to lesion the cerebellar cortex completely without damaging the underlying cerebellar nuclei (Harvey et al., 1993). Thus, re-examining the results of neuropharmacological studies was critical in determining which of these hypotheses is correct.

Experiments described in Chapter 3 (Parker et al., 2009) were conducted with the objective to re-examine the effects of blocking GABAergic cortical projections to interposed nuclei. Based on the critical analysis of available studies we hypothesized that behavioral effects of blocking GABAergic neurotransmission in deep cerebellar nuclei could be dose-dependent. We found that pharmacologically-induced SLRs are indeed replicable and their presence is dependent on the extent of the pharmacological block. While small doses of either the chloride channel blocker picrotoxin or the $GABA_A$ antagonist gabazine induced SLRs, a more complete cortical disconnection with large doses of either of these GABA blockers actually abolished CRs. Surprisingly, these contrasting results fall short of robustly rejecting one hypothesis in support of the other. It became clear that dissociating between the timing and learning hypotheses will require

determining whether SLRs are triggered by the direct CS input from the collaterals of mossy fibers to the IN (**Fig. 2**) or whether they are evoked by residual sensory signals from the incompletely blocked cerebellar cortex (Aksenov et al., 2004). To address this issue, general principles of CS signaling in the cerebellum have to be considered.

1.4.4 Blocking CS inputs to the cerebellum and their involvement in the generation of CRs and SLRs

One of the largest sources of cerebellar afferents are the pontine nuclei, which give rise to a mossy fiber tract, the middle cerebellar peduncle (MCP). The MCP sends direct projections to the cerebellar cortex and some MCP axons also send collaterals that terminate directly in interposed nuclei (Steinmetz and Sengelaub, 1992; Mihailoff, 1993; Brodal and Jansen, 1946; Cicirata et al., 2005). As shown in **Fig. 2**, the MCP is thought to supply the cerebellum with information encoding the CS. In agreement with this postulate, neurons in pontine nuclei respond to the CS (Steinmetz et al., 1986) and MCP lesions abolish CRs evoked by CSs of several modalities (Lewis et al., 1987).

As described above, the cortical timing hypothesis assumes that eyeblink CRs are generated within interposed nuclei in response to direct CS inputs and that the cerebellar cortex regulates their timing. This reasoning suggests a straight-forward test of the cortical timing hypothesis. Namely, if SLRs are poorly timed CRs that are generated by interposed nuclei, then blocking the direct CS input to the nuclei should abolish them. Since collaterals of mossy fibers are glutamatergic, this input to the IN could be blocked by local infusions of AMPA/kainate and NMDA receptor blockers. In our preliminary experiments we attempted to use this approach. Trained rabbits were injected in the IN

with a cocktail of low dose PTX to induce SLRs and γ-D-glutamylglycine (DGG) to block AMPA/kainite and NMDA receptors. We found that this cocktail did not suppress SLRs (data not shown in this thesis, Detweiler, 2004). This preliminary finding was later confirmed by Ohyama et al. (2006) who used a similar approach. They infused the IN of SLR-expressing rabbits with a cocktail of the glutamate blockers NBQX (blocks AMPA/kainate receptors) and AP5 (blocks NMDA receptors). They found that this treatment reduced the amplitude of SLRs, but it did not eliminate them. These data seemed to invalidate the cerebellar cortical timing hypothesis because blocking the direct CS input to the IN did not abolish CRs. Paradoxically, blocking all eyeblink-related inputs in the IN did not abolish CRs. These findings implied that CRs expressed after blocking GABA and glutamate inputs in the IN might be generated by extra-cerebellar circuits. An alternate explanation of the above data could be that SLR-inducing doses of GABA blockers did not block the cerebellar cortical input completely and that SLRs were driven by residual signals mediated via the cerebellar cortex. This interpretation was supported by our study demonstrating that small doses of picrotoxin do not suppress completely the task-related modulation of IN neurons (Aksenov et al., 2004).

There are two possible approaches to testing the cerebellar cortical involvement in the generation of CRs and SLRs. First of all, one could disconnect the cerebellar cortex more completely simply by infusing the IN with larger doses of GABA blockers. This approach, however, is counter-productive because large doses of GABA blockers have major side-effects. Specifically, they dramatically increase the spontaneous firing rate of IN neurons, rendering the cerebellum dysfunctional and abolishing CRs as a non-specific consequence (Aksenov et al., 2004). The second and much more promising approach

would be blocking CS inputs to the cerebellar cortex. We hypothesized that if SLRs are driven by cerebellar cortical CS signals reaching the IN via incompletely blocked GABA inputs, then blocking the cortical CS input should abolish CRs in animals treated with SLR-inducing doses of GABA blockers. We tested this hypothesis in experiments described in Chapter 4 (Parker and Bracha, 2009).

1.4.5 Future studies – testing the network performance hypothesis

In experiments described in Chapter 4 (Parker and Bracha, 2009) we found that blocking all traditionally considered CS signals to the cerebellum by inactivating axons in the MCP abolished both SLRs and CRs. This result, collectively with our inability to abolish learned responses in experiments blocking direct inputs in the IN (see the previous section), indicated that both CRs and SLRs are evoked by signals originating from the cerebellar cortex. These findings support the cerebellar cortical learning hypothesis and argue against the cerebellar cortical timing hypothesis. However, accepting the cerebellar cortical learning hypothesis requires eliminating one additional major alternate explanation – **the network performance hypothesis**.

We formulated the network performance hypothesis and described its details in our recent review (Bracha et al., 2008). The following is a brief description of the hypothesis and its implications for experiments in this thesis and for future studies.

Traditional behavioral neuropharmacology in the eyeblink conditioning field is based on the so called “telecommunications network metaphor” (Bracha et al., 2008). Typically, neurotransmitter receptor or ionic channel blockers are used to block information processing or signal flow in the eyeblink neuronal network. However, due to

the physiology of neurons comprising this network, blocking local neurotransmission affects not only signal transmission and processing, but also the spontaneous firing rate of manipulated neurons. The local changes of spontaneous activity can then propagate through the network, altering its basic functional state. As a consequence of this process, experiments intending to affect the local information processing can incapacitate the operation of the whole network, and this could lead to false conclusions about the role of information processing at the manipulated site. Our laboratory pioneered studies that demonstrated these phenomena (Zbarska et al., 2008).

Pertinent to the results of this dissertation, in Chapters 3 and 4 we have shown that SLRs and CRs are most likely triggered by cerebellar cortical signals. However, this conclusion assumes that blocking CS signals by inactivating the MCP does not significantly affect the general functional state of the IN and the rest of the network. We conclude that this assumption will have to be tested in future experiments. The first steps toward this goal are currently ongoing and preliminary results are described in Chapter 6.

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CHAPTER 2: A LONG RANGE, WIDE FIELD-OF-VIEW INFRARED EYEBLINK DETECTOR

A paper published in the journal *"Journal of Neuroscience Methods"*[1]

Steven B. Ryan[2], Krystal L. Detweiler[2], Kyle H. Holland[3], Michael A. Hord[2] and Vlastislav Bracha[24]

2.1 Abstract

Classical conditioning of the eyeblink response in the rabbit is one of the most advanced models of learning and memory in the mammalian brain. Successful use of the eyeblink conditioning paradigm requires precise measurements of the eyeblink response. One common technique of eyelid movement detection utilizes measurements of infrared (IR) light reflected from the surface of the eye. The performance of current IR sensors is, however, limited by their sensitivity to ambient infrared noise, by their small field-of-view and by short working distances. To address these limitations, we developed an IR eyeblink detector consisting of a pulsing (62.5 kHz) IR light emitting diode (LED) paired with a silicon IR photodiode and circuit that synchronously demodulates the recorded signal and rejects background IR noise. The working distance of the sensor exceeds 20 mm, and the field-of-view is larger than the area of a rabbit's eye. Due to its superior characteristics, the new sensor is ideally suited for both standard eyeblink conditioning

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and for studies that utilize IR-containing visual stimuli and/or that are conducted in an environments contaminated with IR noise.

2.2 Introduction

Classical conditioning of the eyeblink response in the rabbit is an advanced model for investigating the neural substrates of associative learning in mammals. The successful use of this paradigm requires reliable and sensitive recording of coordinated movements associated with the eyeblink including those of the eyeball, nictitating membrane, and the external eyelids. A number of eyeblink recording techniques have been developed to monitor different aspects of the response. The oldest method, still commonly used, monitors the movements of the nictitating membrane or external eyelids with direct mechanical linkage to a displacement transducer lever (Gormezano, 1966; Patterson and Romano, 1987; Bracha et al., 1993; Perrett and Mauk, 1995). Although, these techniques are simple and provide excellent information about the position of the eyelid or nictitating membrane, they require direct mechanical contact with the eyelid. This contact produces mechanical stimulation of the eye which can confound the experiment. In addition, lever systems are sensitive to inertial artifacts.

A second common method of eyeblink recording utilizes infrared (IR) photoelectric sensors. This approach measures IR light reflected from the surface of the eye (VanDercar et al., 1969; Thompson et al., 1994; Li et al., 2003). A typical IR eyeblink measurement device consists of an IR light emitting diode (LED), which illuminates the eye surface, paired with an IR photodiode that detects IR light reflected back from the eye. Since the rabbit (New Zealand White) eyelid reflects more IR light

than does the cornea, eyelid closure corresponds with increased IR reflection, which, in turn, leads to increased current through the photodiode.

An ideal IR eyeblink detector should have several important properties. To detect the full range of eyelid movement, the IR LED should completely illuminate the surface of the fully opened eye, and in addition, the field-of-view of the IR photodiode should encompass the whole eye area. Some currently used detectors rely on commercial proximity sensors that not only must be positioned close to the eye but also emit a narrow IR beam resulting in incomplete coverage of the full range of eyelid motion (Thompson et al., 1994). To increase the area view of the original IR detector, a double sensor head system was developed (Li et al., 2003). Design of a wider, but not well-characterized, field-of-view sensor was reported by (Orlowska-Majdak et al., 2001). Additionally, the ideal IR eyeblink detector should be immune to ambient IR noise and yet maintain frequency characteristics that capture the fast oscillatory components of the eyeblink. Since traditional indoor incandescent and fluorescent lights are strong sources of IR emissions, lighting in the experimental environment introduces significant IR noise. Because the sensor is attached to the head of the rabbit in our experiments, movements of the head change the position of the sensor with respect to room illumination. Thus, ambient IR noise fluctuates dramatically even with constant ambient illumination. The suppression of this noise can be achieved either through IR light source modulation (e.g. (Orlowska-Majdak et al., 2001), or by improving the signal-to-noise ratio with a brighter IR illuminator.

Here we report a new design of a single-sensor head, wide-beam, and long-range IR eyeblink detector that suppresses IR noise both through high-intensity IR illumination

and through high-frequency IR modulation. In addition to characterizing the properties of this sensor by describing its field-of-view, frequency characteristics, and IR noise rejection, we compare it to electro-mechanical, video, and EMG recording methods. To demonstrate the practical application of this sensor, we used it to measure conditioned eyeblinks in rabbits during pharmacological manipulation of the cerebellar interposed nuclei (IN).

2.3 Material and Methods

2.3.1 Animals and the training procedure

New Zealand White rabbits were fitted with a small plastic head stage holder for mounting the eyeblink detector and airpuff delivery nozzle to the head of the subject during the experiment. An injection needle guide tube was stereotaxically aimed at the region of the left IN (Aksenov et al., 2004). Implanted rabbits were trained in a standard delay conditioning paradigm using a tone CS and a co-terminating airpuff US. Trained rabbits were then tested in eyeblink expression experiments in which the IN was microinjected with the $GABA_A$ receptor antagonist gabazine (370 ng/µl). For details of standard injection techniques, see (Aksenov et al., 2004). This study was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and was performed in accordance with the protocol approved by the Committee On Animal Care at Iowa State University.

2.3.2 IR eyeblink detector

Principle of operation. The IR sensor was constructed of a paired IR LED and IR photodiode. The sensor was attached to the implanted holder and positioned in front of the eye (**Fig. 1**). During eyeblink detection, IR light from the LED illuminates the eye and reflected IR light induces an electrical current through the IR photodiode. As the eyelid closes, increasingly more IR light is reflected on the photodiode, since the eyelid has a higher IR reflectivity than the cornea (**Fig. 2C**). The resulting IR signal is amplified, digitized, and recorded on a computer data acquisition system.

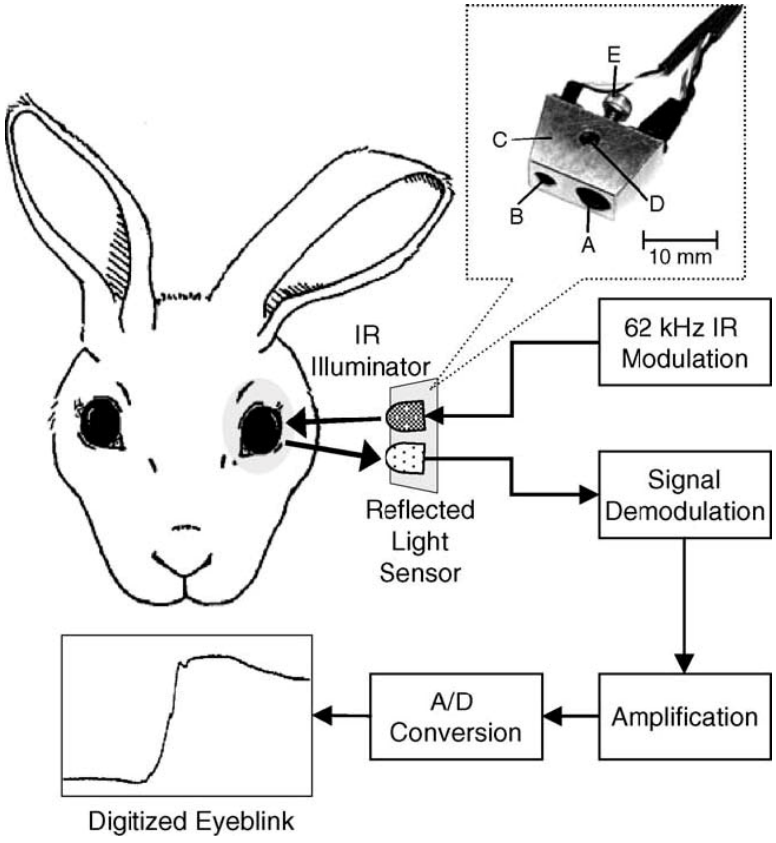

Fig. 1. Block diagram of the main components of the IR eyeblink detector. The inset is a photograph of the IR sensor head. A – LED lens; B – photodiode lens;

C – aluminum housing of the sensor; D – hole for attaching the sensor positioning hardware; E – screw for locking the sensor positioning hardware in place.

The IR sensor head. The sensor is comprised of an infrared LED and a photodiode held by an aluminum block at an angle allowing a working distance to the eye of approximately 20 mm from the front edge of the block (**Fig. 1**) compared to a working distance of 4 mm required with the OPB704 used by Thompson, et al. (1994). Since the Osram SFH485-2 IR LED (Digikey, 475-1112-ND) used in this sensor has a reported 20-degree half-angle, the field of illumination at a working distance of 20 mm has a diameter of approximately 20 mm (**Fig. 2B**). This field of illumination is significantly larger than what is produced by the OPB704 (Thompson et al., 1994; **Fig. 2A**).

The maximum calculated radiant intensity of the SFH485-2 LED is 4.46 mW/cm^2 at the distance of 20 mm from the eye. According to the ICNIRP Statement (2000), exposure to this radiant intensity does not represent any known health risk. Based on our experience, the IR light in the described setup does not produce any discomfort in experimental subjects. The silicon photodiode, an Osram SFH229fa (Digikey, 475-1079-ND), has a reported 17-degree half-angle field-of-view. The sensor is connected to the detector circuit using a pair of 1.5 m thin and flexible shielded cables. A two-conductor cable (Cooner Wire, NMUF 2/30-4046SJ) is used for the photodiode and a four-conductor cable (Cooner Wire, NMUF 4/30-4046SJ) for the LED. To increase the current carrying capacity of the LED cable, two leads were used for both the LED anode and cathode.

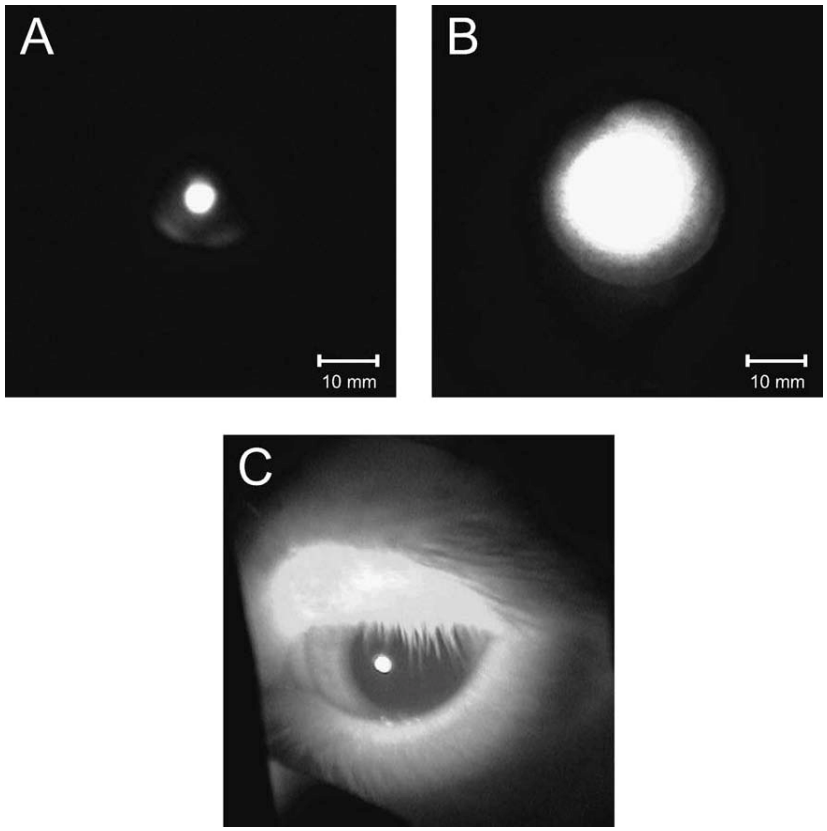

Fig. 2. IR photographs of the area illuminated by the original (Thompson et al., 1994) IR sensor (A) and by the modulated IR sensor (B). The original narrow-field sensor was placed at a distance of 4 mm, and the modulated wide-field sensor was placed 20 mm from the illuminated target. Note that the wide field-of-view sensors illuminate much larger areas. This field-of-view is sufficient to illuminate the whole periocular region of the rabbit (C).

Eyeblink detector circuit. The sensor eliminates ambient IR noise by using high-frequency, pulsed IR illumination, much like a carrier wave in an amplitude-modulated radio (**Fig. 3**). Because the illuminating LED is pulsed at a high frequency (62.5 kHz),

changes in reflectivity of the eye during a blink cause modulation of the amplitude of the reflected IR carrier wave. The modulated output of the photodiode is amplified to a usable level and then run through a product detector circuit. This produces a signal containing the desired demodulated information, as well as undesirable high frequency content around the carrier frequency and also higher harmonics of the carrier. The phase synchronization normally needed with product detector circuits is inherent in the design since the frequency of switching is derived directly from the same oscillator as the LED drive pulses, and hence is perfectly in phase with the LED pulses. The signal is then filtered to remove the higher frequency content, completing demodulation, and is scaled and adjusted for offset as desired by the user.

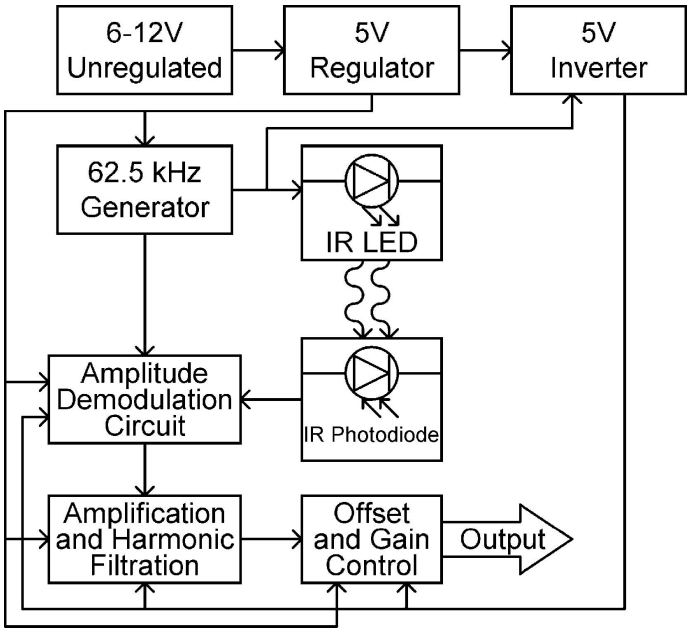

Fig. 3. Block diagram of the modulated circuit operation.

Detailed schematic of the circuit is provided in **Fig. 4**. U3 is a timer, producing pulses at approximately 62 kHz. Transistor Q1 allows the low drive strength timer chip to drive a higher current infrared LED (LED1). U5 and U4:B amplify the signal as received by PD1, the photodiode, as well as filtering the signal to remove content significantly less than the modulation frequency. This is important, as the primary source of interference is likely to be light in the 60Hz range produced by conventional light sources. U7 is a CMOS analog switch, which forms the main demodulation element. U6 provides additional amplification as well as filtration of high frequency noise from the carrier signal. U4: A then provides further filtration and a mechanism for adjusting the gain and offset of the final signal.

U1 and U2 form the power supply; an important feature is the use of the carrier frequency from U3 to drive the switching of voltage inverter U2, which causes the switching noise on the -5V power supply line to match the carrier signal rather than being out of phase with it which would cause destructive interference.

2.3.3 Data acquisition and experiment control

The amplified signal from the IR eyeblink detector, as well as the EMG signal and the signal from the electromechanical sensor were digitized at 25 kHz using a 12 bit A/D PC board. Digitized data were stored on a hard drive of a custom data acquisition PC system and processed off-line using custom data analysis software. The data acquisition PC also triggered the delivery of conditioned and unconditioned stimuli (Bracha et al., 2003).

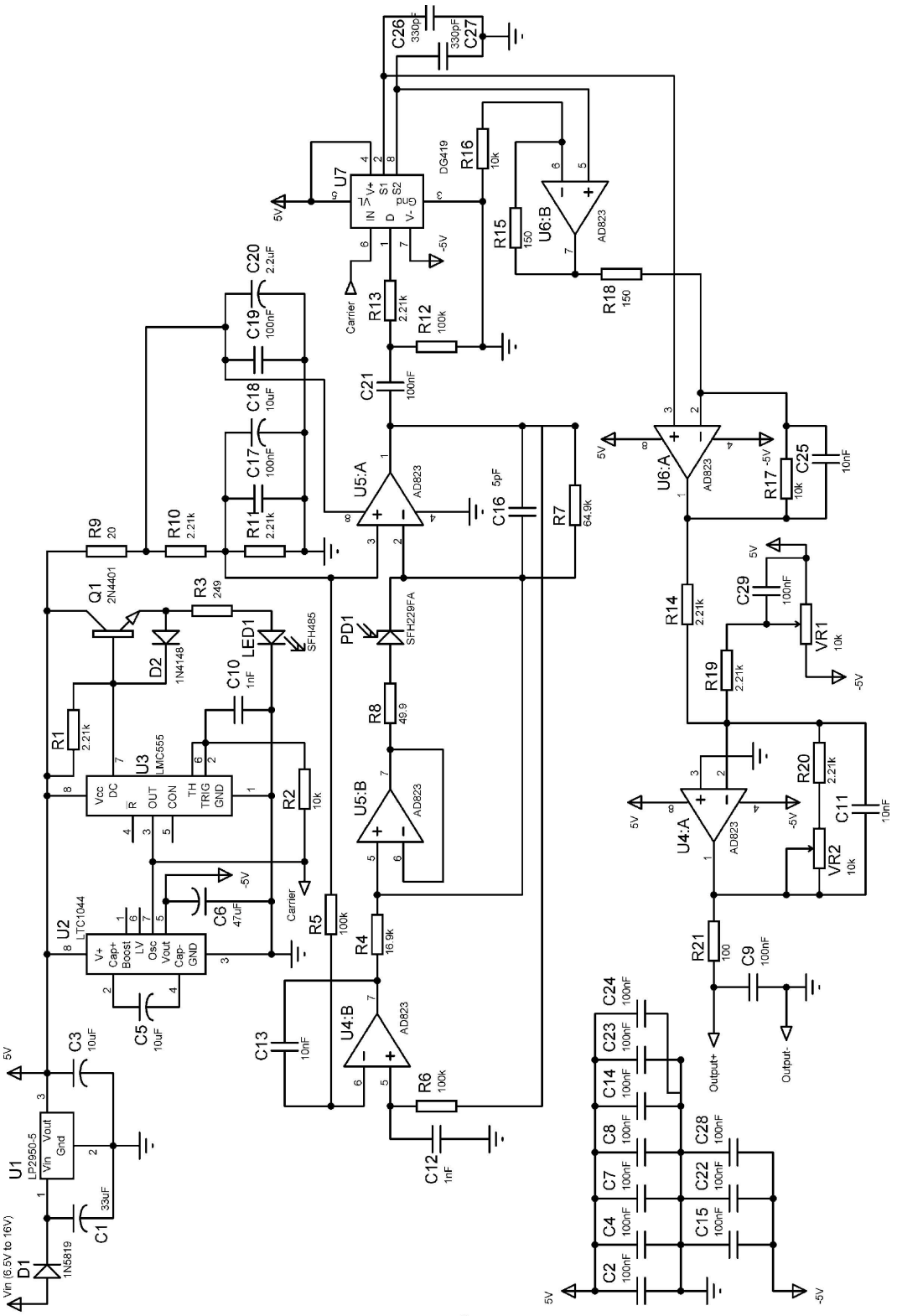

Fig. 4. Wiring diagram of the non-modulated circuit.

2.4 Results

2.4.1 IR eyeblink detector tests

Field-of-view. With an IR photoelectric sensor, eyeblink detection requires the coincidence of reflected IR light with the photodiode's field of sensitivity. Thus, the relative angle between the axis of the emitter and that of the photodiode was chosen to provide optimal overlap at a working distance of 20 mm. Since the geometries of both the emitted IR cone and the photodiode field determine this angle, we mapped the emitted IR light cone and then examined the sensor field in and around this cone.

