Glutamate Neurotransmission in the Cerebellar Interposed Nuclei: Involvement in Classically Conditioned Eyeblinks and Neuronal Activity

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INTRODUCTION

Classical conditioning of eyeblink responses in the rabbit is an associative learning paradigm that is critically dependent on intermediate cerebellar circuits. It has been well established that lesioning or inactivating the interposed nuclei (IN) prevents learning new conditioned responses (CRs) (Hardiman et al. 1996; Krupa et al. 1993) and abolishes the expression of previously learned CRs (Bracha et al. 1994; McCormick and Thompson 1984; Yeo et al. 1985). It has also been shown that cells in the IN fire before the initiation and during the execution of CRs, suggesting that signals from the IN could contribute to a CR-generating motor command (Aksenov et al. 2004; Berthier and Moore 1990; Jimenez-Diaz et al. 2002). Several studies have reported that the cerebellar cortex is not essential for CR expression both in the rabbit and mouse (Chen et al. 1996; McCormick and Thompson 1984; Perrett et al. 1993; Steinmetz et al. 1992). CR dependency on IN integrity, together with the reported capacity of animals to generate CRs in the absence of cerebellar cortical input, led to proposals that the IN is the primary structure involved in CR acquisition and generation (Christian and Thompson 2003; Thompson 1986) or that the IN generates a motor command that is shaped by the cerebellar cortex (Garcia and Mauk 1998). These concepts assume that IN eyeblink-related neuronal activity arises in response to collaterals of mossy and climbing fibers that terminate in the cerebellar nuclei (Christian and Thompson 2003; Garcia and Mauk 1998). It should be noted that, although there is a general agreement about the involvement of the IN in CR expression, studies examining the role of the cerebellar cortex are controversial. In contrast to the above-cited reports, which indicate a nonessential role for the cerebellar cortex in CR expression, several studies demonstrated that permanent lesions or inactivation of cerebellar cortical folium HVI abolishes CRs (Attwell et al. 2001; Hardiman and Yeo 1992). These findings offer an alternative view of the IN and cerebellar cortical involvement in conditioned eyeblinks. Specifically, they implicate that the cerebellar portion of CR motor commands could actually originate in the cerebellar cortex and that the IN only further process this command and transmit it to eyeblink premotoneurons.

To examine the relative contributions of the cerebellar cortex and the cerebellar afferents to IN function, we designed a series of studies in which we pharmacologically blocked either the GABAergic cerebellar cortical projections to the IN (Ito 1984) or the glutamatergic projections from the collaterals of mossy and climbing fibers (e.g., Audinat et al. 1990, 1992). In these experiments, we analyzed the effects of pharmacological manipulations on the activity of IN neurons and on the behavioral expression of CRs. In our previous report, we described the effects of blocking GABAergic cerebellar cortical input by microinjections of picrotoxin (PTX) (Aksenov et al. 2004). In that study, we found that blocking γ-aminobutyric acid-A (GABAA) neurotransmission in the IN profoundly affects both IN neuronal activity as well as the capacity of rabbits to generate CRs. These findings were consistent with the primary role of the cerebellar cortex in CR control. Here we report the electrophysiological and behavioral effects of blocking fast glutamate neurotransmission in the IN. If the input from collaterals of mossy and climbing fibers plays a significant role in the modulation of IN single-unit activity and in CR expression, it would be expected that blocking fast components of glutamate neurotransmission in the IN should disrupt both the...
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expression of CRs and the normal modulation of IN neurons. Contrary to this prediction, we found that injections of the wide-spectrum glutamate receptor blocker DGG had relatively mild effects on learned responses and cerebellar nuclear neuronal activity. On the other hand, simultaneously blocking both glutamate and GABA neurotransmission using a mixture of DGG and PTX abolished behavioral CRs as well as most of the task-related IN neuronal modulation. These data indicate that in the rabbit eyeblink model, glutamate-mediated input from collaterals of major cerebellar afferents into the IN is not essential for CR expression and that IN neuronal activity, and ultimately the behavioral expression of CRs, is primarily under intermediate cerebellar cortical control.

METHODS

Animals injection sites, recording methods, and principles of data analysis used in this paper are the same as described in detail in our previous report (Aksenov et al. 2004). Therefore only the general aspects of the methods are provided in this section. The experiments were performed in 6 male New Zealand rabbits (2.5–3.0 kg, 3–4 mo old at the start of experiments) that were provided with food and water ad libitum. All experiments were performed in accordance with the National Institutes of Health “Principles of Laboratory Animal Care” (National Institutes of Health 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.

Surgery

Animals were anesthetized with a mixture of ketamine (50 mg/kg), xylazine (6 mg/kg), and acepromazine (1.5 mg/kg) and implanted with an assembly of 3 guide tubes that was aimed 0.5 mm dorsal to the left cerebellar interposed nuclei. The guide tube assembly consisted of 3 parallel 26-gauge stainless steel tubes arranged in an equilateral triangle with a distance of 0.8 mm between them. Two of the guide tubes contained a vertically adjustable bundle of 5 microwires. During surgery, lambda was positioned 1.5 mm below bregma and the stereotaxic coordinates were: AP = 0.2–1.2 mm rostral to lambda, ML = 5.2 mm from midline and DV = 14.7 mm ventral to lambda. All animals were treated with antibiotics for 5 days after surgery.