To map IR emissions, IR light from the sensor was projected through a flat, IR-translucent calibration grid at varying distances, and then photographed with an IR sensitive CCD camera. Digital images of the projected IR spot were measured with image editing software (Adobe Photoshop 7.0), scaled using the calibration grid, and visually fit to circles. To map the photodiode field-of-view, the sensor was mounted at varying distances above an IR absorbent target. At each distance, an IR reflective test strip was moved from the periphery toward the presumed center of the photodiode field. The position where the test strip first elicited a deflection in sensor output was marked on the target. By systematically altering the orientation and direction of the test strip, the approximate edges of the photodiode field were delineated. For each sensor distance from the target, the marked field edges were visually fit to a circle. Sensor gain settings were in the range of those used for measuring eyeblinks in rabbits.

We found that the sensor provides an illumination area and field-of-view large enough to cover the area of the rabbit eye when positioned farther then 17 mm from the

surface of the eye (**Fig. 5**). The optimal placement is approximately 25 mm from the eye. At this distance, the field-of-view is a circle with an approximate diameter of 20 mm. This is sufficient to completely cover the eye area without including unnecessary adjacent areas.

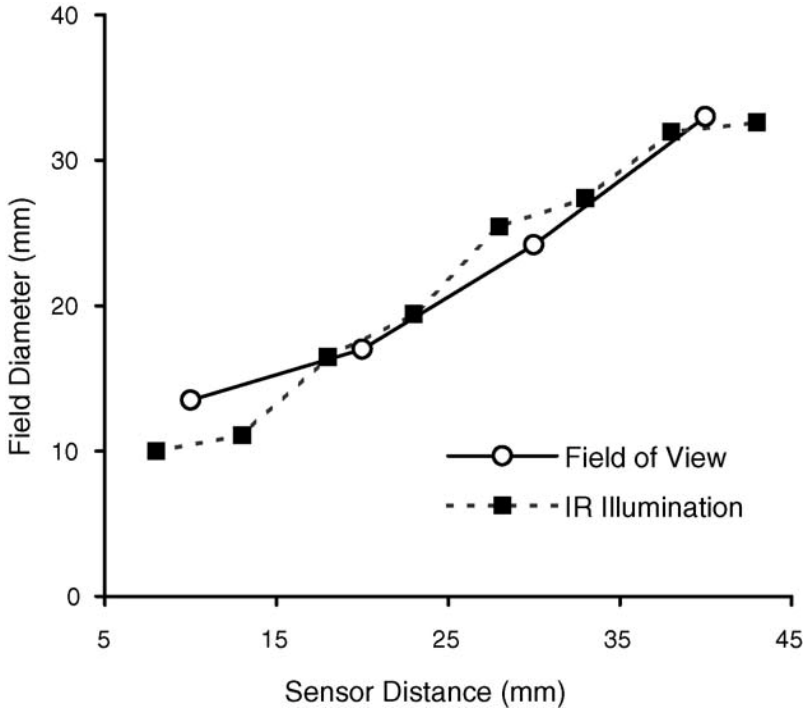

Fig. 5. The field-of-view of the modulated sensor and the area illuminated by this sensor plotted against the distance of the sensor from the observed target. Note that at a distance of 20 mm, both the illuminated area and the field-of-view exceed the average size of a 3.5 kg New Zealand rabbit eye (~15 mm corner to corner).

Frequency characteristics. The eyeblink imposes obvious temporal constraints on any detection schema. Gruart et al. (2000) found that the reflexive lid motion in the rabbit contains oscillatory components reaching frequencies of up to 30 Hz. To test whether the sensor could reliably track fast components of the eyeblink motion, we constructed a test target disc (120 mm diameter) with four IR absorbent sectors and four reflective sectors, each 45°. The target disc was then connected to a variable speed motor. The eyeblink sensor was fixed 20 mm above the disc and the gain of the sensor was set to the same value as during eyeblink measurements. Output from the sensor was monitored on an oscilloscope and digitized for further analysis.

IR sensor response to the reflective sectors on the spinning disk was recorded at sector frequencies of 40, 80, 160, 320, 640 and 800 Hz. The 800 Hz frequency corresponded to the upper limit of our testing device. The sensor response showed no attenuation of the recorded signal amplitude at sector frequencies below 640 Hz, and even at 800 Hz, the signal attenuation did not exceed 1.5%. Since the sensor input was not sinusoidal, the small attenuation observed at 800 Hz sector frequency was most likely due to the attenuation of even higher frequency components of the signal. Determining the true upper cut-off frequency of the sensor was beyond the frequency range of our test system. These excellent frequency properties of the sensor far exceed requirements for eyeblink recording.

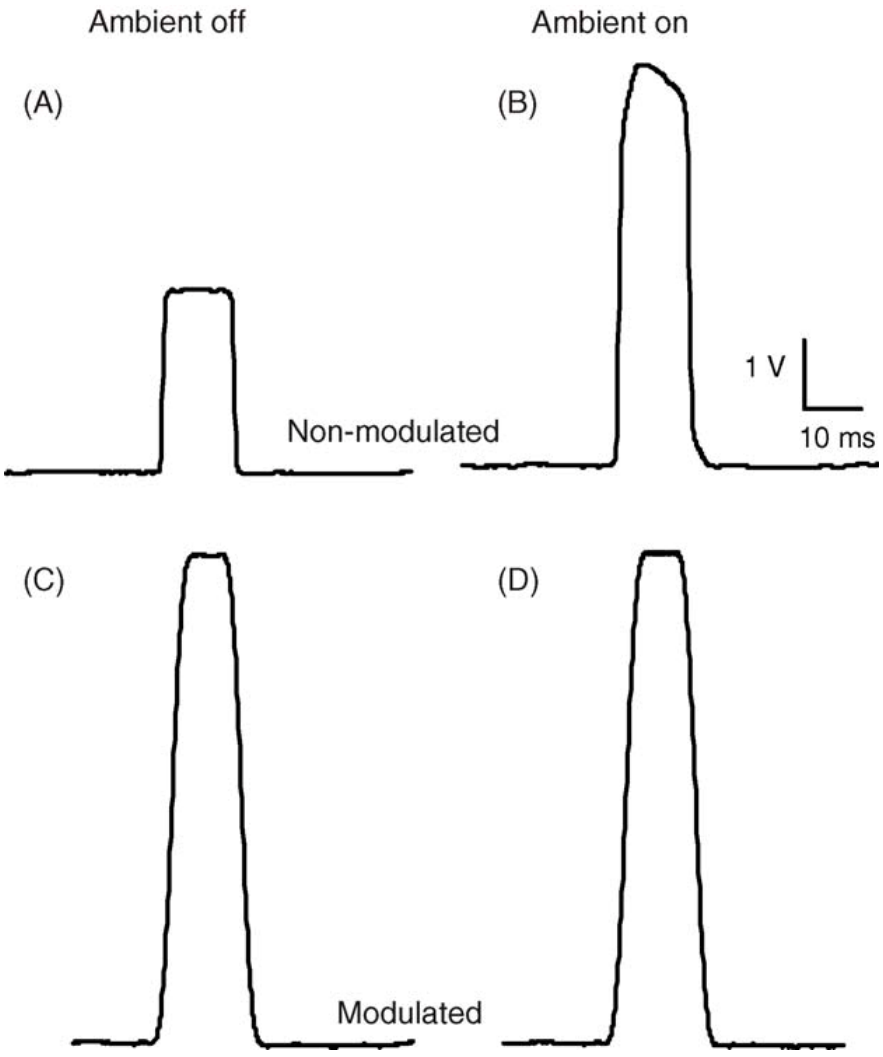

Fig. 6. Sensitivity of the IR sensors to ambient IR light. A non-modulated IR sensor (top traces, A and B, based on Thompson et al., 1994) compared to the modulated version of the new IR detector (bottom traces, C and D) in the absence (left column) and presence (right column) of bright ambient illumination. Signal peaks are responses of sensors to the presence of the IR reflective sector on the test disk. Note the undesirable distortion of the signal from the non-modulated

sensor – its response dramatically increases when the ambient IR light is present. The new modulated sensor is not sensitive to the ambient IR illumination.

Response to ambient IR light. Since the one of primary objectives for development of a modulated IR eyeblink sensor was to reduce the sensor response to ambient IR noise, we examined the output of the modulated IR sensor to the spinning disc in the absence and presence of bright ambient illumination. To do this, we used an IR absorbent target disc with two 45°reflective sectors. The sensor was positioned 20 mm above the disk and an ambient IR light source (150 W halogen bulb) was placed 50 cm above the disc to expose the sensor to the reflected IR ambient emission. Presence of ambient illumination did not significantly affect output of the circuit during exposure to either surface (absorptive or reflective) of the spinning disc (**Fig. 6**).

To gauge the effectiveness of the modulation scheme for ambient IR rejection, we compared this output of the modulated detector to that of a non-modulated IR eyeblink detector based on previously published specifications (Thompson et al., 1994). In contrast to the modulated sensor, the non-modulated sensor showed dramatic sensitivity to ambient light during exposure to the reflective sector (**Fig. 6**). Thus, the modulated sensor could reject ambient IR noise far better than conventional non-modulated detection. The differences in rise time during onset and offset of the reflective sector presumably are related to the differences in field size. Since the modulated detector was placed far (20mm) from the spinning disk, its field of view was correspondingly much larger. Therefore for a given disc speed, the transition across the field of view would occur much more slowly. Since the maximum output of the modulated sensor was

undiminished at much higher sector frequencies (see previous section), the slow rise and fall times (**Fig. 6**) are a direct result of the transition geometry and not due to limitations of the circuit.

2.4.2 Comparison to other methods of eyeblink recording

Although IR sensors are frequently used in eyeblink studies, the relationship of the recorded signal to eyelid movement has not been examined. To aid the interpretation of the recorded signal, we compared the IR detector to other common eyeblink recording methods. Trained rabbits (n=2) underwent paired classical conditioning trials with simultaneous recording of eyeblink data from the modulated IR sensor and one of three other detection methods: an electromechanical system, video image analysis, or electromyography (EMG) recordings. Since detailed descriptions of these recording methods have previously been published, only a short synopsis of their principles is given here.

The electromechanical recording technique relies upon a direct mechanical link (via a thread) between the eyelid and a low mass lever (Bracha et al., 2001). A Hall-effect sensor detects movement of a magnet that is attached to the lever, and converts the eyelid motion to an electronic signal.

Video image analysis (Bracha et al., 2003) utilizes high speed video capture of eyeblink conditioning trials. Prior to simultaneous recording of the modulated IR and video data, markers were temporarily attached to the upper and lower eyelids and a reference marker was attached to the head stage. Automated image analysis routines then computed the vertical distance between the eyelid markers and the reference for each

frame (time resolution 8.3 ms). Also, by applying machine vision algorithms to each grayscale image of the rabbit eye, open eye area (area of exposed corneal surface in pixels) was calculated. To compare video measures of the conditioned eyeblink with those of the IR sensor, all data were normalized to percent maximal response.

EMG recordings were obtained using bipolar electrodes (Basmadjian and Stecko, 1962) that were made from Teflon insulated microwire (30 µm diameter). Electrodes were inserted in the orbicularis oculi muscle in the lateral part of the upper eyelid. The EMG signal was filtered, amplified by a differential amplifier, and digitized. Rectification and additional filtration (first order low pass Butterworth filter) were performed off-line on the digitized data.

Comparing the output of the modulated IR eyeblink detector with the simultaneous signal from the Hall-effect lever system revealed that during eyelid closure, the IR signal exhibited a consistent time lead of 5-10 ms (**Fig. 7**). In **Fig. 7**, also note that the IR signal does not show an inertial artifact (lever bounce, seen as double peak of the eyeblink in the mechanical recording). Following the end of the US, during eyelid opening, the mechanical signal also typically lagged behind the IR signal. It is not clear to what extent this lag reflected the true kinematics of the upper eyelid. Differences in the IR and mechanical signal during eye opening could possibly result from friction-related artifacts that occur during the return of the lever to the initial position. The delay in the mechanical recording during eyelid closure is most likely related to initial inertial forces causing a deformation of the eyelid-thread-lever mechanical link.

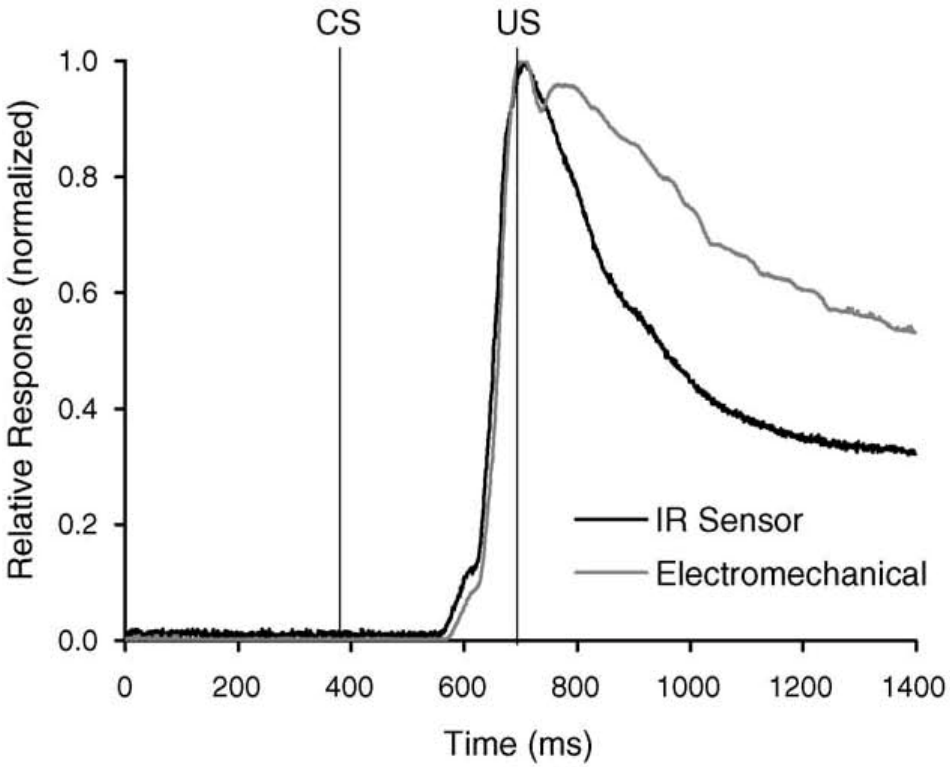

Fig. 7. An example of an eyeblink simultaneously recorded using the modulated IR detector and the lever electromechanical transducer. The lever transducer monitored the movement of the upper eyelid. CS – the time of the conditioned stimulus onset. US – the unconditioned stimulus onset. Note that during eyelid closure (the positive slope of both curves), both sensors produce remarkably similar signals, but the signal from the lever lags slightly behind the IR detector signal. Also note the inertial artifact in the lever signal (the second peak.)

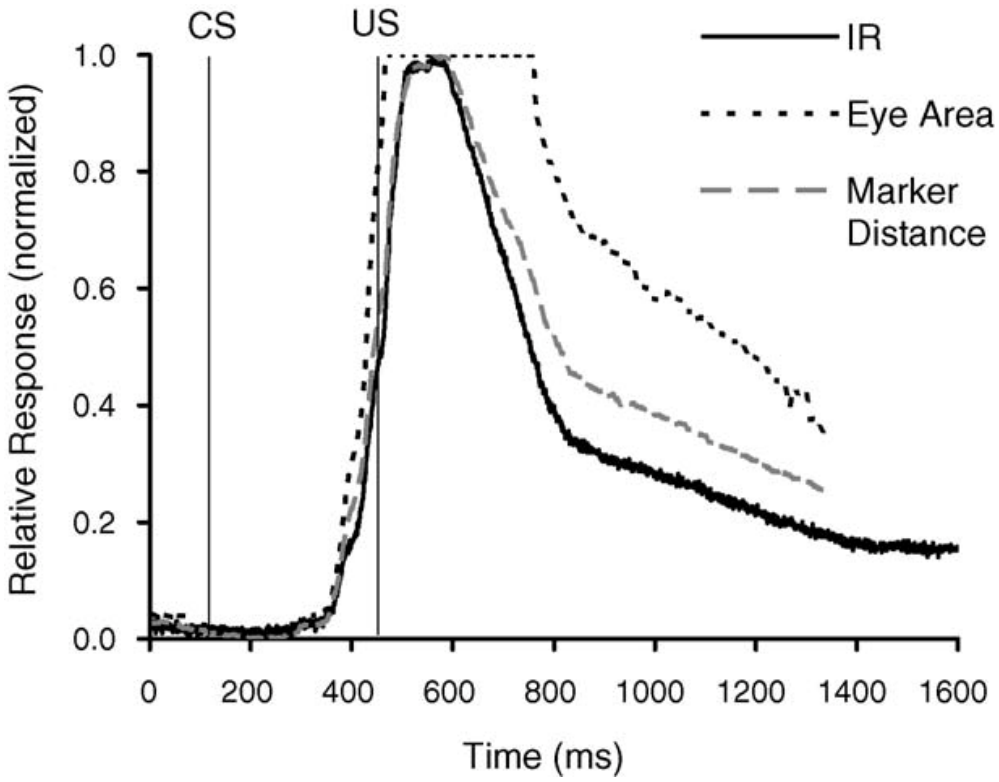

Fig. 8. An example of an eyeblink simultaneously recorded using the modulated IR detector and the high-speed video recording system. The output of the IR detector is compared to the direct measurement of the distance between the upper and lower eyelids and to the exposed corneal area during the blink. Note remarkable overlay of the IR sensor signal with the reconstructed distance between eyelids during the eyelid closure. Also note that the IR signal reflects eyelid contraction that occurs during complete closure of eyelid aperture (plateau of the corneal area plot). For more details, see **Fig. 9**.

Comparing the modulated IR signal with the analysis of simultaneously conducted high-speed video recording revealed a striking resemblance to the reconstructed distance between the upper and lower eyelids (**Fig. 8**). This finding was surprising. Since the IR detector signal is derived from the difference between the reflectivity of the cornea and eyelid, one would expect that the IR signal should change in proportion to the size of exposed corneal surface, and it should stop changing once all parts of the cornea are covered by eyelids. This is clearly not true, since the IR signal continues to increase even after the exposed corneal area measurement levels off (**Fig. 8**, dotted line, the plateau beginning just past the US marker corresponds to complete eye closure and consequent absence of any detectable exposed cornea). Visual inspection of the IR video indicated that folding and deformation of the external eyelids during the late stages of eyelid closure could contribute to the increased IR reflectivity. It can be concluded that both the change in exposed corneal surface and the increase of IR reflection from folding external eyelids contribute to the signal produced by the IR detector. The resulting signal is directly proportionate to the distance between markers placed on the external eyelids.

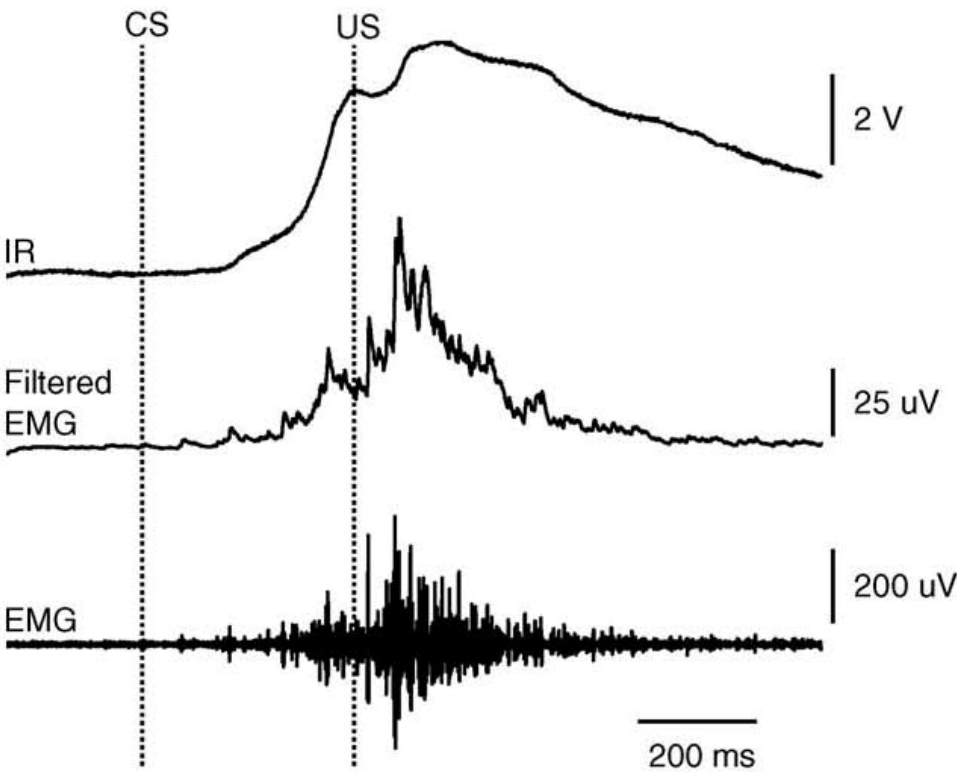

Fig. 9. An example of an eyeblink recorded simultaneously with the modulated IR detector and with EMG electrodes inserted in the *orbicularis oculi* muscle. IR – signal The close relationship between the modulated IR detector signal and eyelid movement is further reinforced by comparing the IR signal with an EMG recording of *orbicularis oculi* muscle activity (**Fig. 9**). Note that each acceleration of the IR signal was preceded by a corresponding peak in the rectified and integrated EMG activity from the IR eyeblink detector. EMG – raw EMG signal. Filtered EMG – rectified and integrated.

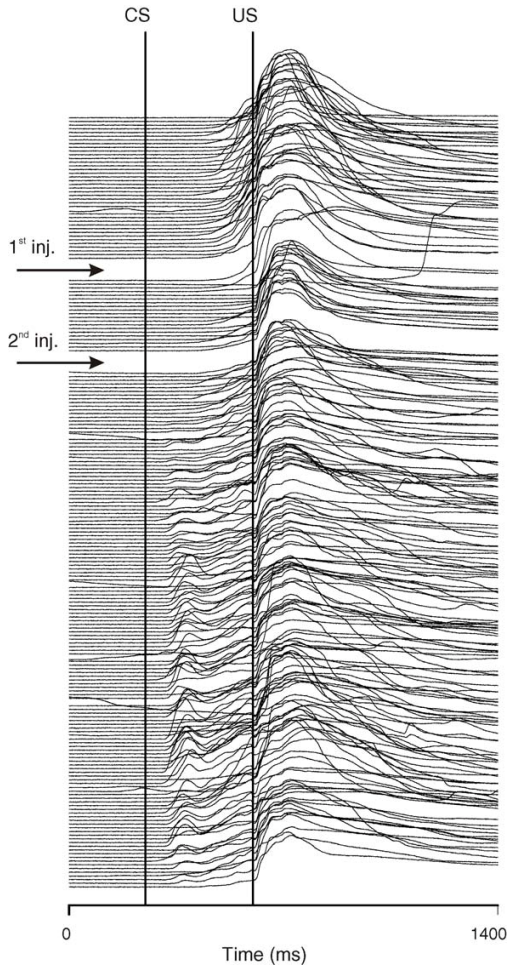

Fig. 10. An example of using the modulated IR sensor in a neuropharmacological experiment in which gabazine was microinjected (2 injections) in the interposed nuclei. Stack plot of eyeblinks recorded in 200 trials of this experiment. Data is filtered to remove the contribution of the tonic position of eyelids at the beginning of each trial. Individual trials are stacked with the first trial at the top and the last trial at the bottom of the plot. Horizontal gaps in the plot indicate times of the gabazine injections. Note that following the second injection of the drug, the latency of conditioned responses (signal deflections between the CS and US markers) dramatically decreased. CS – conditioned stimulus onset. US – unconditioned stimulus onset.

2.4.3 Practical use of the IR detector

The new eyeblink detector has been tested extensively in ongoing experiments in our laboratory. It has proven to be an excellent tool. It is noninvasive and simple to set up. It produces a high quality signal that corresponds to both the phasic and tonic components of eyelid movement. Our ongoing study examining the effects of pharmacological manipulations of the cerebellar interposed nuclei on the expression of classically conditioned eyeblinks provides a good example of how the detector can be used. It is known that activating cerebellar nuclear neurons by antagonizing GABAergic neurotransmission can decrease the latency of conditioned responses under specific conditions (Garcia and Mauk, 1998). The effect of a low concentration of gabazine on the eyeblink is illustrated in **Fig. 10**. In this experiment, a trained rabbit was exposed to paired conditioned tone and unconditioned airpuff stimuli. Following trial 40 and trial 60, the animal was injected with 0.5 µl (370 ng/µl) of gabazine in the cerebellar interposed nuclei. The second gabazine injection shortened the latency of conditioned responses (**Fig. 10**, responses between the CS and US markers).

Notes on IR detector setup for experiments. Several precautions are warranted when setting up the IR eyeblink detector prior to experiments. Since the signal depends on the distance of the sensor from the eye and on the angle under which the IR light reflects from the eye surface, the position of the sensor has to be firmly secured to the implanted hardware to assure a constant position of the sensor relative to the eye throughout the experiment. Also, the sensor has to be positioned to illuminate the whole eye area. Since IR light is invisible to the human eye, an IR-sensitive video camera can be used to assist positioning of the sensor. We use a closed video circuit equipped with a

Toshiba IK-64WDA digital video camera mounted on the wall of the experimental chamber. This highly sensitive camera was modified by removing the IR filter from the CCD chip. This extends the CCD sensitivity to the near IR part of the spectrum so that it can be used for visualizing the IR light emitted by the sensor as well as for monitoring the animal during the experiment.

Notes on the detection of eyeblink responses. Traditional lever-based electromechanical devices for eyeblink measurement can easily be calibrated to convert the recorded signal to units of displacement of eyelid movement (Bracha et al., 2001). This calibration then permits the measurement of response amplitude or setting the threshold for response detection in units of distance (mm). A similar calibration of the IR eyeblink detector is appreciably more complex. Although our tests revealed an almost linear relationship between the sensor output and the distance between the upper and lower eyelids (**Fig. 8**), the slope of this function would change with varying sensor/eye angle, and to a lesser degree, also with the distance of the sensor from the eye. An appropriate metric calibration of the sensor would require a separate calibration for each animal. Furthermore, identical placement of the sensor would be essential for each experiment. Such demands are difficult to achieve in a high throughput lab in which one sensor and its associated positioning hardware are used in several animals with varying anatomical features and varying implant positions. Consequently, we developed an alternate procedure for determining the response amplitude and onset. In each experiment (consisting of 100 or more trials), the lowest and highest values of the IR signal are determined. The lowest value corresponds to the eyelids maximally open, and the highest value to the eyelids maximally closed. The voltage difference between the

lowest and highest values then corresponds to the maximum amplitude of the eyeblink. This maximum eyeblink is assigned a value of 100%, and all amplitudes are normalized to percentage of the maximal eyeblink in a given experiment. It should be noted that this approach is only useful for assessing changes of eyeblinks within one experimental session. If a comparison of eyeblink amplitudes between individual sessions is desired, precautions should be taken to assure the identical position of the sensor in these sessions.

The traditional threshold for eyeblink detection in the rabbit has been a 0.5 mm movement of the nictitating membrane. If one assumes that the nictitating membrane response can reach amplitudes of up to 12 mm, then the 0.5 mm threshold represents approximately 4% of the maximum response. By analogy, a 4% value of a maximum eyeblink determined with the IR detector could be used as the threshold for the detection of eyeblink responses. An alternate approach to eyeblink detection is based on the statistical analysis of biological and electronic noise that precedes the delivery of stimuli. In our laboratory, we calculate the baseline variability of the eyeblink signal (mean, standard deviation) for a 250 ms period prior to the onset of the conditioned stimulus. For identifying eyeblink responses, we use a criterion of 5 standard deviations above the mean baseline level. With the modulated IR detector, this threshold corresponds to approximately 0.3% of the maximum response.

2.5 Discussion

The IR eyeblink detector described here is well suited for eyeblink measurements in the rabbit eyeblink conditioning model. It has several unique features: a wide field-of-

view, large working distance, superb frequency characteristics, low sensitivity to ambient IR noise, and an output signal that is linearly related to the distance between the upper and lower eyelids. These combined features represent significant improvement over the pioneering version of an IR system that utilized a commercial proximity detector (Thompson et al., 1994). Specifically, the significantly wider field-of-view of the present detector assures accurate detection of integral contributions of both the upper and lower eyelids to the eyeblink response, including movements that follow the complete closure of the eyelids. A large working distance avoids stimulating the eyelids by direct contact of the detector with the eyelashes, and also provides additional space for easy placement of additional hardware (e.g. airpuff delivery nozzle) in front of the eye. The lower sensitivity to IR noise, achieved by using a brighter IR illuminator and by implementing an amplitude modulating scheme, suits the new detector well for experiments in environments with bright ambient illumination and/or those involving photic stimuli.

Comparisons with other eyeblink recording systems indicate that the IR eyeblink detector accurately reflects the kinematics of eyelid closure. When compared with the lever-based electromechanical method (Bracha et al., 2001), the IR system does not suffer inertial artifacts and does not require direct contact with eyelids, thus avoiding unnecessary stimulation of the eyelids during recording. Lever systems, on the other hand, are easier to calibrate. Compared to the video-recording system (Bracha et al., 2003), the IR detector signal closely approximates explicitly measured eyelid aperture. Not only is the IR detector significantly less expensive than the video system, it is also simpler to set up. Because the sensor is directly attached to the head of the animal, the IR detector is immune to possible parallax errors plaguing table-mounted video systems.

Video-recording, however, is superior in providing information about the relative contributions of the individual lids to the blink.

In conclusion, this IR eyeblink detector is a non-invasive, remote-sensing instrument that provides integral, yet accurate, measurement of the eyeblink. The setup of this method for experiments is quick and simple. It is well suited for experiments that intend to measure movements of the external eyelids in the rabbit. The application of this instrument in smaller animals would require the miniaturization of the sensor head and cable leads. The wide field-of-view and the size of this detector would allow direct application for experiments involving human subjects.

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CHAPTER 3: BLOCKING $GABA_A$ NEUROTRANSMISSION IN THE INTERPOSED NUCLEI: EFFECTS ON CONDITIONED AND UNCONDITIONED EYEBLINKS

A paper submitted to the journal "Brain Research"[1]

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3.1 Abstract

The interposed nuclei (IN) of the intermediate cerebellum are critical components of the circuits that control associative learning of eyeblinks and other defensive reflexes in mammals. The IN, which represent the sole output of the intermediate cerebellum, receive massive GABAergic input from Purkinje cells of the cerebellar cortex and are thought to contribute to the acquisition and performance of classically conditioned eyeblinks. The specific role of deep cerebellar nuclei and the cerebellar cortex in eyeblink conditioning are not well understood. One group of studies reported that blocking $GABA_A$ neurotransmission in the IN altered the time profile of conditioned responses (CRs), suggesting that the main function of the cerebellar cortex is to shape the timing of CRs. Other studies reported that blocking $GABA_A$ neurotransmission in the IN

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abolished CRs, indicating a more fundamental involvement of the cerebellar cortex in CR generation.

When examining this controversy, we hypothesized that the behavioral effect of $GABA_A$ blockers could be dose-dependent. The IN of classically conditioned rabbits were injected with high and low doses of picrotoxin and gabazine. Both $GABA_A$ blockers produced tonic eyelid closure. A high dose of both drugs abolished CRs, whereas a less complete block of $GABA_A$-mediated inputs with substantially smaller drug doses shortened CR latencies. In addition, low doses of picrotoxin facilitated the expression of unconditioned eyeblinks evoked by trigeminal stimulation. These results suggest that the intermediate cerebellum regulates both associative and non-associative components of the eyeblink reflex, and that behavioral effects of blocking Purkinje cell action on IN neurons are related to collective changes in cerebellar signals and in the excitability of extra-cerebellar eyeblink circuits.

3.2 Introduction

The intermediate cerebellar cortex and interposed nuclei (IN) are important parts of circuits controlling the learning and expression of anticipatory withdrawal responses, such as classically conditioned eyeblinks (CRs). The IN are the output of the intermediate cerebellum, and GABAergic Purkinje cells of the cerebellar cortex are the main source of their innervation (Ito, 1984). As a consequence of this functional arrangement, studies manipulating $GABA_A$ receptor-mediated neurotransmission in deep cerebellar nuclei offer important insights into cerebellar control of eyeblink motoneurons. It is known that activating $GABA_A$ receptors with muscimol suppresses the modulation

and spontaneous activity of IN neurons (Aksenov et al., 2004), and this treatment blocks expression of CRs (Krupa et al., 1993; Bracha et al., 1994). Effects of suppressing IN neuronal activity in rabbits are not restricted to CRs. Cerebellar nuclear microinjections of muscimol also down-regulate the amplitude of airpuff-evoked unconditioned trigeminal eyeblinks (Bracha et al., 1994; Jimenez-Diaz et al., 2004) and block instrumental eyelid closure (Bracha et al., 2001). The inactivation data together with the fact that neuronal activity in intact IN correlates with stimuli and movements during conditioned and unconditioned blinks (Berthier and Moore, 1990; Aksenov et al., 2004; Jimenez-Diaz et al., 2004; Zbarska et al., 2008) suggest that the modulation of neuronal activity in the intermediate cerebellum, coupled with the out-going tonic excitatory drive, control a range of learned and reflexive eyeblink behaviors.

Additional understanding of the intermediate cerebellar role in eyeblink control can be gained from studies that block GABAergic neurotransmission in cerebellar output nuclei. In contrast to muscimol, infusing the IN with the chloride channel blocker picrotoxin (PTX) dramatically elevates the spontaneous firing of IN neurons and decreases modulation of their activity during CR expression (Aksenov et al., 2004). Thus far, behavioral effects of blocking cerebellar nuclear GABA neurotransmission were not studied systematically and several studies of CR performance yielded conflicting results. Mauk and Garcia (1998) reported that IN injections of picrotoxin or gabazine (GZ) invariably decreased CR latency, leading to so called short-latency conditioned responses (SLRs). In contrast, others reported that IN infusions of PTX abolish CRs (Mamounas et al., 1987; Attwell et al., 2002; Aksenov et al., 2004). The cause of these variable PTX effects is not clear.

Based on our previous report of a dose-dependent effect of PTX on IN neuronal activity (Aksenov et al., 2004), we propose that behavioral outcomes of injecting the IN with $GABA_A$ receptor antagonists are related to the extent of the block. We hypothesized that a partial disruption of GABAergic neurotransmission in eyeblink-related neurons should produce SLRs and that a complete block of inhibitory inputs will abolish CRs. To address this hypothesis, we infused the IN of classically conditioned rabbits with low and high doses of GABA antagonists. In the second part of this study we investigated the parallel effects of PTX infusions on CRs and inborn visual and trigeminal unconditioned responses (URs). Here we report that a more complete block of $GABA_A$ receptor-mediated neurotransmission with high doses of PTX and GZ abolished CRs. In contrast, lower doses of PTX and GZ produced SLRs, increased tonic eyelid closure, and facilitated unconditioned trigeminal eyeblinks.

3.3 Results

3.3.1 General observations

When injected in the IN at sites where previous small injections of muscimol abolished CR expression, both GZ and PTX had a dose-dependent effect on eyeblink expression. At small doses, both drugs shortened CR latency and increased tonic eyelid closure. In addition, low-dose PTX increased the amplitude of URs to a weak airpuff US and altered the velocity and duration of URs evoked by the normal airpuff intensity. At higher doses both drugs suppressed CRs. Besides their effect on eyelid movements, both drugs exaggerated responses to the airpuff US causing a more generalized withdrawal response encompassing neck and forelimb movements that drew the animal's head away

from the air stimulation. Notably, tonic eyelid closure and withdrawal-related postural asymmetry disappeared immediately after the animal was removed from the restraint box. All of these effects were observed at injection sites located directly at or in the near vicinity of the left anterior IN (**Fig. 1**).

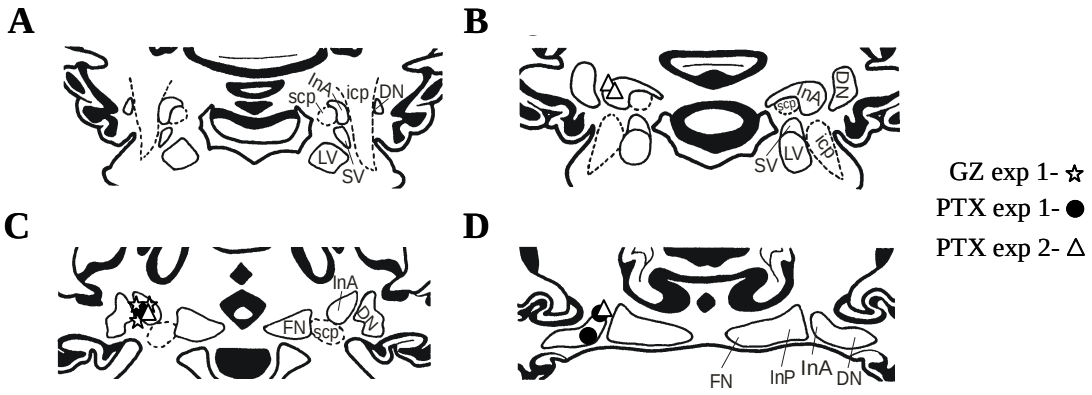

Fig. 1. Reconstruction of injection sites in the IN for GZ (stars, n = 3) and PTX (circles, n = 3) for Experiment #1 and PTX (triangles, n = 4) for Experiment #2. The identified sites were transferred to a set of standardized coronal sections of the rabbit cerebellum. A-D: four adjacent, 0.5-mm sections through the cerebellum, arranged in rostral-caudal order. All injection sites were located directly on or in close proximity to the anterior interposed/dentate nuclear border. InA, anterior interposed nucleus; DN, dentate nucleus; LV, lateral vestibular nucleus; SV, superior vestibular nucleus; InP, posterior interposed nucleus; FN, fastigial nucleus; scp, superior cerebellar peduncle; icp, inferior cerebellar peduncle.

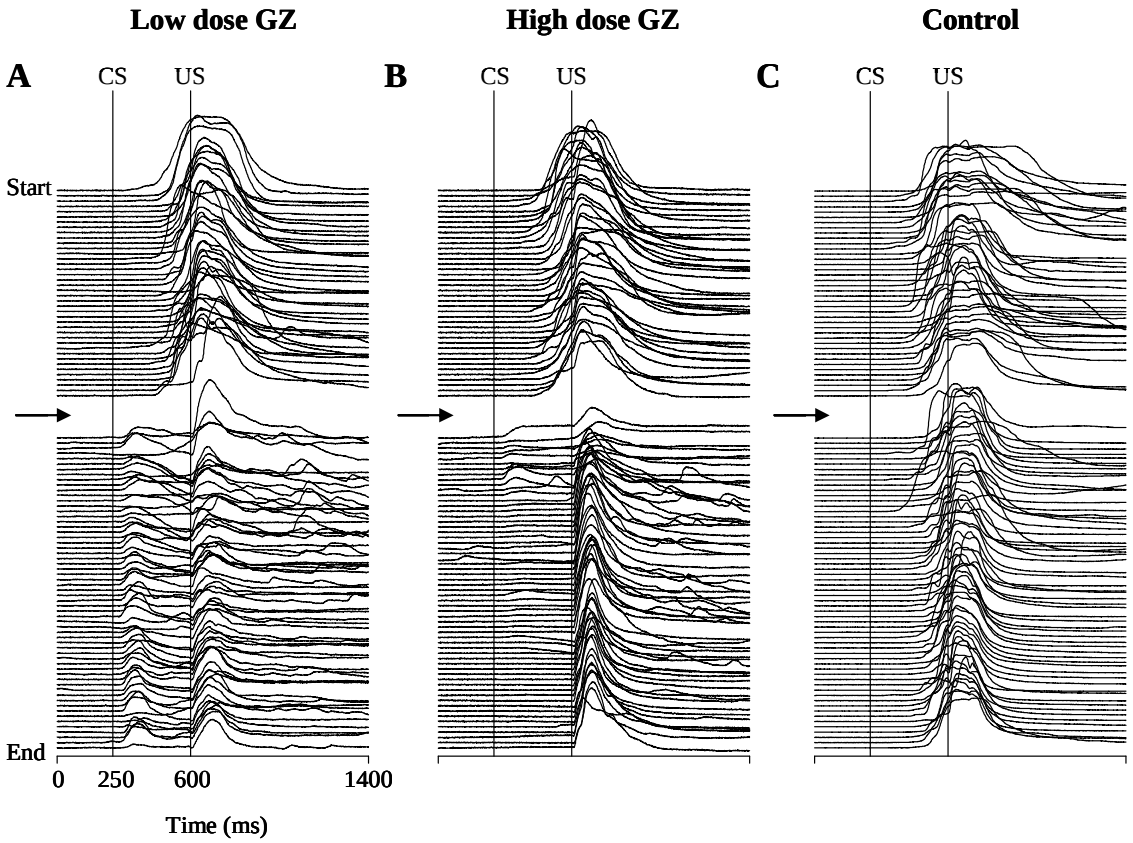

Fig. 2. Examples of stack plots of eyeblink mechanograms showing the effects of GZ on conditioned eyeblink performance when injected in the IN. Each experiment begins at the top with each mechanogram representing 1 trial. All mechanograms were filtered by subtracting the mean pre-stimulation eyelid position in the corresponding trial. A: an experiment with an injection of low-dose GZ. Following the injection (indicated with arrow), the latency of conditioned responses (upward deflections between CS and US onset markers) was shortened for the remaining 60 trials. B: identical to plot A but in this experiment, high-dose GZ was injected. Following the injection, CRs were gradually abolished. C: control for both A and B in which aCSF was injected

following 40 pre-injection trials. There was no vehicle effect on the expression of CRs in the experiment.

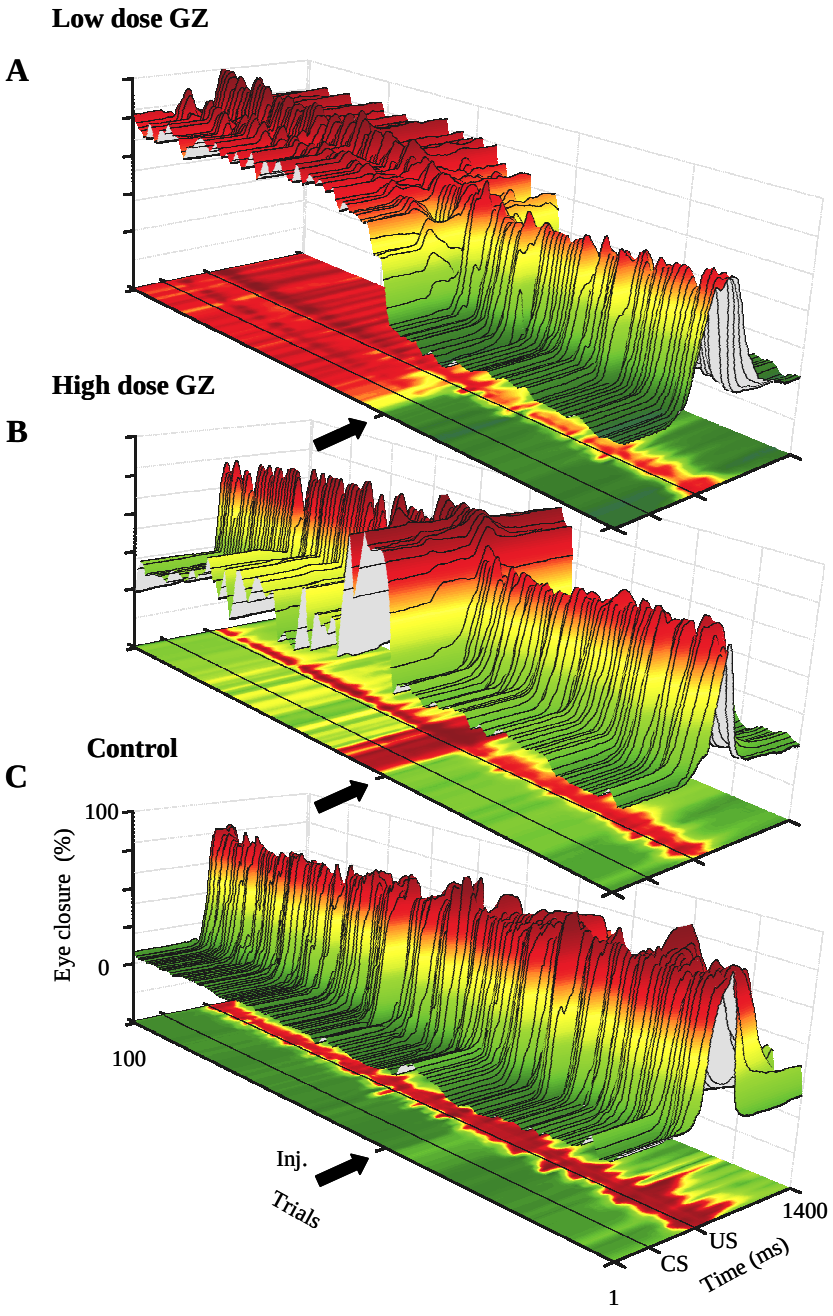

Fig. 3 Three dimensional surface plots of the individual examples of eyeblink mechanograms shown in **Fig. 2**. In this display, eyeblink mechanograms were not filtered to preserve information about the tonic eyelid position. Each cut through the surface plot corresponds to one trial and the colors, together with the z axis, code the eyelid aperture (dark green = open, yellow = intermediate, dark red = mostly closed). A: one hundred trials illustrating the effect of low-dose GZ. The GZ injection (thick arrow) induced a long lasting tonic eyelid closure, coupled with decreased eyeblink amplitude and SLRs. B: effect of high-dose GZ showing SLRs super-imposed on a tonically closed eye. Shortly thereafter, the tonic eyelid closure subsided and CRs were gradually abolished. C: control experiment for both A and B where aCSF, injected after 40 pre-injection trials, had no effect on tonic eyelid closure.

3.3.2 Effects of Gabazine and Picrotoxin on CR expression (Experiment #1)

The effects of both drugs on CR expression were dose-dependent, yielding either SLRs or CR abolition. Drug doses that led to these effects varied between rabbits. For example, a dose that shortened latencies of CRs in one rabbit could abolish CRs in another. Consequently, both the low and high doses were individually titrated for each animal. Our criterion for the high-dose was suppression of CR incidence to 30 % or less in at least one post-injection block of 10 trials. The low-dose was identified based on the appearance of SLRs lasting at least 10 minutes (about 30 trials).

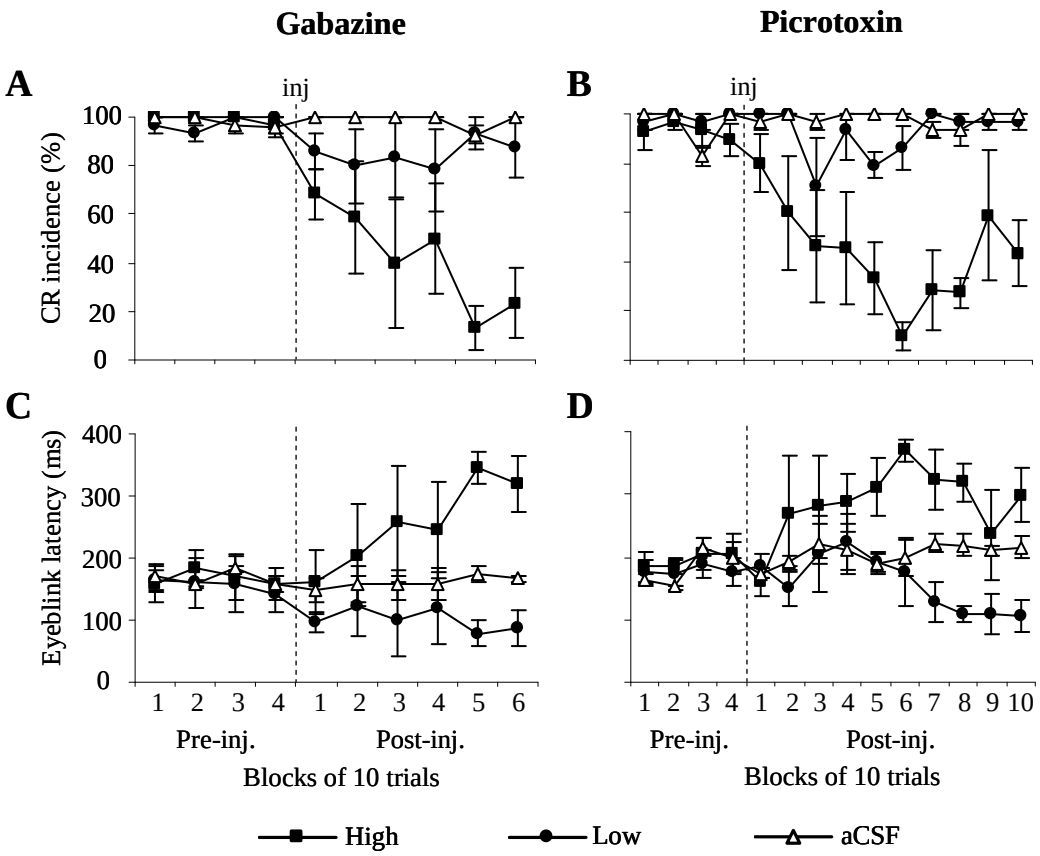

Fig. 4. Effects of low (circle) and high doses (squares) of GZ and PTX on means (± SE, n = 3) for CR incidence and eyeblink latency. A: effect of GZ on CR incidence. While high-dose GZ suppressed CRs, low-dose GZ and control injections of vehicle (triangles) had minor or no effect on CR incidence. B: effect of PTX on CR incidence. Similar to GZ, high-dose PTX suppressed CRs, but CRs had a greater tendency to recover toward the end of the experiment. C: effect of GZ on eyeblink latency. High-dose GZ increased CR latency whereas low-dose decreased the CR onset time. D: effect of PTX on eyeblink latency. Effects of PTX are similar to GZ, except that low-dose PTX took 60 trials to shorten CR latency. In A-D, injections of aCSF (triangles) had no effect on CR expression.

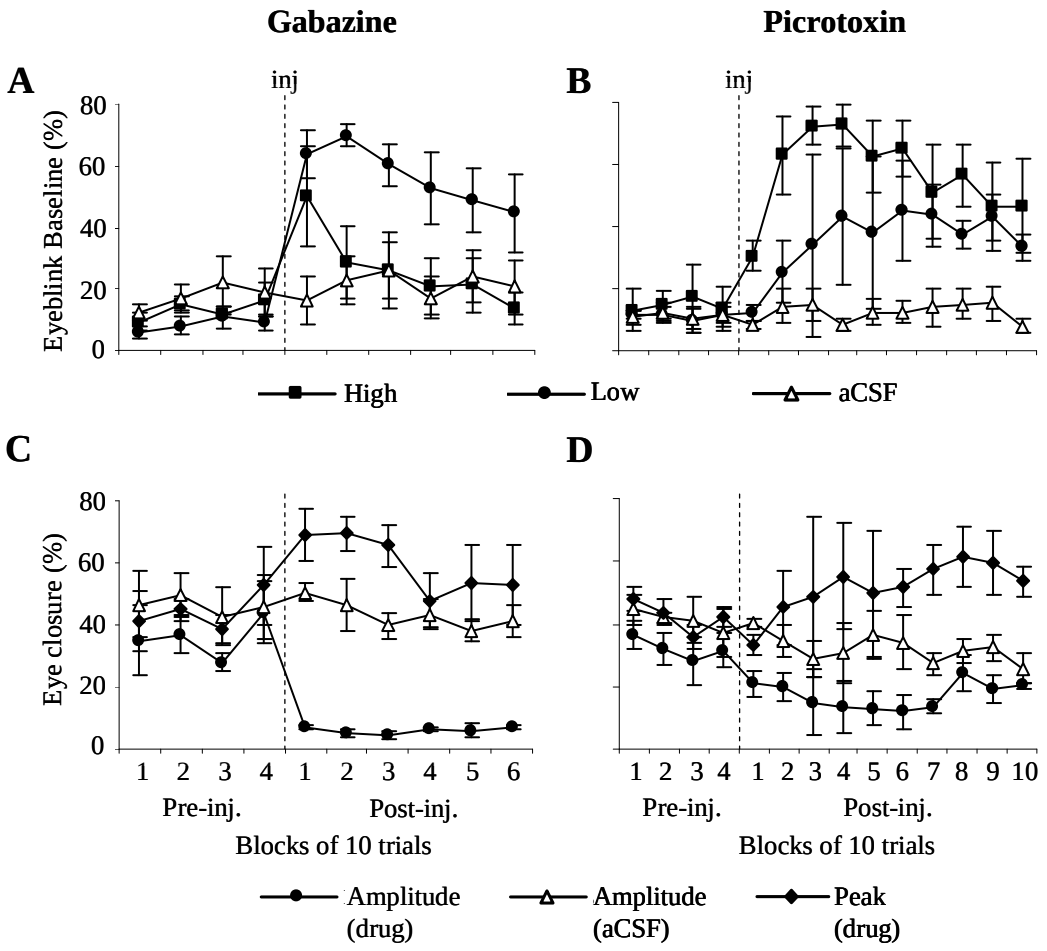

Fig. 5. Effects of low (circle) and high-doses (square) of GZ and PTX on the means (± SE, n = 3) for eyeblink baseline, peak, and amplitude. A: effect of GZ on eyeblink baseline expressed in percentage of maximum eyelid closure. Low-dose GZ produced an immediate and long lasting eyelid closure. The eyelid closure resulting from high-dose GZ was short-lasting and it recovered within 20 trials. B: effect of PTX on eyeblink baseline. Both low and high-dose PTX induced long lasting eyelid closure. Control injections of aCSF (triangles) in A and B did not affect eyeblink baseline. C: effect of a low, SLR-inducing dose of GZ on eyeblink peak (diamonds) and amplitude (circles). Injected (inj.) after 40 pre-injection trials, GZ immediately increased eyeblink peak and decreased

amplitude. D: effect of SLR-inducing, low-dose PTX on eyeblink peak and amplitude. Similar to GZ, PTX increased CR peaks but decreased eyeblink amplitudes.

3.3.3 Low-Dose Effects

When compared to control aCSF injections (**Fig. 2C**), low-dose GZ injections (0.13 – 0.51 nmol) significantly shortened CR latencies (**Figs. 2A, 3A**, $F_{1,2} = 29.91$, $p = 0.032$). The mean pre-injection eyeblink latency was 155.72 ± 15.03 ms and it declined immediately to 96.89 ± 15.80 ms in the first post-injection block of trials, peaking in the fifth at 78.41 ± 20.57 ms (**Fig. 4C**). Typically, SLRs were super-imposed on the background of GZ-induced tonic eyelid closure (**Fig. 3A**). Mean pre-injection eyelid aperture was 8.42 ± 1.28 % of maximum eyelid closure. Following GZ, rabbits had a tendency to 'squint,' significantly reducing their mean pre-eyeblink eyelid aperture nearly 6-fold to 56.69 ± 3.87 (**Fig. 5A**, $F_{9,18} = 6.97$, $p = 0.00025$). In parallel to changes in eyeblink latency, GZ significantly reduced CR amplitude measured relative to eyelid aperture before the eyeblink (**Figs. 2A, 3A, 5C**, $F_{9,18} = 2.96$, $p = 0.024$). However, since these small amplitude CRs were executed on the background of a partially closed eye, the CR peak (measured relative to maximally opened eyelids) actually increased following the injection. Control injections of aCSF had no effect on CR latencies, amplitudes, or on baseline eyelid aperture (**Figs. 4C, 5A, 5C**).

Similar to GZ, injections of low-dose PTX (0.31 – 4.98 nmol) shortened eyeblink latency. However, this effect was notably delayed when compared to GZ and it developed only in the last four blocks of the 10-block post-injection period (**Fig. 4D**,

$F_{13,26}$ = 3.48, p = 0.0032). This difference in the effect onset was most likely related to differences in effectiveness of drug diffusion. Mean eyeblink latency decreased from 177.95 ± 9.53 ms pre-injection to 104.41 ± 25.79 ms by post-injection block 10. Also similar to GZ, PTX increased tonic eyelid closure (**Fig. 5B**, $F_{13,26}$ = 2.13, p = 0.049) and it reduced CR amplitude (**Fig. 5D,** $F_{13,52}$ = 4.09, p = 0.00013) while increasing the absolute eyeblink peak in the CS - US period. Low doses of GZ and PTX had only a moderate effect on CR incidence, being slightly reduced and tending to recover toward the end of experiments (**Figs. 4A-B**).

3.3.4 High-Dose Effects

Effects of high doses of GZ (0.51 – 1.02 nmol) and PTX (0.62 – 7.47 nmol) were remarkably different from low-dose injections of these drugs. Most notably, both GZ and PTX at high doses suppressed CRs. An individual example in **Figs. 2B** and **3B** shows that shortly after the injection of GZ, CRs were abolished, contrasting with the CR latency-shortening effects of low-dose GZ in the same animal and injection site (**Figs. 2A, 3A**).

At the group level, high doses of GZ and PTX gradually, but significantly suppressed CR incidence (**Figs. 4A-B**) when compared to the pre-injection performance and to the control experiment (GZ: $F_{9,18}$ = 9.21, p = 0.00004, PTX: $F_{13,26}$ = 5.25, p = 0.0002). This gradual suppression of CRs was paralleled by a gradual increase of eyeblink latency in the CS - US period (**Figs. 4C-D**, GZ: $F_{9,18}$ = 4.29, p = 0.0041, PTX: $F_{13,26}$ = 5.23, p = 0.00017). While the effect of high-dose GZ on tonic eyelid closure was transient (**Fig. 5A**), high-dose PTX produced sustained eyelid closure, nearly doubling

from 21.25 ± 3.40 % pre-injection to 41.42 ± 4.78 % of the full eyelid closure post-injection (**Fig. 5B**, F 13,26 = 0.64, p = 0.000031).

3.3.5 Effects of Low-Dose Picrotoxin on CR and UR expression (Experiment #2)

In Experiment #1, we demonstrated how low doses of PTX affected CR expression. In considering whether disinhibiting the IN affects URs, four rabbits were injected with an SLR-eliciting dose of PTX so parallel effects of this treatment on CR and UR expression could be examined. In these animals, paired CS + US trials were intermixed with three different types of US trials: light, weak airpuff, and regular airpuff. As expected, injections of low-dose PTX (2.49 to 6.22 nmol) shortened CR latencies (**Fig. 6A**). The repeated-measures ANOVA revealed a significant drug and block-of-trials interaction (**Fig. 7A**, $F_{3,9}$ = 8.061, p = 0.0064). Low-dose PTX shortened baseline CR latency from 197.20 ± 20.85 ms pre-injection to 99.43 ± 13.53 ms in the third block of post-injection trials. The tonic eyelid closure increased from 5.36 ± 0.76 % pre-injection to 20.02 ± 3.42 % in the second post-injection block and to 27.53 ± 8.26 % in the third ($F_{3,9}$ = 4.23, p = 0.04). In parallel with tonic eyelid closure, the CR peak increased from 32.70 ± 7.41 % in pre-injection trials to 59.05 ± 12.04 % in the third post-injection block (**Fig. 8B**). This CR peak finding was revealed as main effects for the within-subject factor, blocks ($F_{3,9}$ = 4.70, p = 0.031), and for the between-subject factor, PTX vs aCSF ($F_{3,9}$ = 11.81, p = 0.041). Injections of vehicle had no effect on CR latency or tonic eyelid position. Since CR amplitudes were not significantly affected by PTX

(**Fig. 8A**), it is likely that changes in the CR peak amplitude were due to increased tonic eyelid closure.

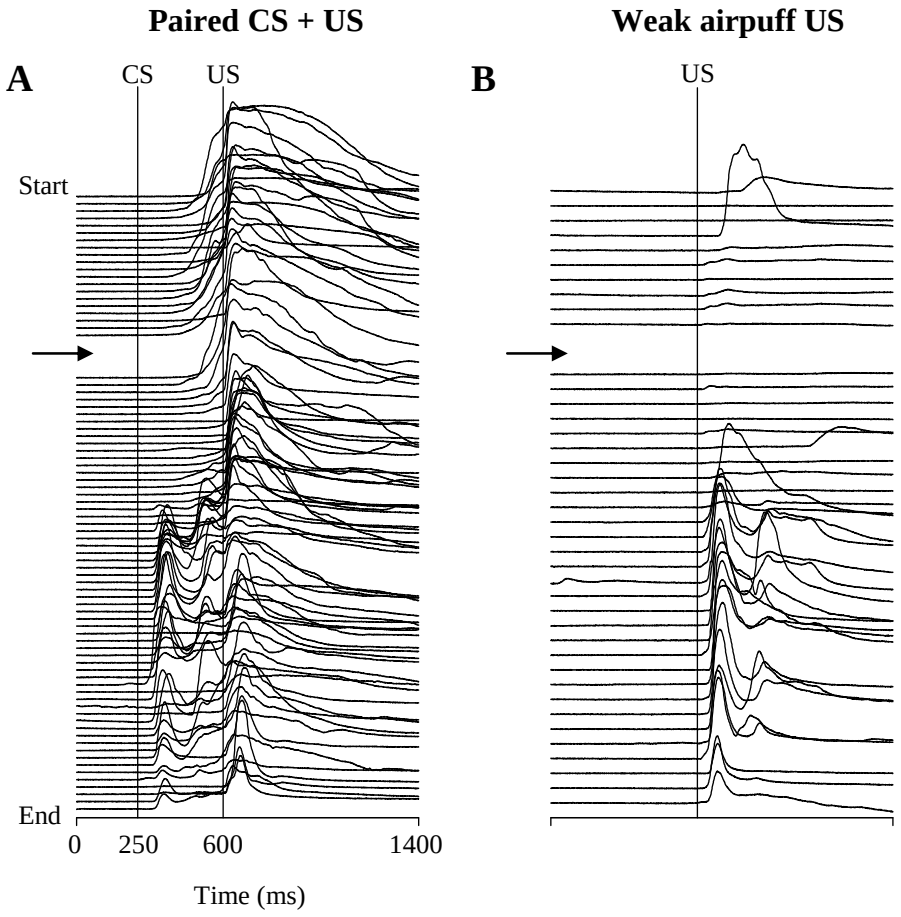

Fig. 6. Example of effects of PTX injections in the IN on the performance of conditioned eyeblinks and on unconditioned responses evoked by weak airpuffs. Both stack plots of eyeblink mechanograms are a complete printout from the same experiment. The experiment begins at the top, with each trace representing one trial, and the time of injection is indicated by an arrow. A: in paired CS + US trials, PTX shortened the latency of CRs (positive trace deflections between the CS and US markers). B: in weak airpuff-alone trials, PTX increased UR amplitude approximately at the same time when SLRs were observed in A.

3.3.6 URs to the weak airpuff

Among the three types of URs tested, eyeblinks to the weak airpuff were the most affected. Most notably, PTX increased UR amplitude during the same time period in which the drug affected CR expression (**Figs. 6A-B**). The mean amplitude of URs to the weak airpuff increased from 9.01 ± 2.65 % pre-injection to 45.88 ± 4.48 % during the third block of post-injection trials (**Fig. 8C**, $F_{3,9} = 10.75$, $p = 0.00025$). Mean UR peaks to the weak airpuff likewise increased from 13.66 ± 2.42 % during the pre-injection block of 10 trials to 67.64 ± 8.45 % during the third block of post-injection trials (**Fig. 8D**, $F_{3,9} = 11.18$, $p = 0.0022$). Similar to the PTX effect on CR latencies, mean UR latencies to the weak airpuff were shortened 50 % post-injection (**Fig. 7B**, $F_{3,9} = 5.095$, $p = 0.025$), steadily decreasing from 68.43 ± 9.80 % pre-injection to 33.80 ± 1.91 % in the third block of post-injection trials. Control injections of vehicle had no effect on latency or amplitude of URs to the weak airpuff (**Fig. 7B, 8C**).

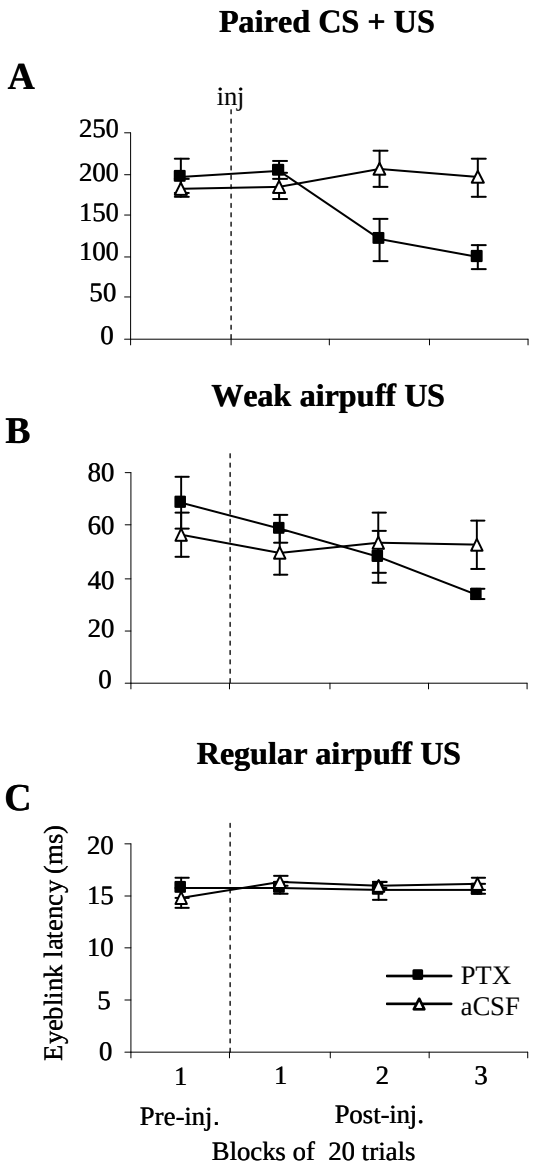

Fig. 7. Effects of PTX (squares) injections (inj.) on the means (± SE, n = 4) for CR and UR latency. A: effect of PTX on eyeblink latency in paired CS + US trials. PTX reduced CR latency. B: effect of PTX injections to the IN on UR latency during weak airpuff trials. The UR latency gradually decreased when compared to the pre-injection level. C: PTX had no effect on UR latency during

regular intensity airpuff trials. Injections of aCSF (triangles) had no effect on eyeblink latency during either type of trial.

3.3.7 URs to the strong airpuff and to the light

While PTX did not significantly affect UR amplitude to the strong airpuff (**Fig. 8E**), the profile and peak of these eyeblinks were affected. The mean peak of URs to the strong airpuff increased from 54.33 ± 11.74 % pre-injection to 91.34 ± 3.84 % during the third block of post-injection trials. (**Fig. 8F**, $F_{3,9} = 5.61$, $p = 0.019$). The maximum instantaneous velocity of eyelid closure during eyeblinks to the strong airpuff nearly doubled from 0.99 ± 0.26 % per ms pre-injection to 1.93 ± 0.23 % per ms during the third block of post-injection trials (**Fig. 9D**, $F_{3,9} = 4.49$, $p = 0.035$). Besides its effect on eyelid closure velocity, PTX also delayed eye re-opening which was manifested as greater eyelid closure still present at the end of the 1400-ms recording period. Delayed eye re-opening was apparent both in raw and normalized eyeblink averages (**Figs. 9A, 9C**). At the end of the 1400 ms recording period, raw eyeblinks had increased from 9.44 ± 1.30 % in pre-injection to 53.28 ± 12.76 % during the third block of post-injection trials (**Fig. 9A**, $F_{3,9} = 9.65$, $p = 0.00036$). Normalized responses showed the same tendency (**Fig. 9C**). Injections of vehicle had no effect on the peak, profile, or velocity of URs to the strong airpuff (**Figs. 8E-F, 9B**). Furthermore, PTX did not significantly affect URs to light.

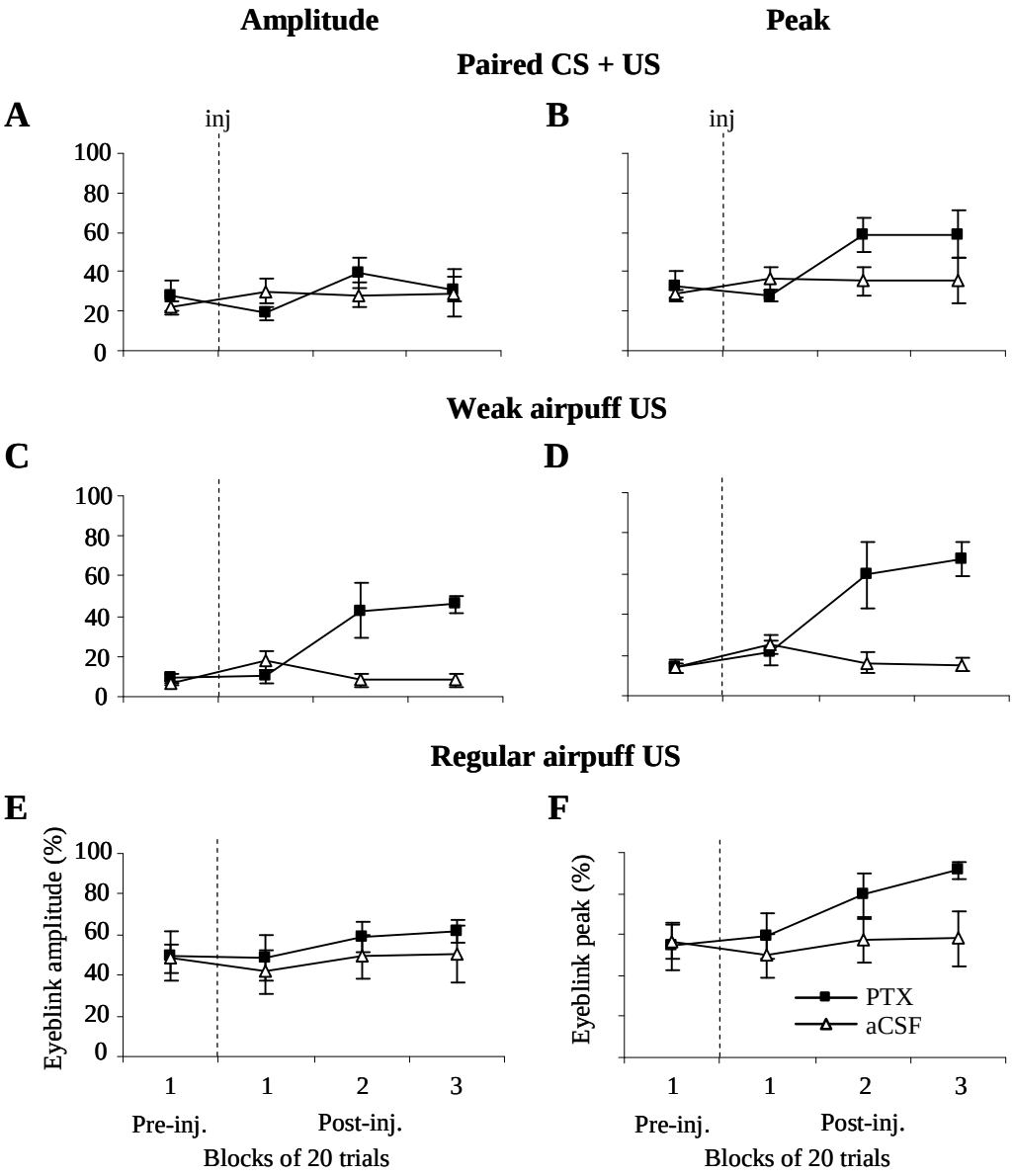

Fig. 8. Effects of PTX (squares) on the means (± SE, n = 4) of amplitudes (left column) and peaks (right column) of CRs and airpuff-evoked URs. A: PTX injections (inj.) did not significantly affect CR amplitude in CS + US trials. B: PTX injections increased the CR peak in CS + US trials. C: the amplitude of responses to the weak airpuff US increased following PTX injections. D: the peak of responses to the weak airpuff US also increased following PTX

injections. E: the amplitude of regular airpuff-evoked URs was not affected by PTX. F: the peak of regular airpuff URs slightly increased following PTX. Neither peaks nor amplitudes of responses in all three conditions were affected by control injections of aCSF (A-F).

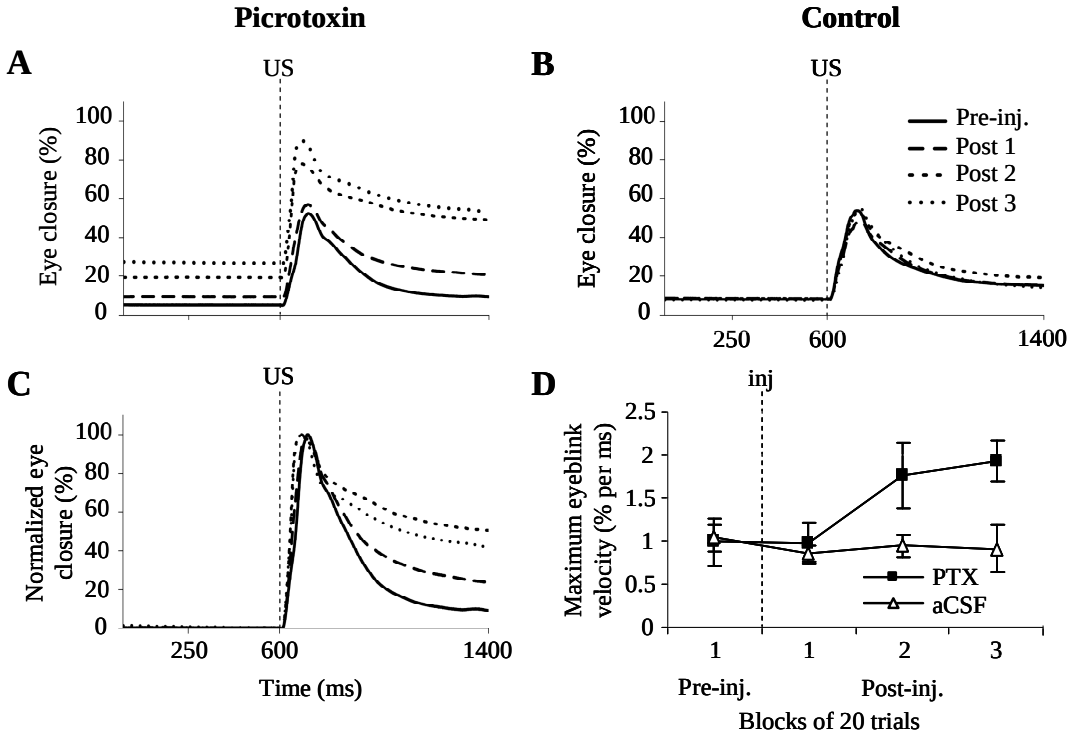

Fig. 9. Effects of PTX on the profile and instantaneous velocity of URs evoked by strong airpuff. A: average eyeblinks (n = 4) to the strong airpuff in one block of trials before (Pre-inj.) and three blocks of trials following (Post 1-3) PTX injections. PTX increased tonic eyelid closure seen as increased signal levels before the US onset. Also, eyelid re-opening was delayed in post-injection trials as indicated by the higher signal at the end of the recording period. C: eyeblinks from (A) were normalized to compare their time profiles. This analysis revealed a

slight decrease in UR peak time as well as a clear delay in post-blink eyelid re-opening in post-injection trials. B: average eyeblinks to the strong airpuff before and after control injections of aCSF. Injections of vehicle had no effect on the shape and velocity of strong airpuff-evoked eyeblinks. D: maximum velocity of URs to the strong airpuff (mean ± SE, n = 4). PTX significantly increased the maximum instantaneous velocity of strong airpuff URs.

3.4 Discussion

The present study demonstrated that blocking $GABA_A$ neurotransmission in the IN with PTX or GZ affects CRs in a dose-dependent manner. While high doses of $GABA_A$ blockers suppressed CR expression, lower drug doses shortened CR latency. Besides their effects on CRs, the SLR-inducing doses of PTX also affected non-associative components of eyelid movements; they increased tonic eyelid closure and facilitated URs evoked by trigeminal stimulation.

3.4.1 Effects on CR expression

We hypothesized that prior variance in results of blocking $GABA_A$ receptor-mediated neurotransmission in the IN on CR expression could be related to the extent of the $GABA_A$ block. Our data presented here confirm this notion. High amounts of PTX and GZ suppressed CRs. This finding confirmed previous reports of CR abolition (Mamounas et al., 1987; Attwell et al., 2002; Aksenov et al., 2004). On the other hand, lower amounts of both drugs, when administered at sites where high doses abolished CRs, had a minor effect on CR incidence, but significantly shortened their latency. This

supports previous reports of SLRs induced by cerebellar nuclear injections of PTX (Garcia and Mauk, 1998; Medina et al., 2001) or GZ (Ohyama et al., 2006). Furthermore, the amount of PTX and GZ required to produce SLRs varied among individual animals. Although all injection sites in this study were located in the IN region (**Fig. 1**), and their proximity to eyeblink-related parts of cerebellar nuclei was functionally confirmed with muscimol injections abolishing CRs, effective doses of $GABA_A$ blockers had to be titrated to optimize effects. In general, smaller amounts of drugs were required for SLRs and CR abolition at sites with the best muscimol effects, suggesting a dependency on the amount of drug diffusing around the eyeblink representation in deep cerebellar nuclei.

Differences between the effects of high and low drug doses could result from drug spreading to the overlying cerebellar cortex. Blocking $GABA_A$ neurotransmission in the cerebellar cortex increases the tonic firing rate of GABAergic Purkinje cells (Thomsen et al., 2004). Assuming the high drug dose did diffuse into the non-targeted cerebellar cortex, increased Purkinje cell activity would inhibit the IN and abolish CRs. However, this effect would be prevented by the simultaneous suppression of $GABA_A$ neurotransmission in the targeted deep cerebellar nuclei. Thus, conjectured drug diffusion to the cerebellar cortex does not explain CR abolition by high doses of $GABA_A$ blockers.

In agreement with previous reports (Medina et al., 2001), SLR-inducing doses of both blockers reduced CR latency and changed the temporal profile of CRs, which frequently peaked before the US onset (**Figs. 2A, 3A**). GZ reduced CR amplitude (**Figs. 2, 5**) when measured relative to the eyelid position before application of the CS. Effects

of PTX on CR amplitude were less pronounced, ranging from a small decrease in Experiment #1 (**Fig. 5**) to no change in Experiment #2 (**Fig. 8**). On the other hand, CR peaks measured relative to the maximally open eye, increased following both drugs and in both experiments (**Figs. 5, 8**). These seemingly contradictory effects were due to a drug-induced tonic eyelid closure. Following drug injections, CRs were evoked on a background of tonic eyelid closure and the same or smaller blinks resulted in absolute eyelid closure larger than baseline blinks recorded before injections or those recorded after control.

3.4.2 Effects on non-associative components of blinking

High and low doses of PTX and low-dose GZ elicited sustained tonic eyelid closure during which animals maintained partially closed eyelids both during and between trials. This finding confirms and extends our previous reports of PTX effects on tonic eyelid position in instrumental and classical conditioning tasks in the rabbit (Bracha et al., 2001; Aksenov et al., 2004). In our study of instrumental eyelid behavior we found that inactivating the IN with the $GABA_A$ agonist, muscimol, disrupts instrumentally conditioned tonic eyelid closure (Bracha et al., 2001). Since down-regulating the neuronal firing rate in the IN with muscimol produces tonic eyelid opening, whereas increasing the IN neuronal activity with $GABA_A$ antagonists increases tonic eyelid closure, it appears that tonic IN activity controls tonic eyelid aperture. In this regard, it was surprising that high doses of GZ produced only transient tonic eyelid closure, followed by eyelid opening at later stages of the experiment. Pertinent to this finding, in earlier work we observed that injecting the IN with the $GABA_A$ antagonist bicuculline at

low concentrations increased the tonic activity of IN neurons and at high concentrations evoked bursting followed by long periods of inactivity (Bayev and Bracha, unpublished observations). If GZ has similar properties, then low doses would increase the tonic activity of IN neurons (Chen and Evinger, 2006) and enhance tonic eyelid closure. On the other hand, high doses would reduce the IN spontaneous firing rate, leading to eyelid opening and CR abolition.

To determine whether SLR-inducing doses of PTX affect URs, rabbits were presented with trigeminal and visual stimuli. Analyses of latencies, amplitudes, and velocity profiles showed no effect on photic URs. In contrast, PTX facilitated URs evoked by airpuffs. This was most pronounced in weak airpuff trials, where PTX shortened UR latencies and increased UR amplitudes. In strong airpuff trials, PTX increased the maximum instantaneous velocity of eyelid closure and delayed eyelid re-opening following the blink. These findings corroborate observations of GZ effects on URs in anesthetized rats (Chen and Evinger, 2006) and complement reports of opposite effects of IN lesions and inactivations in the rabbit (Welsh and Harvey, 1989; Welsh, 1992; Bracha et al., 1994). Here we have shown that SLR-inducing levels of $GABA_A$ neurotransmission affect non-associative eyelid movements. These data collectively demonstrate IN involvement in the control of tonic eyelid closure and trigeminal stimulation-evoked URs.

3.4.3 Implications for cerebellar control of eyeblinks

The present findings illuminate the controversy about PTX's effect on CR expression (Garcia and Mauk, 1998; Attwell et al., 2002). We have shown that blocking

$GABA_A$ neurotransmission affects CRs in a dose-dependent manner. Blocking either $GABA_A$ receptors or chloride channels with low drug doses induces SLRs. On the other hand, administering higher drug doses at the same injection sites abolishes CRs. In their original reports, Mauk and colleagues suggested that SLRs are evoked when GABAergic Purkinje cells are functionally disconnected from eyeblink representation in the deep cerebellar nuclei (Garcia and Mauk, 1998; Medina et al., 2001). Our results do not support this notion. We propose that during SLRs, cerebellar cortical projections are not disconnected completely because increasing the drug dose further aggravates behavioral effects, resulting in CR abolition.

An important contribution of the present study is showing that SLR-inducing injections of PTX affect non-associative components of blinking. This confirms previous suggestions that the intermediate cerebellum controls both classically conditioned and unconditioned eyeblink reflexes (Welsh and Harvey, 1989; Bloedel and Bracha, 1995; Delgado-Garcia and Gruart, 2006; Chen and Evinger, 2006). It is known that neurons in the interposed nuclei respond to both the tone CS and trigeminal US (Berthier and Moore, 1990; Aksenov et al., 2004; Jimenez-Diaz et al., 2004; Chen and Evinger, 2006). Consequently, it is possible that effects of PTX and GZ could be related to changes of IN task-related signals. However, the effects of IN pharmacological manipulations are also very likely related to changes in tonic IN activity. It is paramount to note that PTX and GZ dramatically enhance the spontaneous firing rate of IN neurons (Aksenov et al., 2004; Chen and Evinger, 2006). These IN neurons then send excitatory projections to the red nucleus and other mesencephalic eyeblink-related targets. In addition, neurons in the red nucleus receive CS and US information (Desmond and Moore, 1991) and project to

sensory trigeminal (Davis and Dostrovsky, 1986; Godefroy et al., 1998) and facial nuclei (Holstege and Tan, 1988). Thus, it is possible that the elevated spontaneous IN activity increases excitability of extra-cerebellar eyeblink pre-motoneurons and this could affect CR and UR performance in a manner unrelated to learning. Since PTX and GZ affect both neuronal modulation and spontaneous activity simultaneously, dissociating contributions of these two processes to CR and UR performance is difficult and the present study cannot resolve this question (for review, see Bracha et al., 2008).

The most plausible explanation of the effects of low doses of PTX and GZ is that they partially block the inhibitory drive of Purkinje cells and IN GABAergic interneurons. This enhances the spontaneous firing rate of IN neurons, and reduces their depth of modulation (Aksenov et al., 2004; Chen and Evinger, 2006). The elevated IN firing in turn increases the activity of eyeblink pre-motoneurons and motoneurons, and modulates transmission of sensory information in the sensory trigeminal system. The high spontaneous firing within eyeblink circuits enhances tonic eyelid closure. Importantly, this tonic effect on eyelid position is context-dependent, because removing the animal from the restraining box restores normal eyelid aperture. This suggests a so far unknown and context-dependent gating mechanism that can cancel the influence of the high IN firing rate on pre-motoneurons. The reduced modulation of IN neurons is transmitted to mesencephalic pre-motoneurons, which themselves are now more excitable and respond more vigorously to IN signals as well as to direct CS and US inputs. The collective changes both inside and outside of the cerebellum are then responsible for facilitating responses to the CS and trigeminal US. Notably, the described facilitation of eyeblink circuits does not affect optic URs. This indicates that CR / UR facilitation is not

due to increased excitability of motoneurons, because this process would affect blinks to all modalities.

In our previous study we have shown that large doses of PTX dramatically increase IN firing rates and suppress neuronal responses to the CS and US (Aksenov et al., 2004). It is likely that this over-excitation of IN neurons, together with the associated high excitability in their efferent targets, saturate the circuit's capacity to respond to the CS and this suppresses CRs on the background of pronounced eyelid closure. As addressed above, the mechanism of high GZ doses is different – it appears to suppress IN activity. The resulting suppression of cerebellar task-related signals and the decreased excitatory drive to eyeblink pre-motoneurons counter-balance tonic eyelid closure and suppresses CRs.

3.5 Material and Methods

3.5.1 Subjects

The experiments were performed on 10 male New Zealand White Rabbits (Harlan; Indianapolis, IN) weighing 2.5-3.0 kg (3-4 months old at time of surgery). Rabbits were housed individually on a 12-hour light/dark cycle and provided food and water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health's "Principles of Laboratory Animal Care" (publication No. 86-23, revised 1985), the American Physiological Society's "Guiding Principles in the Care and Use of Animals," and the protocol approved by Iowa State University's Committee on Animal Care.

3.5.2 Surgery

Using aseptic techniques, surgery was performed on naive rabbits anesthetized with a mixture of ketamine (50 mg/kg), xylazine (6 mg/kg) and acepromazine (1.5 mg/kg). The head was secured in a stereotaxic apparatus with lambda positioned 1.5 mm ventral to bregma. A stainless steel injection guide tube (28-gauge thin-wall tubing) was stereotaxically implanted 0.5 mm dorsal to the expected location of the left anterior IN ((0.69x + 4.8) - x mm rostral from lambda, x being the horizontal distance between bregma and lambda in mm: 5.3 mm lateral and 13.5 mm ventral to lambda). A 33-gauge stainless steel stylet was inserted into the guide tube in-between experiments to protect its patency. The guide tube, anchor screws, and a small Delrin block designed to accommodate an airpuff delivery nozzle and eyeblink sensor were secured in place with dental acrylic. All animals were treated with antibiotics for 5 days during recovery from surgery.

3.5.3 Training procedures

Following recovery from surgery, rabbits were adapted to a restraint box in three daily 30-minute sessions. Adapted rabbits were trained in the standard classical conditioning paradigm until they reached at least 90 % CRs for 3 consecutive days. The conditioned stimulus (CS) was an 85-db, 450-ms, 1-kHz tone, super-imposed on a continuous 70-db white noise background. The CS co-terminated with a 40-psi, 100-ms airpuff unconditioned stimulus (US) directed to the left eye. The inter-stimulus interval was 350 ms and each training session consisted of 100 trials presented in pseudorandom,

15-25 sec inter-trial intervals. All experiments were conducted in a sound-attenuated chamber.

Animals tested in the UR performance experiments (Experiment #2) were adapted to a mixed paradigm following training. The paired presentation of the CS + US was alternated with three different types of US in Experiment #2: a normal airpuff US, a weak airpuff US (100 ms, 4-5 psi at the source), and a photic US (30 ms flash of four white LEDs positioned in front of the left eye; light intensity was dimmed to only elicit near-threshold URs). This mixed-stimulation paradigm consisted of repeated blocks of 10 trials: 4 paired CS + US, 2 normal US, 2 weak airpuff US, and 2 light US trials were pseudorandomly intermixed.

3.5.4 Injection procedures

Injections were delivered via a 33-gauge stainless steel injection needle which was connected via transparent Tygon tubing to a 10-µL Hamilton syringe. The injection tubing was first filled with nanopure water, and then a small bubble was drawn into the end of the injection needle before drawing in drug. The bubble was used for monitoring the injected volume relative to gradation marks on the tubing. The injection needle was inserted in the guide tube prior to beginning the experiment. A pre-injection period of 40 trials (or 50 trials in Experiment #2) was presented to rule out needle insertion effects and to assess baseline eyeblink performance. Following the pre-injection period, drug micro-injections were manually administered at a rate of 0.5 µL/min. To assess the drug effect, training continued for 60-150 additional trials.

The present study had two objectives. In the first group of rabbits (n = 6), CR performance was examined following injection of two GABA antagonists, picrotoxin (PTX, chloride channel blocker; Sigma-Aldrich, USA) and gabazine (GZ, $GABA_A$ receptor antagonist; Ascent Scientific, Weston-super-Mare, UK). Of the 6 animals, 3 were used in the PTX group and 3 were used in the GZ group. In preliminary experiments we found that effects of both drugs were dose-dependent besides being animal and injection site-dependent. For this reason, effective injection sites and drug doses in each animal were determined. The starting doses for PTX and GZ were 0.62 nmol and 0.51 nmol, respectively. If CRs were abolished after the injection, this drug concentration was considered the 'high-dose,' and the drug dose was progressively decreased on consecutive days until SLRs were observed (the 'low-dose' for the drug) or until no drug effect was detected. If no effect on CR performance was found following the initial drug injection, the drug dose was progressively increased until SLRs (the 'low-dose') and CR abolition (the 'high-dose') were detected. Only one drug was injected on any given experimentation day. Both GZ and PTX were dissolved in artificial cerebrospinal fluid and their pH was adjusted to 7.4 ± 0.1. All injections of PTX and GZ were performed at CR expression-related deep cerebellar nuclear sites where 0.5 µL of muscimol (1.75 nmol) completely suppressed conditioned eyeblinks (Bracha et al., 1994).

In the second group of animals (n = 4), the parallel effects of PTX on CR and UR expression were examined (Experiment #2). In this group of rabbits, PTX was injected in 0.5-µL (0.3 nmol) increments beginning immediately following 50 pre-injection trials. These injections were administered every 20 trials until SLRs were observed or until 2.5 µL of PTX had been cumulatively administered. In control experiments for both

Experiment #1 and Experiment #2, an equal volume of drug vehicle (aCSF) was injected using the same injection protocol.

3.5.5 Data recording and analysis

Rabbit behavior was monitored using an infrared video system installed in the experiment chamber. Eyelid movements were recorded by a frequency-modulated infrared sensor that measures infrared light reflected from the eye and peri-orbital region (Ryan et al., 2006). The sensor, attached to an aluminum stage, was secured to the Delrin block on the rabbit's head before every experiment. The output of the sensor was amplified, digitized (25 kHz, 12-bit A/D converter), and stored in a PC-based data acquisition system. During each trial, 1400 ms of the signal was recorded, beginning with 250 ms of baseline before the CS onset and extending for 800 ms beyond the US onset.

Eyeblink responses from each trial were examined off-line for the presence of CRs within the time window between CS and US onsets and for the presence of URs in US-alone trials. The threshold for eyeblink detection was set to 5 standard deviations of the baseline signal noise, which in the present setup corresponded to an approximately 0.15 mm decrease in eyelid aperture. The following response parameters were measured in each trial: baseline eyelid aperture, response latency, response amplitude and response peak. Response amplitude was defined as the difference between the baseline eyelid aperture and the maximum eyelid closure in the corresponding response time window for each trial. Response peak was calculated as the difference between the experiment-wide maximum eyelid aperture (openness) within every injection experiment (the signal value

corresponding to completely open eyelids) and the maximum eyelid closure in the corresponding response time window for each trial. All amplitudes were first measured in A/D units of the recording system. Typical eyeblinks in rabbits consist of eyelid closure and subsequent folding of external eyelids. Both of these response components were detected by our IR sensor (Ryan et al., 2006). The native amplitude measurements were normalized by converting them to a percentage of maximum eyeblink, assuming that the difference between minimum and maximum sensor signals in a particular experiment captures the eye both maximally open and closed. Means of eyeblink measures were calculated for consecutive blocks of 10 trials in Experiment #1. In Experiment #2, means of eyeblink measures were calculated for blocks of trials as follows: 20 paired CS + US, 10 light, 10 weak airpuff, and 10 strong airpuff trials, which were all randomly presented during blocks of 50 trials. In addition, instantaneous velocities were calculated in a sliding window of 20 msec as the first derivative of rise-to-peak velocities of URs to light and to the strong airpuff in Experiment #2. To compare time profiles of URs to the light and strong airpuff, response averages were normalized by expressing them as a percentage of their amplitude. We tested unique hypotheses about dose dependence by conducting separate repeated measures ANOVAs for PTX and GZ at each dose (high concentration: abolition-inducing, and low concentration: SLR-inducing). Response variables, divided into blocks of 20 trials as the within-subject repeated measures, were modeled against a two-factor treatment (drug vs vehicle) together with subject as a blocking factor. Reported F-ratios and their p-values refer to main effects only when there was no significant interaction between treatment and blocks-of-trials. All group data were reported as mean ± standard error of mean, and

significance was declared by an alpha level = 0.05. All statistical analyses were performed using Statsoft Statistica software.

3.5.6 Histology

Upon the conclusion of experimentation, rabbits were deeply anesthetized with a cocktail of ketamine (100 mg/kg), xylazine (12 mg/kg), and acepromazine (3 mg/kg). Injection sites were marked by injecting 1 µL of tissue-marking dye. Animals were perfused transcardially with 1 L of a phosphate-buffered saline followed by 1 L of a tissue fixative (10 % buffered formalin). Carefully excised brains were stored in a solution of 30 % sucrose and 10 % formalin and subsequently sectioned coronally at 50 µm on a freezing microtome. The sections were mounted onto gelatin-coated slides, and once dry, stained with luxol blue and neutral red. Using bright light microscopy, injection locations were determined and plotted on standard sections of the rabbit cerebellum.

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CHAPTER 4: INACTIVATING THE MIDDLE CEREBELLAR PEDUNCLE ABOLISHES THE EXPRESSION OF SHORT LATENCY CONDITIONED EYEBLINKS.

A paper submitted to the journal *"Brain Research"*[1]

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4.1 Abstract

The interposed nuclei (IN) of the cerebellum play a crucial role in the classically conditioned eyeblink circuit. It has previously been shown in well trained animals that injecting the IN with $GABA_A$ antagonists produces short latency conditioned responses (SLRs). The mechanism underlying SLR generation is not clear. According to one concept, SLRs originate in cerebellar nuclei in response to direct inputs from collaterals of mossy fibers. An alternate explanation is that SLRs are produced by extra-cerebellar circuits that are excited by increased tonic activity in cerebellar nuclei or by the combined action of inputs to cerebellar nuclei from mossy fiber collaterals and incompletely blocked Purkinje cells. In the present study, we examined whether cerebellar afferent axons in the middle cerebellar peduncle (MCP) participate in SLR expression. We hypothesized that if SLRs are evoked by the sensory mossy fiber input to the IN and cerebellar cortex, then blocking the MCP should abolish these responses. Well trained

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animals, which had been implanted with dual injection cannulae in the left IN and the left MCP, were injected with GZ into the IN to produce SLRs followed by an injection of the sodium channel blocker TTX into the MCP. TTX infusions in the MCP suppressed both CRs and SLRs. These findings suggest that the expression of SLRs depends on both direct and cerebellar cortex-mediated sensory information from the mossy fiber system.

4.2 Introduction

The cerebellar interposed nuclei (IN) are a pivotal component in the eyeblink conditioning circuit. The IN receives two major inputs. The first is a massive GABAergic projection from Purkinje cells of the cerebellar cortex (Ito, 1984). The second and appreciably weaker input is from presumably glutamatergic collaterals of cerebellar afferent mossy and climbing fibers (Cicirata et al., 2005; Voogd, 1995). Important insights into the operation of eyeblink conditioning networks were gained in studies manipulating the GABAergic IN input. It has been shown that activating cerebellar cortical projections to the cerebellar nuclei by injecting $GABA_A$ agonists into the IN prevents the acquisition and expression of eyeblink conditioned responses (CRs) (Krupa et al., 1993; Bracha et al., 1994; Hardiman et al., 1996). On the other hand, blocking $GABA_A$ neurotransmission in the IN dramatically increases the spontaneous firing rate of IN neurons and suppresses their task-related responses (Aksenov et al., 2004). This physiological effect is associated with the abolition of CRs (Mamounas et al., 1987; Attwell et al., 2002; Aksenov et al., 2004). An incomplete disruption of $GABA_A$ neurotransmission elevates the IN neuronal firing rate, but it does not eliminate task-related neuronal responses (Aksenov et al., 2004). This physiological state alters CR

expression by shortening their latencies and by modifying their temporal profile (Garcia and Mauk, 1998). The neurophysiological mechanisms of these short-latency CRs (SLRs) are not clear. According to one concept, SLRs could be triggered by direct inputs from collaterals of mossy fibers to cerebellar nuclei. Ohyama and colleagues (2006) examined this proposal by blocking direct mossy fiber inputs by infusing the IN of rabbits generating SLRs with blockers of fast glutamate receptors. This treatment reduced the amplitude of SLRs, but it did not eliminate CRs. The persistence of CRs following blocks of glutamate- and GABA-mediated IN inputs in these experiments seems to suggest that the residual CRs were generated by extra-cerebellar circuits. This suggestion, however, is inconclusive because the SLR-inducing $GABA_A$ blockade is most likely incomplete (Aksenov et al., 2004; Parker et al., 2009) and therefore conditioned stimulus (CS) signals could still enter the IN via the cerebellar cortex.

In the present study, we examined the cerebellar dependency of SLRs by blocking both direct CS input to the IN and CS input to the cerebellar cortex. This was achieved by inactivating the middle cerebellar peduncle (MCP). The MCP is a fiber tract originating in the pontine nuclei and it relays information encoding the CS to the cerebellum. The MCP projects directly to the IN via mossy fiber collaterals, but its main target is the cerebellar cortex which processes the CS and the unconditioned stimulus (US) information and conveys the results to the IN. We hypothesized that if SLRs are driven by cerebellum-mediated CS signals, then blocking the MCP should abolish these responses. Well trained animals, which had been implanted with dual-injection cannulae in the left IN and the left MCP, were injected with gabazine (GZ) into the IN to produce SLRs, and subsequently, tetrodotoxin (TTX) was injected into the MCP to block mossy

fiber inputs to the cerebellum. Here we report that blocking the MCP prevented the expression of both CRs and SLRs.

4.3 Results

4.3.1 General Observations

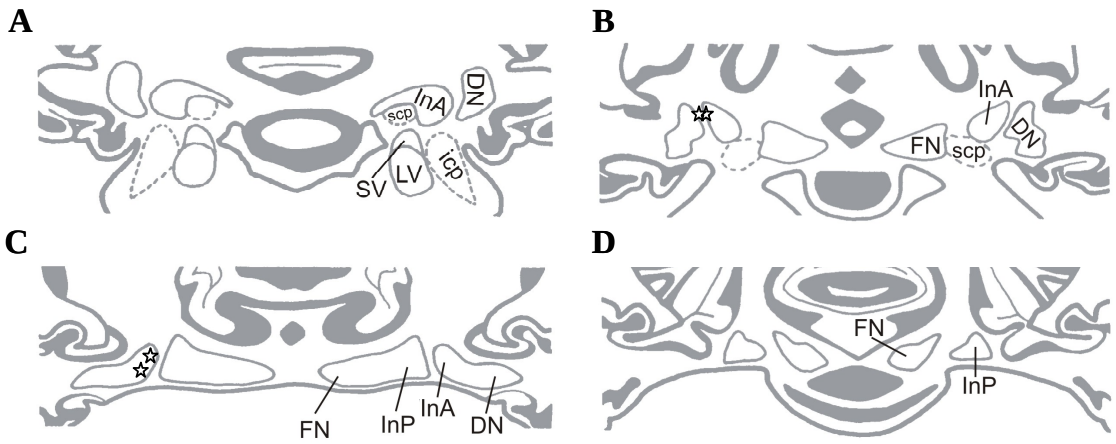

Fig. 1. Reconstruction of injection sites in the IN for rabbits injected with GZ (stars). A-D: 4 rostral-caudal cerebellar sections spaced 0.5-mm apart. All injection sites were located directly in the anterior IN or in close proximity to the anterior interposed/denate nuclear border. InA, anterior interposed nucleus; DN, dentate nucleus; LV, lateral vestibular nucleus; SV, superior vestibular nucleus; InP, posterior interposed nucleus; FN, fastigial nucleus; scp, superior cerebellar peduncle; icp, inferior cerebellar peduncle.

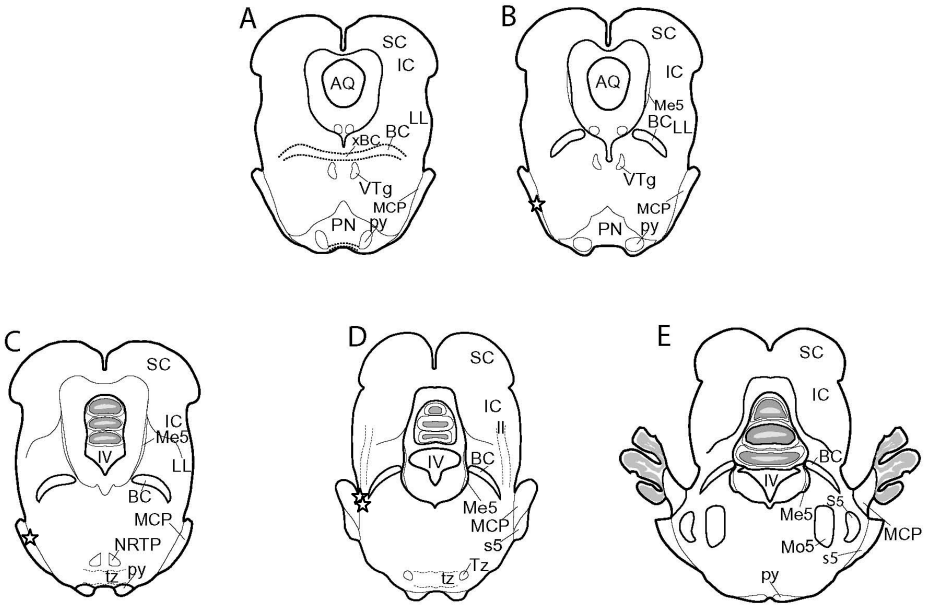

Fig. 2. Reconstruction of injection sites in the MCP for rabbits injected with TTX (stars). A-D: 4 rostral-caudal cerebellar sections spaced 0.5-mm apart. IV, fourth ventricle; BC, brachium conjunctivum; IC, inferior colliculus; ll, lateral lemniscus; Mo5, motor trigeminal nucleus; MCP, middle cerebellar peduncle; Me5, mesencephalic trigeminal nucleus; NRTP, pontine tegmental reticular nucleus; PN, pontine nuclei; py, pyramidal tract; S5, sensory trigeminal nucleus; s5, root of the sensory trigeminal nerve; SC, superior colliculus; tz, trapezoid body; Tz, nucleus of the trapezoid body; VTg, ventral tegmental nucleus; xBC, decussation of the brachium conjunctivum.

Injections of GZ in the IN shortened CR latencies. In the absence of this GZ action, administering TTX in the MCP suppressed CR expression. Injections of TTX in the MCP of animals exhibiting GZ-induced SLRs suppressed all responses to the CS. Similar to our previous observations (Parker et al., 2009), GZ injections also increased

tonic eyelid closure. All of these effects were observed at injection sites located directly at or in the near vicinity of the left anterior IN (**Fig. 1**) and the left MCP (**Fig. 2**).

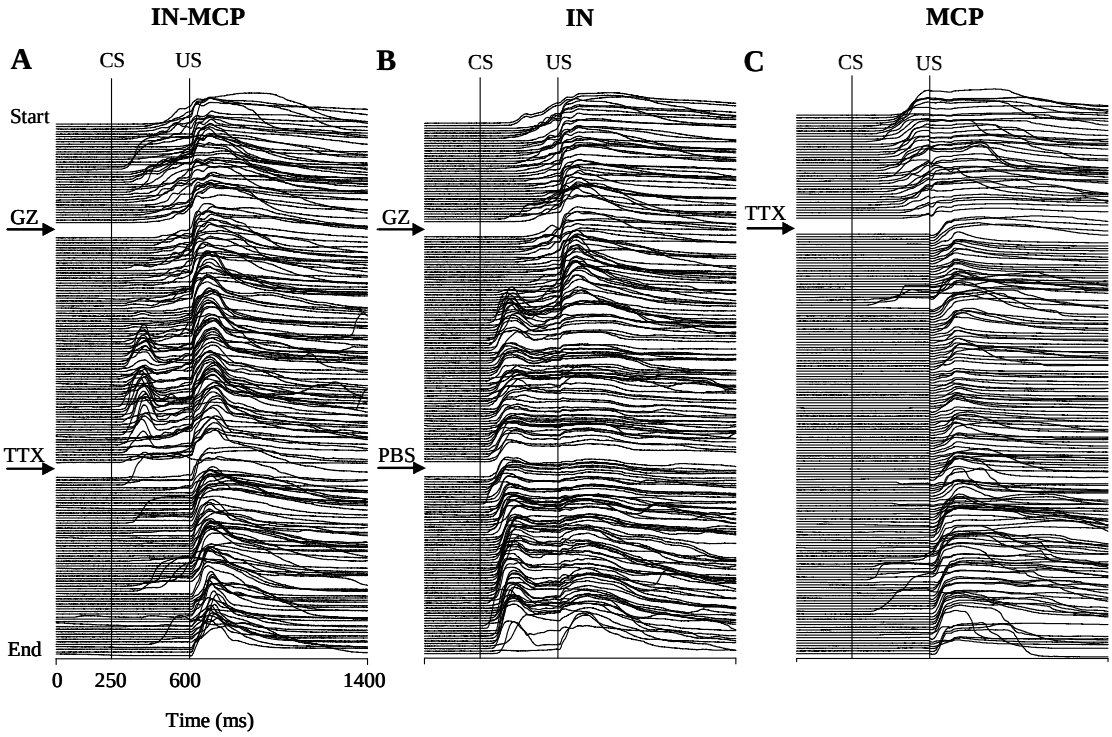

Fig. 3. Individual examples of combined GZ-TTX and individual GZ and TTX effects on CR expression. All three examples were recorded from the same animal. A: example of effects of combined injections of GZ to the IN and TTX to the MCP on CR expression. Following GZ, CR latency was shortened and the resulting SLRs were suppressed after injecting TTX. The experiment begins at the top. Each plot in the stack plot represents one trial and positive deflections in each trace correspond to eyelid closure. B: a stack plot of eyeblink records in an experiment where GZ was injected into IN and a control injection of PBS was administered to the MCP. The MCP vehicle injection had no effect on GZ-

induced SLRs. C: a stack plot of eyeblink records in an experiment where TTX was injected in the MCP, producing long-lasting abolition of CRs.

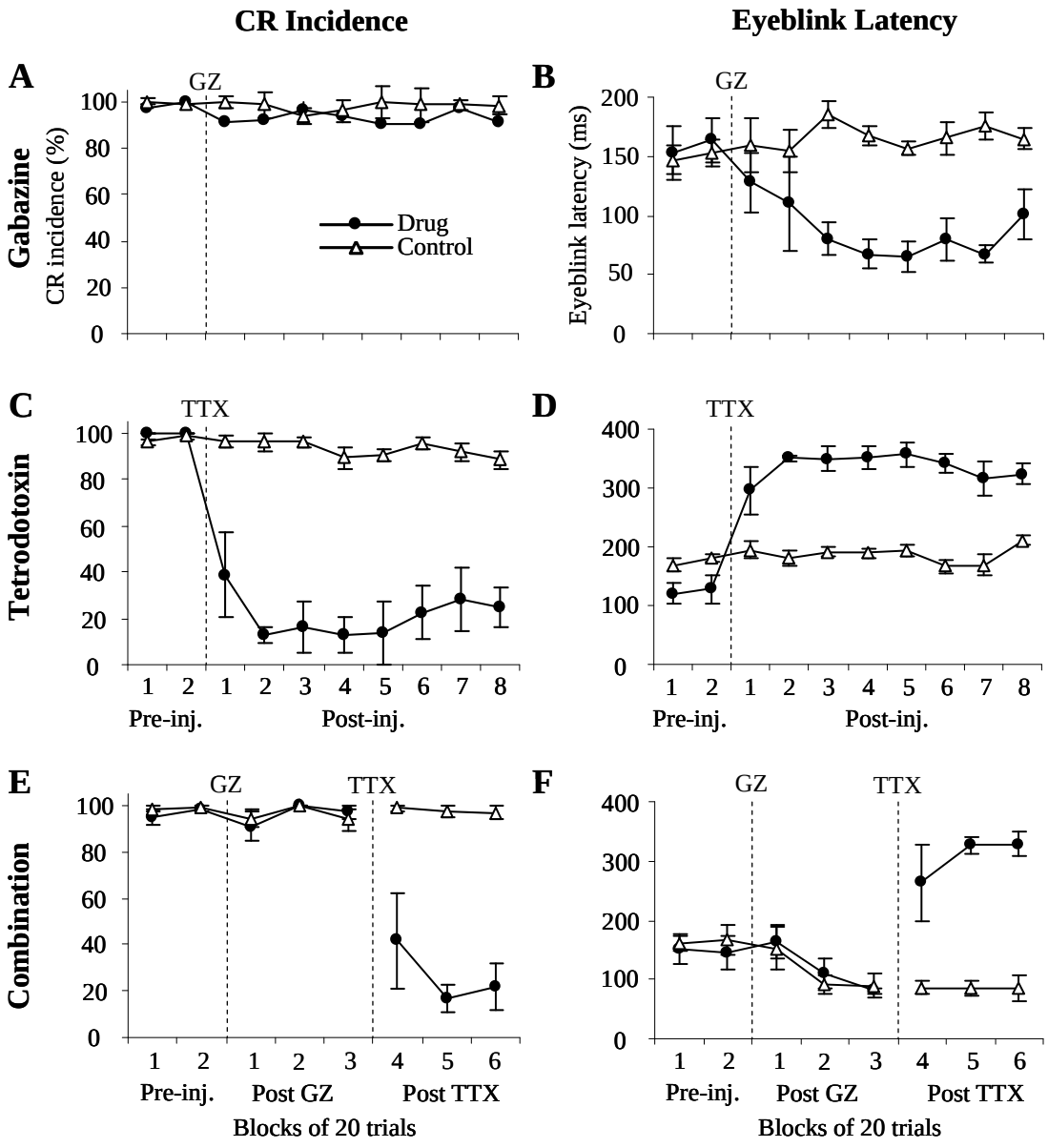

Fig. 4. Group effects of GZ, TTX, and combined GZ+TTX injections on CR incidence and eyeblink latency. All data points represent group means ± SEM (n = 4). A: effect of GZ on CR incidence. Neither GZ (circles) nor vehicle (triangles) injections affected CR incidence. B: effect of GZ on eyeblink latency.

Following GZ injections into IN, CR latency dramatically decreased. C: effect of TTX on CR incidence. Injections of TTX into MCP area suppressed CR expression. D: effect of TTX on eyeblink latency. Coupled with the decreased CR incidence (C), TTX in the MCP increased eyeblink latency. E: effect of TTX on incidence of CRs facilitated by prior administration of GZ into IN. TTX clearly suppressed GZ-evoked SLRs, similar to that shown in plot B with normal CRs. F: effect of TTX on SLR latency. As a consequence of SLR suppression, the latency of the responses increased following TTX injections. Pre-inj. – pre-injection blocks of trials; Post-inj. – post-injection blocks of trials.

4.3.2 Effect of Gabazine on CR expression

Injections of GZ in the IN were administered in well trained animals at the location determined during the mapping phase of the experiment. The effective dose of GZ differed among animals, varying from 0.125 – 4.11 nmol, and it was carefully chosen to shorten CR latency (**Fig. 3A-B**). On the other hand, larger doses of GZ abolished CRs (not shown). When compared to control injections of aCSF, infusions of SLR-inducing doses of GZ significantly shortened CR latency (**Fig. 4B**, $F_{9,27} = 4.17$, $p = 0.0019$) without affecting CR incidence in all four animals (**Fig. 4A**). The onset of GZ effects on CR latency varied between rabbits, and at the group level this was manifested as a gradual decline in CR latency during the first 3 blocks of the post-GZ period (**Fig. 4B**). Prior to the injection, CR latency was 158.23 ± 13.87 ms, becoming shortest in post-injection block 5 with an average of 65.47 ± 13.08 ms.

4.3.3 Effect of TTX on CR expression

The MCP is a pathway that is understood to supply the cerebellum with the sensory CS signal. Consistent with this concept, inactivating the MCP with TTX severely suppressed CRs (**Fig. 3C**). Typically, TTX-injected animals ceased responding to the CS with an infrequent exception of isolated CRs or CS-triggered eyelid opening. When compared to control injections of PBS, micro-injections of TTX (3.13 – 9.39 pmol) significantly suppressed CR incidence in all four rabbits (**Fig. 4C**, $F_{9,27} = 6.85$, $p = 0.000001$). Prior to the injection, animals expressed 100 % CRs. Following TTX, CR incidence quickly declined to less than 15 % and despite a slight increase during post-injection blocks 6-8, it remained depressed throughout the experiment (**Fig. 4C**). As a consequence of CR suppression, eyeblink latency increased TTX injections (**Fig. 4D**, $F_{9,27} = 10.66$, $p = 0.000001$). Before the injection, the mean eyeblink latency was 124.67 ± 14.27 ms, and it lengthened to a mean above 350 ms for the majority of post-injection trials (**Fig. 4D**).

TTX is a sodium channel blocker and as such it can inactivate not only neuronal axons but also neuronal bodies. Consequently, it can not be excluded that besides its effects on the MCP, TTX could also inactivate nearby neurons potentially involved in CR expression. Although no known CR expression-related neurons are located in the vicinity of our MCP injection sites, to examine this possibility, one rabbit was injected with 3.5 nmol of muscimol at the site where TTX suppressed CRs. This treatment had no effect on CR incidence or latency.

4.3.4 Effect of TTX on SLR expression

The combined injections of GZ and TTX examined the involvement of the MCP in the expression of GZ-induced SLRs. All animals were injected first with GZ to induce SLRs and then with TTX to inactivate axons in the MCP. All drug injections in this experiment were administered at sites and with drug doses that were shown in prior experiments to induce SLRS (for GZ) and to abolish CRs (for TTX). As expected, GZ did not affect CR incidence but it did decrease eyeblink latency (**Figs. 3A, 4E-F**). Following TTX injections, mean CR incidence quickly decreased and remained suppressed through the end of the experiment (**Fig. 4E**, $F_{9,27} = 6.08$, $p = 0.00012$). In parallel to this effect, eyeblink latency significantly increased (**Fig. 4F**, $F_{9,27} = 7.35$, $p = 0.000044$). Control experiments consisted of GZ in the IN, but instead of TTX in the MCP, PBS was injected at the same time, depth, and volume as was TTX in each animal. In these experiments, GZ shortened CR latency as expected for the entirety of the experiment with no intervening effect of PBS on CR expression (**Fig. 3B, 4E**) or eyeblink latency (**Fig. 3B, 4F**).

4.4 Discussion

Our data demonstrated that injections of low doses of the $GABA_A$ antagonist GZ into the IN shorten the latencies of classically conditioned eyeblinks. Both SLRs and normal CRs were suppressed by inactivating the MCP. These findings suggest that normal sensory inputs to the cerebellum are essential for the expression of normal CRs and SLRs.

The intermediate cerebellum is involved in the acquisition and expression of conditioned eyeblinks. Current concepts suggest that the cerebellar cortex and deep cerebellar nuclei contribute to motor commands that generate CRs (Christian and Thompson, 2003; De Zeeuw and Yeo, 2005; Bracha et al., 2008). Since the intermediate cerebellar cortex and IN are serially connected and both receive CS and US information, the individual contribution of these two structures to CR generation have been difficult to establish. Garcia and Mauk (1998) proposed that the function of the IN could be uncovered by blocking the GABAergic input the IN receives from the cerebellar cortex. They reported that infusing the IN with the chloride channel blocker picrotoxin (PTX) evokes short-latency CRs, or SLRs. Ohyama and colleagues (2006) hypothesized that SLRs represent "nuclear CRs" that are evoked by direct sensory inputs to deep cerebellar nuclei. To test this hypothesis, they blocked collaterals of cerebellar mossy fibers with glutamate antagonists, resulting in suppressed SLR amplitude. Surprisingly, even though blocking the mossy fiber input in the IN suppressed the SLR amplitude, it did not eliminate small amplitude SLRs, and similar to previous reports (Attwell et al., 2002; Aksenov et al., 2005), it did not abolish normally timed components of CRs. These findings suggest that the residual CRs were mediated either by extra-cerebellar circuits or by incompletely blocked cerebellar cortical projections to the IN. The feasibility of the latter scenario was hinted to by observations showing that SLR-inducing doses of PTX or GZ do not completely block cortical projections to the IN (Aksenov et al., 2004; Parker et al., 2009).

Testing cerebellar cortical contributions to SLRs requires blocking CS sensory signals to both cerebellar nuclei and the cerebellar cortex. CS signals are conveyed to the

cerebellum from pontine nuclei via the middle cerebellar peduncle (Brodal and Jansen, 1946; Steinmetz et al., 1986; Steinmetz and Sengelaub, 1992; Hesslow et al., 1999). Consistent with this notion, we found here that inactivating the MCP with TTX severely suppressed normal CRs as well as SLRs, which are CRs facilitated by GZ application to the IN. Before drawing conclusions from these observations, several alternate explanations of observed results should be considered. First of all, since TTX can inactivate neurons, did TTX inactivate eyeblink expression-related neurons that are not involved in cerebellar information processing? This seems unlikely not only because no known group of such neurons resides in the expected radius of TTX spread (Nilaweera et al., 2006), but also because the control injection of muscimol performed in one subject had no effect on CRs. Another possibility is that TTX inactivated not only the MCP, but also some other fiber tract that is involved in CR expression. Indeed, injection sites in two rabbits were at places where spread of TTX to the superior cerebellar peduncle could not be excluded. However, injection sites in the other two animals were more rostral and ventral - at locations where drug diffusion to the brachium conjunctivum is unlikely. Another possibility is that TTX could have spread to the rubro-spinal tract which carries information from the red nucleus to eyeblink motoneurons. The rubro-spinal fibers, however, also appear to be located beyond the expected spread of TTX (Rosenfield et al., 1985).

In summary, our findings suggest that SLRs are not “nuclear CRs” unmasked by the missing input from the cerebellar cortex. Instead, SLRs seem to be CRs that are facilitated by an increased tonic firing of IN neurons (Aksenov et al., 2004) and that are at least partly driven by sensory signals and/or motor commands from incompletely

blocked cerebellar cortical projections. Consistent with this notion, a more complete block of $GABA_A$ neurotransmission in deep cerebellar nuclei abolishes all blinking to the CS (Attwell et al., 2002; Parker et al., 2009). Importantly, this conclusion assumes that inactivating the MCP does not significantly affect the tonic rate of IN neurons in a manner similar to the consequences of inactivating the inferior olivary cerebellar input (Zbarska et al., 2008). Our main conclusion also predicts that inactivating the MCP should suppress CS and CR-related neuronal activity in the GZ pre-treated IN. These important issues will be examined in future studies.

4.5 Methods

4.5.1 Subjects

Experiments were performed on 4 male New Zealand White Rabbits (Harlan; Indianapolis, IN) weighing 2.5-3.0 kg (3-4 months old at time of surgery). Rabbits were housed individually on a 12-hour light/dark cycle and provided food and water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health's "Principles of Laboratory Animal Care" (publication No. 86-23, revised 1985), the American Physiological Society's "Guiding Principles in the Care and Use of Animals," and the protocol approved by Iowa State University's Committee on Animal Care.

4.5.2 Surgery

Using aseptic techniques, surgery was performed on naive rabbits anesthetized with a mixture of ketamine (50 mg/kg), xylazine (6 mg/kg) and acepromazine (1.5

mg/kg). The head was secured in a stereotaxic apparatus with lambda positioned 1.5 mm ventral to bregma. Two stainless steel guiding tubes (28 gauge thin-wall, 1 mm apart) were stereotaxically implanted 0.5 mm dorsal to the expected location of the anterior IN ((0.69x + 4.8) - x mm rostral from lambda, x being the horizontal distance between bregma and lambda in mm; 5.3 mm lateral and 13.5 mm ventral to lambda). Targeting the MCP was accomplished using the following coordinates measured from bregma: 12.5 mm caudal, 4.0 mm lateral, and 17 mm ventral. To protect the patency of guide tubes, a 33 gauge stainless steel stylet was inserted into each guiding tube in-between experiments. The guide tube, skull anchor screws, and a small Delrin block designed to accommodate an airpuff delivery nozzle and eyeblink sensor were secured in place with dental acrylic. All animals were treated with antibiotics for 5 days during the recovery period following surgery.

4.5.3 Training procedures

Following recovery from surgery, rabbits were adapted to a restraint box for three 30 minutes/day sessions. Box-adapted rabbits were trained in the standard classical conditioning paradigm until they reached at least 90 % CRs for 3 consecutive days. The conditioned stimulus (CS) was an 85-db, 450-ms, 1-Hz tone, superimposed on a continuous 70-dB white noise background. The CS co-terminated with a 40-psi, 100-ms airpuff unconditioned stimulus (US) directed to the left eye. The inter-stimulus interval was 350 ms and each training session consisted of 100 trials presented in pseudorandom, 15-25 sec inter-trial intervals. All experiments were conducted in a sound-attenuated chamber.

4.5.4 Injection Procedures

Injections were delivered via a 33-gauge stainless steel injection needle which was connected via transparent Tygon tubing to a 10-µL Hamilton syringe. The injection needle was inserted in the guide tube prior to beginning each experiment. A pre-injection period of 40 trials was presented to assess baseline eyeblink performance and to detect any insertion effect. Following the pre-injection period, drug micro-injections were manually administered at a rate of 0.5 µL/min. To assess the drug effect, CS + US training continued for an additional 160 trials.

The present study consisted of three parts. The animals were first injected in the interposed nuclei (IN) to determine the location and the dosage of the $GABA_A$ antagonist gabazine (GZ) that would shorten the onset latency of CRs, i.e., produce SLRs (Parker et al., 2009). GZ was injected at locations in the IN where muscimol (1.75 nmol) completely suppressed conditioned eyeblinks (Bracha et al., 1994). Once the optimal GZ dosage and location were found in the IN, the MCP was injected the next experiment day with the sodium channel blocker TTX to determine a site at which MCP inactivation abolished CRs. In the third part, these two experiments were combined to test the effect of blocking the MCP on SLR expression. First, GZ was injected at locations in the IN where muscimol (1.75 nmol) completely suppressed conditioned eyeblinks (Bracha et al., 1994) and then TTX was injected where 3 µL of 4% Lidocaine had the same effect in the MCP. Immediately prior to each injection experiment, GZ was dissolved in artificial cerebral spinal fluid (aCSF), TTX was dissolved in phosphate-buffered saline (PBS), and the pH of both drugs was adjusted to 7.4 ± 0.1. In control experiments, an equal volume of drug vehicle was injected using the same protocol.

4.5.5 Data recording and analysis

Each rabbit's behavior was monitored using an infrared video system installed in the experiment chamber. Eyelid movements were recorded using a frequency-modulated infrared sensor that measures infrared light reflected from the eye and peri-orbital region (Ryan et al., 2006). The sensor was attached to the head implant before every experiment. The output of the sensor was amplified, digitized (25 kHz, 12-bit A/D converter), and stored in a PC-based data acquisition system. During each trial, 1400 ms of the signal were recorded, beginning 250 ms before the onset of the CS and extending for 800 ms beyond the US onset.

Eyeblink responses from each trial were examined off-line for the presence of CRs within the time window between CS and US onsets. The threshold for eyeblink detection was set to 5 standard deviations of the baseline signal noise, which in the present setup corresponded to approximately a 0.15 mm decrease in the eyelid aperture. Means of eyeblink latency and CR incidence were calculated for consecutive blocks of 20 trials. The pooled data from individual rabbits were statistically analyzed using repeated measures ANOVA. Reported F-ratios and their p-values refer to the differences between drug and vehicle in the pre-injection period versus the overall drug effect post injection and in some cases versus a given post-injection block. All group data were reported as mean ± standard error of mean, and an alpha level = 0.05 was used to declare significance. All statistical analyses were performed using Statsoft Statistica software.

4.5.6 Histology

Upon the conclusion of experimentation, rabbits were deeply anesthetized with a concentrated cocktail of ketamine (100 mg/kg), xylazine (12 mg/kg), and acepromazine (3 mg/kg). The injection sites were marked by injecting 1 µL of tissue-marking dye. Animals were perfused transcardially with 1 L of PBS followed by 1 L of tissue fixative (10 % neutral buffered formalin, NBF). Carefully excised brains were stored in a solution of 30 % sucrose and 10 % NBF and subsequently sectioned coronally at 50 µm on a freezing microtome. The sections were mounted onto gelatin-coated slides, and once dry, stained with luxol blue and neutral red. Using bright light microscopy, injection locations were determined and plotted on standard sections of the rabbit cerebellum.

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CHAPTER 5: THE CEREBELLUM AND EYEBLINK CONDITIONING: LEARNING VERSUS NETWORK PERFORMANCE HYPHOTHESES

A paper published in the journal *"Neuroscience"*[1]

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5.1 Abstract

Classical conditioning of the eyeblink reflex is a form of motor learning that is uniquely dependent on the cerebellum. The cerebellar learning hypothesis proposes that plasticity subserving eyeblink conditioning occurs in the cerebellum. The major evidence for this hypothesis originated from studies based on the telecommunications network metaphor of eyeblink circuits. These experiments inactivated parts of cerebellum-related networks during the acquisition and expression of classically conditioned eyeblinks in order to determine sites at which the plasticity occurred. However, recent evidence revealed that these manipulations could be explained by a network performance hypothesis which attributes learning deficits to a non-specific tonic dysfunction of eyeblink networks. Since eyeblink conditioning is mediated by a spontaneously active, recurrent neuronal network with strong tonic interactions, differentiating between the cerebellar learning hypothesis and the network performance hypothesis represents a major experimental

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challenge. A possible solution to this problem is offered by several promising new approaches that minimize the effects of experimental interventions on spontaneous neuronal activity. Results from these studies indicate that plastic changes underlying eyeblink conditioning are distributed across several cerebellar and extra-cerebellar regions. Specific input interactions that induce these plastic changes as well as their cellular mechanisms remain unresolved.

5.2 The cerebellar learning hypothesis and classical conditioning of eyeblink responses

The classically conditioned eyeblink or nictitating membrane reflex is a unique type of associative learning in which the cerebellum plays a major role (Thompson, 1986, for review). In the delay conditioning paradigm, the conditioned stimulus (CS), a stimulus that normally does not evoke the reflex, is paired over successive trials and at a specific interval with an ensuing unconditioned stimulus (US), which is capable of eliciting the unconditioned response (UR) before the conditioning is initiated. In each trial the CS and US co-terminate (**Fig. 1**). As conditioning continues, a new eyeblink response, the conditioned response (CR), gradually develops in the interstimulus interval, and the peak of this response becomes progressively time-locked to the onset of the US. In addition, once acquired, the CR can be evoked by applying the CS alone.

In the early eighties of the last century, a very exciting observation was reported implicating the cerebellum in this type of learned behavior. Lesioning a specific region of the cerebellar nuclei disrupted the performance of the classically conditioned eyeblink reflex in the rabbit (Clark et al., 1984; Yeo et al., 1985). Subsequently, several reports

were published demonstrating that the modulation of neurons in the critical regions of the cerebellar cortex and nuclei is associated with the CS, US and CR (Berthier and Moore, 1986; Berthier and Moore, 1990).

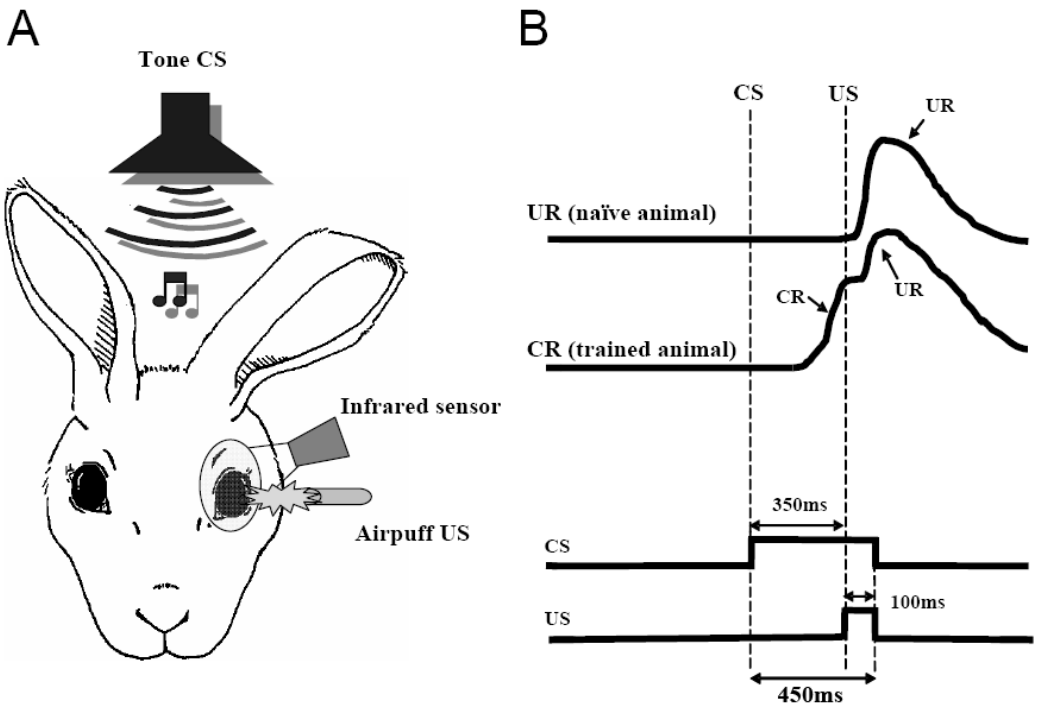

Fig. 1. Schematic of the eyeblink conditioning paradigm. A: rabbits are presented with a paired tone conditioned stimulus (CS) and airpuff unconditioned stimulus (US). Evoked eyeblinks are recorded with an infrared sensor. B: idealized eyeblink records in naïve and trained animals and the pulse diagram denoting the timing of stimuli. In the delay classical conditioning paradigm, the onset of the CS precedes the onset of the US and the stimuli co-terminate. Naïve animals don't respond to the CS, but the US evokes reliably the hard-wired trigeminal unconditioned blink (UR, top eyeblink trace). Over time, rabbits associate the CS with the US, and they learn to blink in anticipation of the

upcoming aversive US. These associatively learned responses are called conditioned responses (CR, the second trace from the top).

In addition, substantial cerebellar involvement in this type of learning has been shown in other species (e.g. Skelton, 1988; Chen et al., 1996; Voneida et al., 1990), including humans (Lye et al., 1988; Solomon et al., 1989). Since these seminal observations, temporary and permanent lesion experiments have implicated the cerebellum in multiple processes underlying the classical conditioning of this reflex system, including acquisition, retention and consolidation (Bracha and Bloedel, 1996; Christian and Thompson, 2003; De Zeeuw and Yeo, 2005, for review). Finally, this dependence was found to extend to other types of conditioned reflexes. Manipulations of the cerebellar circuitry or permanent lesions in cerebellar patients disrupted instrumentally conditioned eyelid closure (Bracha et al., 2001) and classically conditioned withdrawal reflexes in the extremities of cats (Kolb et al., 1997; Bracha et al., 1999) as well as humans (Timmann et al., 2000). Because of the extensive data from several laboratories dealing with the classically conditioned eyeblink reflex in the rabbit, our review will focus on data acquired from this species.

It is generally agreed that eyeblink conditioning in the delay paradigm is controlled by a combination of brainstem eyeblink reflex circuits and the intermediate cerebellar network, which is super-imposed over the UR system (**Fig. 2**). It has been proposed that the ipsilateral cerebellar interposed nuclei (IN) and the cerebellar cortex are essential and perhaps sufficient sites of plastic changes for generating the cerebellar CR motor command. This theoretical position, the cerebellar learning hypothesis, has been

extensively reviewed (Thompson, 1986; Christian and Thompson, 2003; Ohyama et al., 2002; Attwell et al., 2002; Bracha and Bloedel, 1996), and therefore, it will only be briefly outlined here. The primary tenet of this hypothesis is derived from original concepts posited by Albus (1971) and Marr (1969), who deduced testable predictions based on the cerebellum's unique anatomical structure and synaptic organization. It is assumed that information about the CS and US arrives to the cerebellum via two distinct routes. The CS is conveyed through mossy fibers originating in pontine nuclei, whereas the US is coded in the discharge of climbing fibers originating in the inferior olive (IO). Information from the mossy and climbing fibers eventually converges on cortical Purkinje cells and cerebellar nuclear neurons. It is presumed that the hetero-synaptic interaction at the points of convergence triggers local cellular plastic processes resulting in the changed responsiveness of Purkinje and/or nuclear cells. These plastic changes cause the network to respond to the CS mossy fiber signal by issuing a cerebellar nuclear "motor command" that triggers the CR.

Despite almost three decades of research examining the cerebellum's contribution to the acquisition, retention, and expression of the classically conditioned eyeblink reflex, a consensus regarding how this structure plays its important role in this behavior has not been reached. For example, in spite of numerous optimistic claims, specific contributions of the cerebellar cortex, cerebellar nuclei and extra-cerebellar substrates to plasticity that underlies learning are not known. This fundamental issue remains unresolved, mostly because of the lack of tools needed to interfere with learning without affecting both the local and global properties intrinsic to underlying circuits.

In this review we will first examine the conceptual underpinnings of experiments that tested the cerebellar learning hypothesis using local inactivation or manipulations of neurotransmitter signaling. We will outline the telecommunications network metaphor of eyeblink circuits, and we will show that some of the findings from studies that were designed based on this metaphor seem to disprove the cerebellar learning hypothesis or at the very least challenge some of its basic tenets. Then we will demonstrate that traditional cerebellar manipulations affect the spontaneous activity of neurons at the site of intervention and downstream from it, and that tonic interactions associated with this change can radically alter the functional state of the entire network. The tonic interactions in cerebellar systems have been largely overlooked in most discussions of the cerebellar learning hypothesis. We will argue that some of the pivotal observations that were declared to support this hypothesis can be ascribed to the effects that experimental manipulations had on the tonic activity of cerebellar circuitry and/or to methodological aspects of the experiments on which this view is based. We will present promising new data further supporting these arguments, and lastly, we will discuss approaches that could be used to address the function of eyeblink conditioning circuits more effectively.

5.3 The telecommunications network metaphor of eyeblink circuits

In most discussions of the neural substrates for the classically conditioned eyeblink reflex, it is often implicitly assumed that cerebellum-related eyeblink conditioning circuits (**Fig. 2**) operate as a telecommunications network. Telecommunications networks consist of links and nodes arranged so that messages may be passed from one part of the network to another over multiple links and through various

nodes. To employ the metaphor, eyeblink circuits consist of nodes (nuclei) and links (inter-connecting axons). Nodes in eyeblink conditioning circuits, individual nuclei or parts of the cerebellar cortex, are viewed as input-output processing units that transform input messages into output signals. Importantly, properties of this signal transformation can change during learning. Based on this metaphor, experimental interventions in eyeblink circuits, such as local inactivation, are viewed as means for disrupting local information processing and for depriving the rest of the circuit of locally generated or transmitted task-specific signals. However, this concept neglects the fact that nodes in eyeblink networks also exchange a continuous stream of spontaneous activity that shapes the functional state of the network. In our view, this omission has led to several surprising and misleading conclusions.

The telecommunications network metaphor can be implicated in several of the early inactivation studies. For example, Krupa et al. (1993) proposed that systematically inactivating individual nodes in the network during training sessions could be used to find places where learning-induced plasticity occurs. They speculated that successful CR acquisition during inactivation of a particular node would signify that plastic changes develop not at this node, but at some other up-stream parts of the network. On the other hand, failure of CR acquisition would mean that learning occurred either at the manipulated node or at some of its down-stream efferent targets. With this logical reasoning one could methodically examine individual nodes until all potential sites of learning were found. Over time, it has been shown that blocking glutamate neurotransmission in the cerebellar cortex (Attwell et al., 2001) or inactivating cerebellar nuclei (Krupa et al., 1993; Hardiman et al., 1996) during learning prevents CR

acquisition. In contrast, inactivating a major intermediate cerebellar efferent target, the red nucleus, had no effect on learning (Clark and Lavond, 1993; Krupa et al., 1993). These findings suggested that a significant site of plasticity related to CR acquisition is contained in the ipsilateral cerebellum.

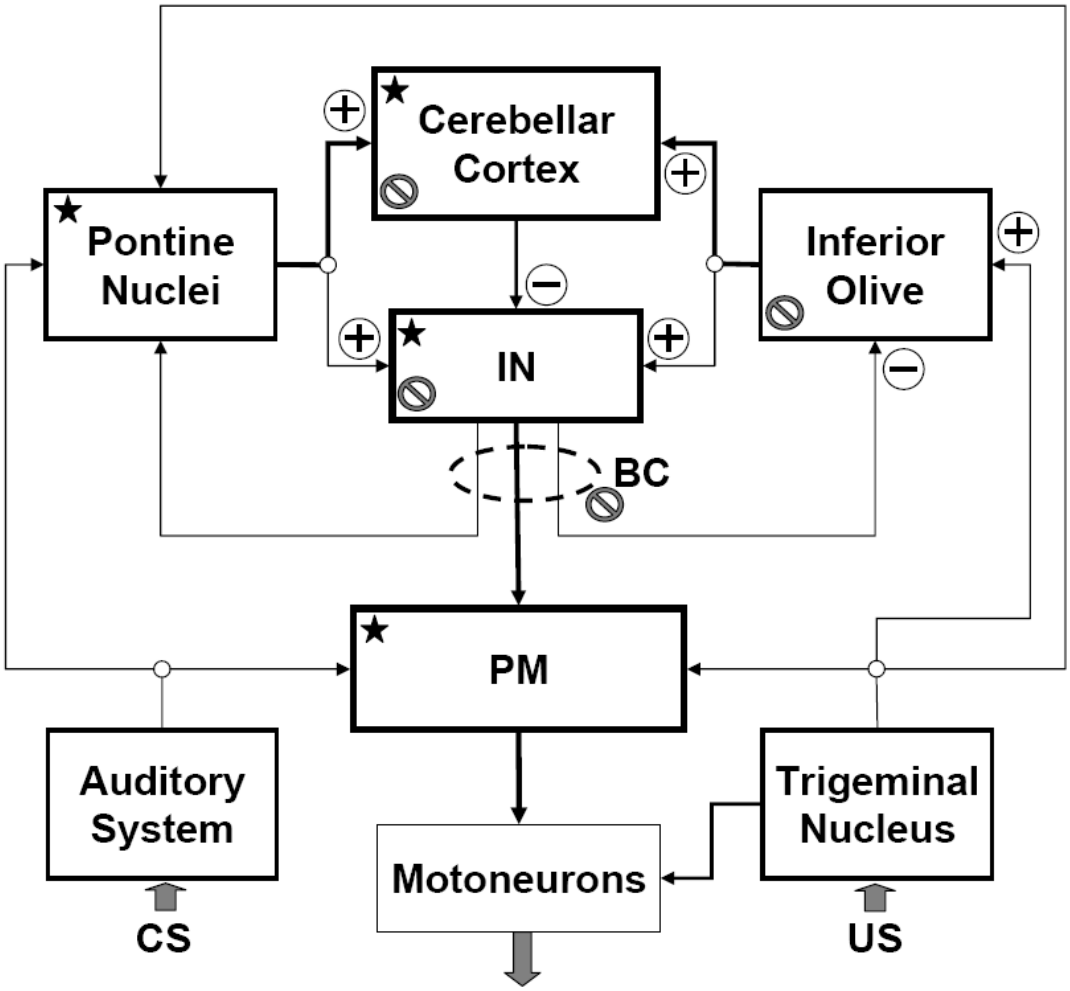

Fig. 2. A conceptual block diagram of the cerebellum-related circuitry involved in acquisition and expression of classically conditioned eyeblinks in the rabbit. This diagram is a highly simplified representation of relevant structures and connectivity. Information regarding the conditioned stimulus (CS) and

unconditioned stimulus (US) information enters the network via auditory and sensory trigeminal systems. These inputs are supplied in parallel to the serially connected pontine nuclei, cerebellar cortex, cerebellar interposed nuclei (IN) and brainstem nuclei contributing to projections to eyeblink premotoneurons and supplying motor commands to them. Since all these sites (labeled with a star) receive CS and US information, they should be considered as putative sites of learning. Output of eyeblink premotoneurons supplies motor commands to eyeblink motoneurons. Backslashed circles denote nodes at which inactivation during training disrupts CR acquisition. Boxes with bold borders represent structures among which are in our view distributed plastic changes underlying eyeblink conditioning. BC – brachium conjunctivum; PM – nuclei containing eyeblink premotoneurons that include the red nucleus. The plus symbols mark excitatory glutamatergic inputs and minus signs label inhibitory GABAergic inputs.

However, this conclusion was strongly contradicted by our recent discovery that a carefully placed, more extensive inactivation of the brachium conjunctivum (outgoing axons of deep cerebellar nuclei, BC) actually *prevented* CR acquisition (Nilaweera et al., 2006). Using the logic derived from the metaphor, this surprising finding leads to an important inference: the learning essential for CR acquisition occurs most likely outside of the cerebellum, in extra-rubral cerebellar efferent targets! Before accepting this unexpected scenario, the possible role of cerebellar feedback circuits has to be considered.

Besides projections to pre-motoneuronal parts of eyeblink circuits, the BC contains axons projecting to cerebellar afferent sources in the IO and in the pontine nuclei and thus participates in cerebellar feedback loops (**Fig. 2**). It is known that inactivating the IN does not prevent the pontine nuclei from transmitting CS signals (Cartford et al., 1997). Therefore, inactivating the BC should not affect learning via its effect on CS signals from the pontine nuclei. Perhaps more important are the implications derived from the cerebello-olivary-cerebellar feedback loop. The cerebellar learning hypothesis assumes that the IO supplies the cerebellum with a learning-inducing US signal (error signal) and that the inhibitory projection to the IO from the cerebellar nuclei suppresses this signal when learning is near completion (e.g. Medina et al., 2002). If so, then inactivating the BC would disinhibit the IO, thereby preventing the suppression of US signals. This condition, however, should not prevent learning (Kim at al., 1998). Consequently, it is unlikely that the effects of BC inactivation on CR acquisition were related to changes in transmission of US signals by the IO. At a minimum, we can conclude that employing all the assumptions underlying the telecommunications network metaphor, together with data from our recent BC inactivation studies, implicate extra-cerebellar sites as additional structures subserving eyeblink conditioning.

A second unexpected conclusion emerged from studies that inactivated the IO during CR expression. As reviewed above, the cerebellar learning hypothesis postulates that cerebellar plasticity is induced by inferior olivary US error signals that enter the IO via glutamatergic projections from the trigeminal nuclei. Correspondingly, lesioning or inactivating the IO prevents CR acquisition (McCormick et al., 1985; Welsh and Harvey, 1998). Given the assumption that IO error signals are required for the maintenance of

cerebellar plasticity, the cerebellar learning hypothesis predicts that blocking US responses in the IO should lead to the gradual suppression of CRs – an "unlearning" of CRs analogous to CR extinction training in which the CS is repeatedly presented without the US (McCormick et al., 1985). The seemingly ultimate support for this concept came from Medina et al. (2002), who reported that blocking glutamate neurotransmission in the IO indeed produces the predicted extinction-like suppression of CRs. However, in our investigation of neurophysiological mechanisms of this phenomenon we found that the gradual suppression of CRs following the block of glutamate receptors in the IO is related to the gradual diffusion of the drug and not to unlearning (Zbarska et al., 2007; Zbarska et al., 2008). Moreover, we determined that precise injections of glutamate antagonists in the IO suppress CRs immediately. These behavioral results are clearly inconsistent with the cerebellar learning hypothesis.

The above findings, when viewed through the lens of the telecommunications network metaphor, contradict the cerebellar learning hypothesis. In the following sections we will argue that, before these contradictions can be considered as solid evidence favoring an alternative view incorporating extra-cerebellar sites of plasticity, a major flaw inherent in this metaphor needs to be exposed: the metaphor ignores the fact that experimental manipulations of cerebellar circuitry at the nodes of the eyeblink conditioning network generate coupled modifications of spontaneous activity capable of altering the functional state both within and beyond the targeted node, potentially cascading throughout the network. The inclusion of these tonic interactions in models of eyeblink circuits offers an alternate interpretation of the available experimental data.

5.4 Tonic interactions in cerebellar circuits

A fundamental feature of cerebellum-related eyeblink conditioning circuits is their spontaneous activity that can be observed in the absence of overt stimuli or movements. The spontaneous activity of individual neurons is a collective product of the intrinsic ability of some neurons (e.g. Purkinje cells, IO and IN neurons) to self-generate action potentials and of the drive from excitatory, inhibitory and modulatory synaptic inputs (Hausser et al., 2004). Spontaneous firing rates differ across individual nodes of the network. For example, the typical spontaneous firing rates of Purkinje cells, IN neurons and IO neurons are about 50 Hz, 10-40 Hz and 1-2 Hz, respectively. The spontaneous activity of individual neurons is propagated through the network affecting cells in other nuclei, and these effects are further sculpted by a number of excitatory and inhibitory recurrent loops. These dynamic, non-linear processes determine the self-regulating functional state of the network.

The importance of tonic activity in cerebellar circuits to the learning and execution of a specific motor behavior should not be a surprise. Several experimental studies over decades of research demonstrated that lesions within the cerebellum or its afferent or efferent systems produce significant tonic effects throughout the motor system, including modifications of a variety of spinal reflexes (for reviews see Dow and Moruzzi, 1958; MacKay and Murphy, 1979; Bloedel and Bracha, 1995). This is particularly clear when manipulating the olivo-cerebellar projection. The early experiments of Carrea et al. (1947) demonstrated that the effects of IO lesions on behavior are so profound that they actually mimic the effects of ablating major portions of the cerebellum itself. Later studies revealed that IO lesions and IO cooling have

profound effects on the spontaneous activity of Purkinje cells. Considering the very low firing rate of IO neurons, it was surprising that removing the IO excitatory input to Purkinje cells led to a high, long lasting increase of their spontaneous discharge (e.g., Montarolo et al., 1982). Central to the arguments we will present, this tonic effect emerging from IO inactivation affected the spontaneous firing rate of cells at downstream sites. Since Purkinje cells are GABA-ergic, their sustained high activity was shown to suppress activity in their target cerebellar nuclear neurons (Batini et al., 1985). In turn, the decreased firing of nuclear neurons suppressed activity in the red nucleus (Billard et al., 1988), which is the main target of excitatory IN projections. In summary, these studies uncovered two important principles:

a) spontaneous activity in cerebellar network nodes can regulate tonic activity in their efferent targets;

b) suppressing spontaneous activity in one node can trigger related tonic changes capable of spreading through large portions of the network, negatively impacting its general functional state.

How relevant are these principles to eyeblink conditioning research?

5.5 Tonic cerebellar interactions in classically conditioned rabbits

Although speculations had been made in a number of studies regarding tonic phenomena and their importance (e.g. Bracha and Bloedel, 1996; Welsh and Harvey, 1998; Attwell et al., 2001), their experimental demonstration in the rabbit eyeblink conditioning model was reported only recently. Characterizing tonic interactions in cerebellar circuits requires combining local circuit manipulations with recording of

neuronal activity. For that purpose, we developed a unique, microwire-based, multi-channel recording system that is well suited for long-term isolation of single units. The long-term stability of unitary recording is paramount for experiments that require monitoring cellular activity for at least 1-2 hours in animals with a freely moving head. In our initial studies, we focused on analyzing electrophysiological consequences of neurotransmitter manipulations in the IO and IN. Results of these experiments offer illuminating insights into the mechanisms through which cerebellar manipulations affect eyeblink conditioning.

As explained in previous sections, demonstrating that US signals from the IO are required for the maintenance of CRs constitutes a pivotal test of the cerebellar learning hypothesis. In a frequently cited study, Medina et al. (2002) proposed that this prediction could be tested by blocking trigeminal projections to the IO by infusing the IO of trained rabbits with a fast glutamate receptor blocker, NBQX. They reported that NBQX indeed produced an extinction-like, gradual suppression of CRs.

To investigate the neurophysiological mechanisms of this process, we injected NBQX in the IO of trained rabbits while simultaneously recording single-unit activity of IN neurons (Zbarska et al., 2008). Based on the prediction of Medina et al., which invokes the telecommunications network metaphor, one would expect NBQX to gradually "extinguish" CRs with a correlated gradual decrease of the CR-related modulation of neuronal activity in the IN. On the other hand, if NBQX decreases the spontaneous IO firing rate (Lang, 2002), one could also expect tonic suppression of IN activity. We not only found that NBQX immediately abolishes CRs without the need for CS presentations (a condition required for extinction), but this behavioral response

coincided with the immediate suppression of both spontaneous IN activity and task-related modulation (**Fig. 3**).

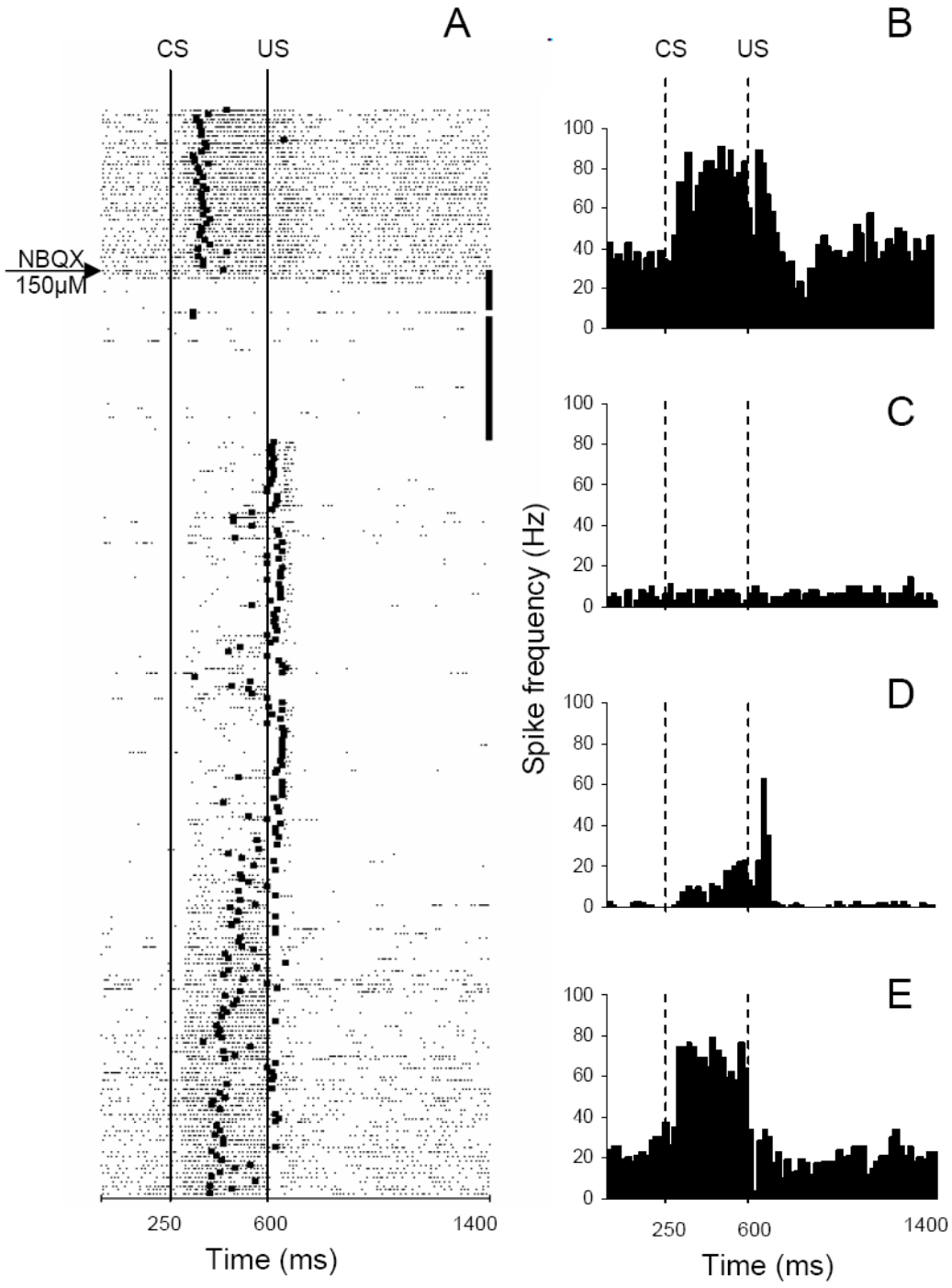

Fig. 3. An example of the parallel effects of inferior olivary NBQX infusion on CR performance and on the activity of a task-modulated IN cell. This experiment consisted of 260 trials. After 40 baseline trials, NBQX was injected at the beginning of a 40-trial no-stimulation period. A, Raster plot of IN cell activity during this experiment. The experiment starts at the top with each row representing one trial, and each dot marking the occurrence of an action potential. The black square in each row corresponds to the onset of the eyeblink in that particular trial. Consequently, CRs have onset markers between lines denoting the CS and US onsets. Eyeblinks initiated past the US onset occur in trials in which the animal failed to produce the CR. Black squares at the ends of the 40 trials following the NBQX injection marker denote the no-stimulation waiting period that was inserted to allow for drug diffusion. Before the injection, this cell responded with excitation during the CS–US interval and with a combined excitatory/inhibitory response to the US. During the drug diffusion period, the firing rate of this cell's activity precipitously declined. When stimulation was resumed, CRs were abolished immediately as evidenced by the blink onset marks on the right side of the US onset line. The baseline activity remained suppressed, and modulation during the CS–US interval was severely reduced whereas the relative excitatory modulation to the US became more distinct. The neuronal activity gradually recovered toward the end of the experiment in parallel with the recovery of behavioral CRs. B–E, Peri-stimulus histograms of the same IN unit constructed for 40 trials before the injection (B), for 40 post-injection drug diffusion trials when stimulation was paused (C), for 40 trials following the

waiting period when stimulation was resumed (D), and for the last 40 trials from the remaining 140 trials of the experiment (E). Bin width for histograms in B–E is 20 ms. CS, onset of conditioned stimulus; US, onset of unconditioned stimulus. (Reprinted with permission from Zbarska et al., 2008)

In the framework of the telecommunications network metaphor, the behavioral part of our study argued against the cerebellar learning hypothesis. However, our electrophysiological data revealed that this metaphor had a major shortcoming affecting the interpretation of the findings – its failure to recognize the importance of a fundamental variable, the tonic interactions in cerebellar networks. The intent of the above IO studies was to observe the consequences of blocking the IO error signal to the cerebellum. However, the IO injection of NBQX also made the cerebellar cortex and nuclei dysfunctional by blocking the cerebellar output, which in turn results in the abolition of CRs, precluding any conclusions about the mechanisms and sites involved in establishing plasticity.

Additional evidence for cerebellar tonic interactions following manipulations of the cerebellar circuitry emerged when we examined GABA and glutamate neurotransmission in the IN of trained rabbits. Individual contributions of the cerebellar cortex and IN to CR acquisition and expression have been the subject of a long-standing debate. In an attempt to resolve this issue, some investigators proposed that nuclear components of learning could be revealed by blocking GABA-ergic projections of Purkinje cells to the IN (Medina et al., 2001; Ohyama et al., 2006). They reported that blocking GABA-A receptors either with picrotoxin or with gabazine shortens the latency

of CRs. The authors proposed that short-latency CRs are a manifestation of nuclear plasticity that is revealed in the absence of cerebellar cortical input. In their computer simulations, Medina et al. (2001) predicted that injecting picrotoxin in the IN should affect the time profile of IN neuronal responses but should have no effect on their spontaneous activity. Relevant to the subject of this review, these simulations are based on the telecommunications network metaphor because they did not consider tonic interactions.

To examine these proposals, we injected the IN of trained rabbits with GABA agonists and antagonists and then measured their effect on IN single-unit activity and on CRs (Aksenov et al., 2004). In our experiments, we could not confirm their prediction that effects would be limited to IN neuronal response timing. Instead, we found that a partial block of chloride channels with picrotoxin dramatically increased IN tonic activity. A more complete block of GABA neurotransmission with larger amounts of picrotoxin further increased IN spontaneous activity. It became so high that practically all modulation was suppressed, and behavioral CRs were abolished (**Fig. 4**). Although the goal of these experiments was to block signals embedded in Purkinje cell firing without altering normal IN activity, our recordings demonstrated that the functional state of the IN was dramatically altered. Furthermore, it is also likely that the excitability of IN efferent targets was also modified. This distributed functional abnormality prevented any conclusions about the cerebellar cortical and IN roles in CR expression. We conclude that simply blocking GABA neurotransmission in the IN can not address these questions.

The examples of tonic interactions in cerebellar circuits demonstrate that local inactivation and pharmacological manipulation are imperfect tools when used to interfere with task-related signals or to block local information processing. This is because local interventions will inevitably alter normal spontaneous activity of manipulated structures, and this abnormality will spread to down-stream parts of the network. This problem is further exacerbated by participation of feedback loops that provide a path for the propagation of the tonic change to up-stream parts of the network. The spread of tonic changes via the cerebello-olivary-cerebellar feedback loop has been documented. Hesslow and colleagues recorded a dramatic reduction of spontaneous Purkinje cell activity when inactivating IN axons in the brachium conjunctivum of decerebrate ferrets (Svensson et al., 2005; Bengtsson et al., 2004), findings which are consistent with results from our laboratory. We found that inactivating the BC in classically conditioned rabbits elevates the tonic activity of upstream IN neurons (Nilaweera et al., 2002). In summary, combined microinjection and recording studies demonstrate that local manipulations alter spontaneous activity and that this change can spread via tonic interactions to both down-stream and up-stream parts of the network.

The down-stream and recurrent propagation of tonic changes is highly pertinent to the interpretation of studies in which components of the cerebellar circuits are inactivated during learning. In previous sections we have shown that, when viewed through the perspective of the telecommunications network metaphor, the collective results of experiments in which this methodology was used point to the existence of extra-cerebellar sites for plasticity underlying this behavior. However, given the potential for recurrent and downstream spread of tonic changes, the failure to acquire CRs during BC

inactivation does not prove extra-cerebellar learning. It is possible that this treatment also affected cerebellar learning via tonic malfunction within the cerebello-olivary-cerebellar feedback loop. As a result, despite the initial optimism regarding the use of reversible lesions, inactivating parts of cerebellar circuits during training actually tells us surprisingly little about the location of plastic changes.

5.6 Cerebellar learning vs. network performance hypotheses

As argued above, the telecommunications network metaphor can't explain the results of experiments that inactivate neurons or block neurotransmission in eyeblink conditioning circuits. A more realistic conceptualization of how these circuits function has to include tonic phenomena. Eyeblink circuits should be viewed not only as a network of nodes that process and transmit task-related signals but also as a spontaneously active, recurrent neuronal network with strong tonic interactions.

Nevertheless, addressing such concepts as the cerebellar learning hypothesis can not proceed without using intervention methods if we are to understand the fundamental mechanisms responsible for motor learning phenomena like the classically conditioned eyeblink reflex. Only interfering with information processing within the putative learning-related substrates can confirm or disprove this view and evaluate its importance for learning at a systems level. The problem is that traditional intervention experiments, such as local inactivation or blocking specific neurotransmitter systems, up- or down-regulate spontaneous neuronal activity, thus complicating the interpretation of findings. For example, based on the telecommunications network metaphor, our study in which CR acquisition was blocked by BC inactivation (Nilaweera et al., 2006) suggests that extra-

cerebellar learning definitely occurs. However, when the associated tonic changes of spontaneous activity are considered, an alternate, more parsimonious explanation – *the network performance hypothesis* – emerges as a basis for the majority of observations. This hypothesis proposes that the effects of manipulating cerebellar circuits are related to a non-specific, wide-spread malfunction of the networks responsible for acquiring and/or expressing CRs. Interestingly, we know that in the case of BC inactivation, the network performance hypothesis is correct, because blocking the BC alters tonic activity in the IO, cerebellar cortex (Bengtsson et al., 2004) and IN (Nilaweera et al., 2002), and most likely also in mesencephalic targets of cerebellar efferents. Does this mean that these BC inactivation studies disprove the cerebellar learning hypothesis? Not necessarily. In fact, both hypotheses could be correct because the cerebellar learning and performance hypotheses are not mutually exclusive nor are they truly antithetical. Rather, the network performance hypothesis proposes that the relevant findings are due to an abnormal functional state of the cerebellar circuitry. Consequently, no definite conclusions about cerebellar learning can be inferred. Advancing our understanding of the neural circuitry subserving eyeblink conditioning will require intervention methods that eliminate the abnormalities on which the network performance hypothesis is based.

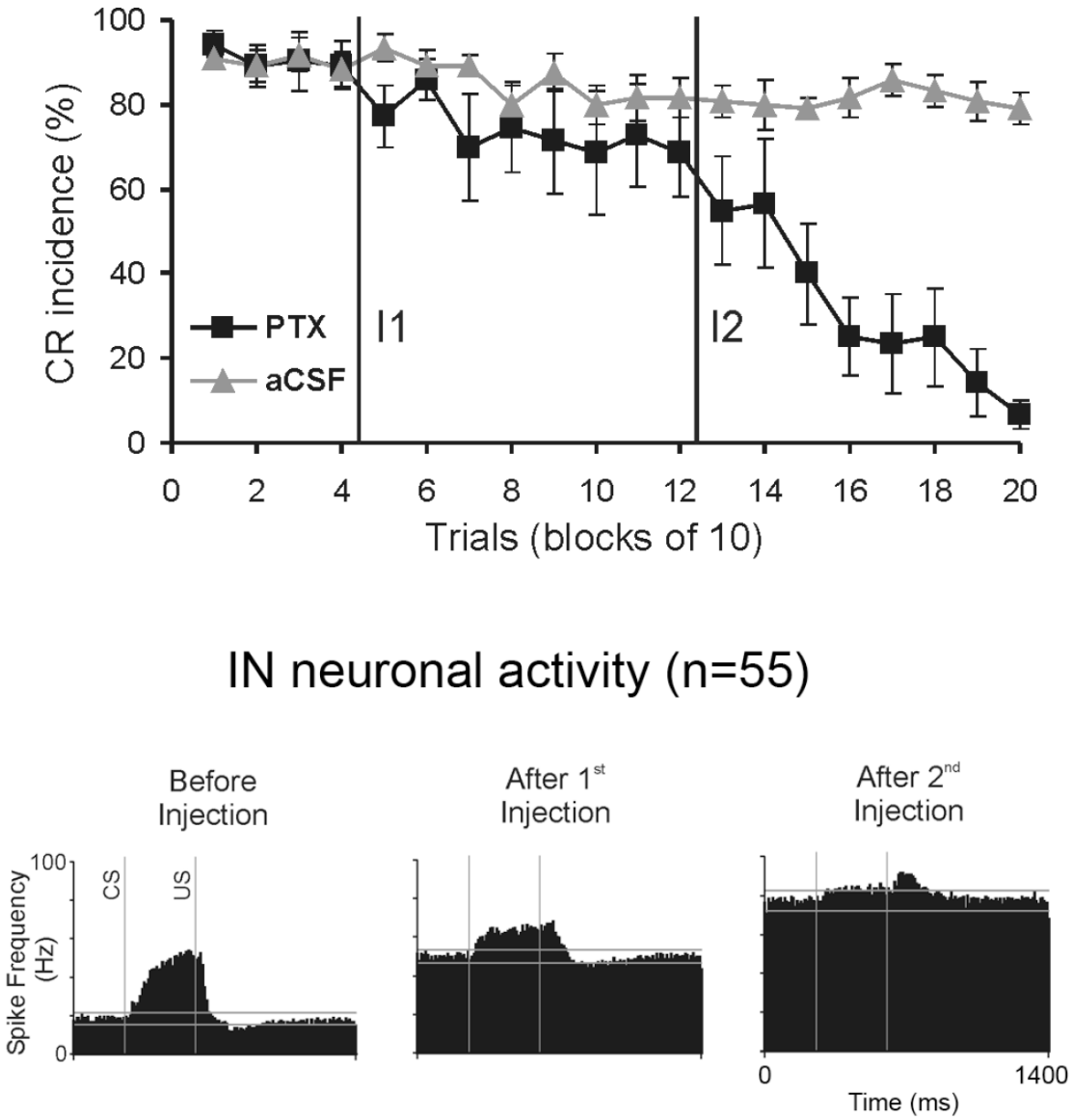

Fig. 4. Effects of injecting the IN with the chloride channel blocker, picrotoxin (PTX), on the expression of CRs and on IN neuronal activity. Top panel: CR incidence in 15 injection experiments in which two injections of PTX were applied to the IN. The first injection (I1) had only a small effect on the frequency of CRs. A more extensive block of GABA-ergic neurotransmission with the

second PTX injection (I2) gradually abolished CRs. Control injections of vehicle (aCSF) did not affect CR incidence. Bottom panel: population peri-stimulus histograms of 55 neurons recorded during PTX injections. Before injections, this population exhibited approximately a 25 Hz spontaneous firing rate and an excitatory response in the CS-US interval. The first PTX injection doubled the spontaneous discharge of IN neurons and reduced their CS-related modulation. Following the second injection, the spontaneous activity further increased, and the responses in the CS-US interval were almost completely attenuated. Two horizontal lines in each histogram denote tolerance limits used for detecting significant levels of neuronal modulation relative to mean baseline activity (Adapted from Aksenov et al., 2004).

5.7 Dissociating learning from network performance-related phenomena

An obvious solution for dissociating learning from network performance abnormalities would be the use of approaches that preserve spontaneous neuronal activity or that target processes insensitive to tonic changes. In our view, three promising research strategies are compatible with these goals.

In theory, not all cerebellar learning-related processes have to be sensitive to cerebellar tonic malfunction. A possible candidate for such a process could be memory consolidation which takes place after signaling events that induce learning in eyeblink circuits have already occurred and therefore could not be perturbed by tonic phenomena.

Attwell et al. (2002) reported that infusions of muscimol in the eyeblink area of the cerebellar cortex following training sessions prevented CR acquisition by interfering with memory consolidation. Interestingly, injecting muscimol in the deep cerebellar nuclei did not affect CR consolidation. Based on these results, Attwell at al. concluded that a muscimol-sensitive memory consolidation process in the cerebellar cortex is required for eyeblink conditioning. This finding, however, does not exclude consolidation of plastic changes in the IN or in extra-cerebellar sites that could be insensitive to IN inactivation and associated tonic changes in the circuit.

Another promising approach involves methods that could interfere with the putative cellular substrates of learning without affecting normal neuronal activity. An example of the successful use of this strategy is our study in which we blocked the synthesis of new proteins in the IN during CR acquisition sessions (Bracha et al., 1998). We found that infusing the IN with anisomycin suppressed CR acquisition. Since anisomycin has been reported to have minimal effects on spontaneous neuronal activity, this finding can be considered among the best evidence for cerebellar learning. Besides their potential for success, tools targeting possible cellular mechanisms of learning also have their limitations. The most important limitation of methods in this category is that they are not suited for analyzing the role of task-related signals during learning. For instance, blocking protein synthesis in the IN can't determine which inputs to the IN trigger the protein synthesis-dependent learning mechanism.

The third category of approaches that could separate learning from abnormalities in network performance consists of combined applications of receptor agonists and antagonists. The tremendous potential of this approach was shown for the first time by

Bao et al. (2002), who discovered that CRs suppressed by infusing a GABA-A agonist, muscimol, in the IN can be recovered by the subsequent infusion of the chloride channel blocker, picrotoxin. We were able to replicate these data (Aksenov et al., 2004; Aksenov et al., in preparation). Our single-unit recordings in injected animals confirmed that muscimol-induced CR suppression was accompanied by inhibition of IN neurons: their spontaneous firing was suppressed. The subsequent infusion of picrotoxin reduced the inhibition and restored spontaneous firing to its near normal rate, but the amplitude of event-related responses was reduced (Aksenov et al., 2004). Surprisingly, this group of observations was paralleled by the partial restoration of CRs. Because these studies blocked Purkinje cell input to the IN without markedly disrupting IN spontaneous activity, they were the first to eliminate the network performance hypothesis in experiments blocking network communication. The fundamental implication of these observations is that the IN can support CR expression in the absence of cerebellar cortical inputs. Did the remaining modulation of IN neurons generate the recovered CRs? To examine this issue, we again infused the IN with muscimol to block cerebellar cortical input, but in addition, a fast glutamate receptor blocker DGG was injected to block direct IN input from collaterals of mossy and climbing fibers. This treatment suppressed both IN activity and CR expression. Follow-up injections of PTX restored IN spontaneous firing, but all event-related modulation was suppressed. Yet, even in this condition CRs were partially restored (Aksenov et al., 2004). This finding strongly suggests that the modulation of IN neurons is not required for the expression of these residual CRs. Such CRs do not seem to require motor commands from the cerebellum, supporting the

argument that these CRs are most likely controlled by extra-cerebellar components of eyeblink circuits.

5.8 Conclusion

In conclusion, we have shown that the basis for the dependency of eyeblink conditioning on cerebellar circuits is not completely understood. The most investigated concept, the cerebellar learning hypothesis, assumes learning occurs in the cerebellar cortex, the deep cerebellar nuclei, or more recently – in both of these locations. However, because of difficulties in dissociating the learning and network performance hypotheses, the cerebellar learning hypothesis has not been supported by unequivocal, direct evidence from lesion or inactivation studies. Unless future intervention studies successfully manipulate putative conditioned eyeblink substrates without altering levels of spontaneous activity, the specific roles of the cerebellum in acquiring, expressing, and retaining classically conditioned eyeblinks will remain elusive.

Recent developments have identified new approaches that could minimize the impact of cerebellar tonic phenomena. Although the development of these new tools is still in its infancy, efforts in that direction have already revealed very promising results indicating plasticity in several cerebellar and extra-cerebellar parts of eyeblink circuits. Previously we proposed, mostly based on indirect evidence, that plastic changes supporting eyeblink conditioning are distributed across several components of eyeblink conditioning networks (Bracha and Bloedel, 1996; Bracha et al., 2001). We speculated that all nodes that receive the information about the CS and US, including sites within the cerebellum, could be sites of plasticity underlying learning (**Fig. 2**, sites labeled with a

star). This position appears to be supported by the available data: (1) consolidation experiments suggesting cerebellar cortical involvement; (2) the dependency of CR acquisition on the synthesis of new proteins in the IN suggesting an important role for cerebellar nuclei; and (3) combined infusions of GABA and glutamate receptor ligands in the IN indicating that CRs are supported at least partially by extra-cerebellar substrates. These data mandate that future research should focus on examining the role of both cerebellar and extra-cerebellar sites in the classical conditioning of the eyeblink reflex. The continued application of novel approaches will lead to the resolution of these questions in the relatively near future.

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CHAPTER 6: CONCLUSIONS

6.1 Study conclusions

The research presented in this dissertation made a major advance toward understanding the role of the cerebellar cortex and interposed nuclei in the generation of classically conditioned eyeblinks. We developed a new, highly sensitive device for eyeblink measurement. In experiments examining the effect of $GABA_A$ blockers on CR expression we successfully reconciled the long-standing controversy by showing that both CR abolition and SLRs could be evoked from the same injection site, simply by varying the drug dose. Specifically, we found that changes in the timing of CRs are associated with an incomplete block of cortical projections to the IN. We also discovered that SLR-inducing doses of picrotoxin affect non-associative components of blinking, such as tonic eyelid position. This important finding suggests that the intermediate cerebellum controls the excitability of extra-cerebellar eyeblink circuits. In contrast to low doses of $GABA_A$ blockers, a more complete suppression of cortical projections with high doses of these drugs abolished CRs. This fundamental finding critically weakened the cerebellar cortical timing hypothesis and suggested that SLRs are driven either by signals from the cerebellar cortex or by extra-cerebellar circuits.

To test whether SLRs are driven by cerebellar inputs, we blocked the direct mossy fiber input to the IN and also the middle cerebellar peduncle. We found that while blocking direct IN inputs did not suppress CRs, inactivating the MCP abolished them. This pivotal finding further weakened the cerebellar cortical timing hypothesis and supported the cerebellar cortical learning hypothesis.

In summary, our data strongly support the cerebellar cortical learning hypothesis and also provide ideas for the next logical step toward future experiments. Most importantly, implications drawn from neuropharmacological studies are always inconclusive and have to be confirmed by electrophysiological experiments. As addressed in the Section 1.4.5, the major task for future experiments will be testing a major methodological hurdle to the cerebellar learning hypothesis – the network performance hypothesis. In previous studies, our laboratory demonstrated that blocking signals in cerebellar circuits can affect CRs in a non-specific manner by producing a tonic malfunction of the network (Aksenov et al., 2004; Zbarska et al., 2008). Is it possible that blocking the MCP (Chapter 4) induced a tonic cerebellar malfunction?

To address this question, we initiated a new series of ongoing experiments and here we provide preliminary results. The ultimate test of the network performance hypothesis would be to record single-unit activity in the IN of SLR-producing animals before and after the MCP inactivation. However, this is a very complex and difficult experiment. To simplify the design, we decided first to analyze effects of MCP inactivation in otherwise intact subjects. Rabbits were implanted with an adjustable array of microelectrodes in the IN and with injection cannulae in the MCP region. We reasoned that if the network performance hypothesis is correct, then blocking the MCP would cause an up-regulation of IN single-unit activity. On the other hand, if the network performance hypothesis is not correct, we expect that only the CS- or CR-related modulation of IN neurons would be greatly reduced. We found that, indeed, the MCP inactivation elevated the spontaneous firing rate of IN neurons (**Fig. 1**).

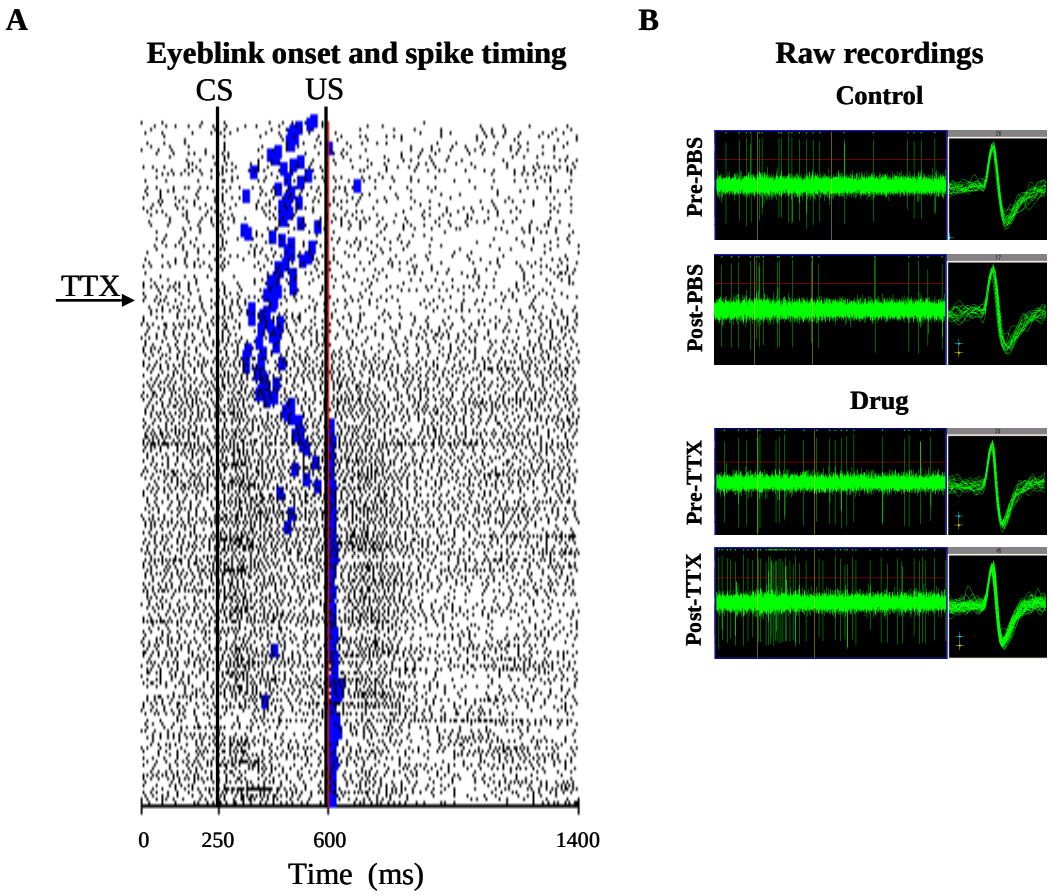

Fig. 1. Example of the effects of TTX injected in the MCP on the performance of conditioned eyeblinks and on the activity of cells in the interposed nuclei. A: a raster plot of action potentials of an IN neuron in an experiment where TTX was injected in the MCP (injection at arrow marked TTX). This experiment begins at the top and ends at the bottom with the CS and US onsets marked. Each row of dots represents one trial. Each large blue dot corresponds to the onset of the behavioral eyeblink response while each small black dot indicates when the recorded cell fired an action potential. Following the injection of TTX, CRs are gradually abolished (eyeblink onset shifts past the US onset) and this effect corresponds with an increased tonic firing frequency. In addition, the cell

continued to respond to the CS and US following the injection. B: an example of raw recordings of IN neurons. Two trial records of these cells are shown for both control and drug injections: one trial before and one trial after the injection. The left side of each panel shows the complete signal record in the 1400 ms-long trial. The right portion of each panel shows overlaid action potentials in this trial. Note that the single unit recording was exquisitely stable as demonstrated by the uniform shape and amplitude of discriminated action potentials during the experiment. The stability of single unit recording is a precondition for reliable detection of the drug effect on neuronal activity.

This crucial finding represents the first demonstration of the tonic effects of MCP inactivation and it is consistent with the network performance hypothesis. The second and more surprising finding in these experiments was that inactivating the MCP did not abolish CR-related neuronal activity in the IN (**Fig. 2**). This indicates that the intermediate cerebellum receives the CS information not only via the MCP, but probably also from axons in the inferior cerebellar peduncle. If this finding would be confirmed, it would require major modifications in the current concepts of cerebellar involvement in this model of associative learning. At present, it is not clear if these results could be extrapolated to animals with the cortical input into the IN blocked. This issue should be the next subject of future investigations.

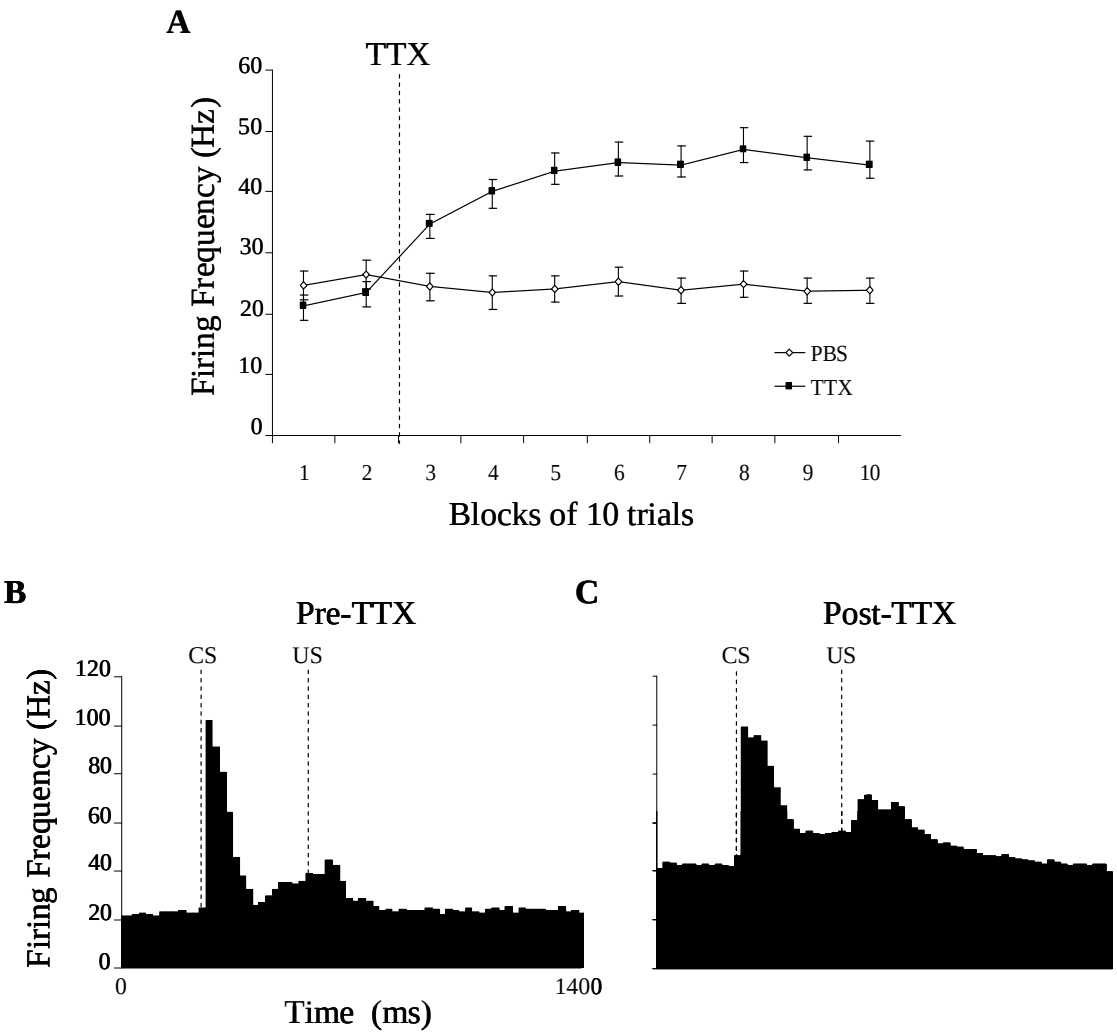

Fig. 2. Group data for effects of the MCP inactivation on the IN neuronal activity. A: the effect of TTX (blocks of 10 trials) on the spontaneous firing rate (mean ± SEM) of IN neurons. Following the injection of TTX (circles), the firing frequency of cells in the IN (n = 72) nearly doubled. There was no effect on firing frequency following control injections of PBS (diamonds, n = 62). B: population peri-stimulus histogram of IN cell activity (n = 72) during pre-TTX trials. These cells vigorously responded to the CS and were also modulated during the CS/US period. C: population peri-stimulus histogram of the same

group of cells as in B, following the injection of TTX. Inactivating the MCP elevated the spontaneous firing rate (bins in the period before the CS onset). Surprisingly, the population of IN neurons continued to respond to the CS and US.

6.2 References

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