Training procedures

After surgery, rabbits were adapted to the restraining box and experimental environment for 3 days, 30 min per day. After adaptation, rabbits were conditioned in the standard delay paradigm using a 100-ms airpuff (210 kPa at the source) applied to the left eye as the unconditioned stimulus (US) and a 450-ms, 80-dB SPL, 1-kHz tone as the CS superimposed on 65 dB SPL of white noise. The interstimulus interval was 350 ms, and the intertrial interval varied pseudorandomly between 17 and 23 s. All animals were trained until they produced CRs in more than 90% of the trials for 3 consecutive days.

Injection and recording experiments

All trained rabbits were tested in experiments in which the cerebellar interposed nuclei were microinjected with solutions of either muscimol (3.5 nmol/μl; MP Biomedicals), γ-D-glutamylglycine (DGG, 0.098 μmol/μl; Tocris), or DGG mixed together with picrotoxin (PTX, 2.5 nmol/μl; Sigma), and the effects of these drugs on eyblinks as well as on the activity of IN neurons were monitored. Only one drug was injected on any given experimental day. The main objective of muscimol injections was to functionally test whether the guide tube for the injection needle was implanted in the IN region related to CR control. Based on previous studies (e.g., Bracha et al. 1994), it was expected that muscimol would abolish CRs in successfully implanted animals. During muscimol experiments, a single 0.5-μl injection was applied after 40 baseline trials. In the other drug experiments, there were two 0.5-μl injections applied at a rate of 0.05 μl/min. Conditioning trials were resumed immediately after each injection. These injections divided each experiment into 3 blocks of trials: 40 trials before any injections (baseline period), 80 trials after the 1st injection, and an additional 80 trials after the 2nd injection. Equal volumes of vehicle [artificial cerebrospinal fluid (aCSF)] were injected following the same procedure in control experiments. The order of DGG, DGG + PTX, and aCSF injections was counterbalanced between animals and experiments.

In this study, we present data only from experiments that yielded stable recordings of IN single-unit activity. The first injections in each animal were functional muscimol tests. In the muscimol injection experiments, no efforts were made to find and record cells that would respond in the CS–US interval. Other drug injection experiments were performed in 3–4 animals in which a single set of muscimol completely blocked CR expression. Before these experiments, the electrodes were advanced to positions yielding cells that responded in the CS–US interval. Once a set of neurons was recorded successfully during drug and/or control experiments, the electrodes were repositioned to search for a new set of IN cells. Consequently, several sets of cells were recorded in the vicinity of each injection site.

Data recording and analysis

The eyeblink was recorded by monitoring the upper eyelid position with an electromagnetic lever system (Bracha et al. 2001) using the eyeblink detection criteria described previously (Aksenov et al. 2004). The multiple single-unit signals from the microwires were amplified, band-pass filtered (300 Hz–3 kHz), and digitized (25 kHz/channel) using a custom data-acquisition system. Unit discrimination was performed off-line. Raster and peri-event histograms were constructed for each unit and experiment. Separate histograms were constructed for baseline trials, trials after the 1st injection, and trials after the 2nd injection. In each histogram, the baseline firing rate (25 ms before CS onset) and the time occurrence of significant excitatory and inhibitory changes were computed. Cell responses were considered significant if the modulation of the firing rate in the post-CS period exceeded the mean baseline ± tolerance limit for 2 consecutive 10-ms bins. Individual normalized cell histograms (spike frequency in Hz) were pooled together for each cell type, drug, and period of time to construct average population histograms. Baseline means of individual cell histograms were pooled together for each type of drug, concentration, and part of the experiment and statistically analyzed. Also, all histograms were binarized, with the logical one representing bins significantly exceeding the baseline rate. The binarized histograms were summarized to construct modulation frequency histograms depicting the relative number of cells showing significant excitatory and inhibitory modulation at each given time during the trial.

The statistical analysis of individual parameters of eyeblink responses, of the baseline firing of IN neurons, and of population histograms was conducted using 2-factor ANOVA with the following factors: drug (3 levels: DGG, DGG + PTX, and aCSF) and time/dose (3 levels: before injection, after the 1st injection, and after the 2nd injection). A separate analysis was performed on the results of the muscimol injection experiments, which included only one injection per experiment. ANOVA was followed by Newman–Keuls post hoc analysis. All significant results (see RESULTS) are based on the post hoc analysis unless specified otherwise. The provided numerical results are group means ± SE. Also, all reported significant effects of drugs were significant when compared both to the preinjection level and to the corresponding time period in the control experiment, unless specified otherwise. All statistical analyses were performed using J Neurophysiol • VOL 93 • JANUARY 2005 • www.jn.org

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Statsoft Statistica (a commercial analytical software package). The level for the rejection of null hypotheses was set to \( P < 0.05 \) in all tests.

**Histology**

After completing the experiments, animals were deeply anesthetized, and injection sites were marked by injecting 1 \( \mu l \) of tissue marking dye. The location of electrodes was marked by passing a 10-\( \mu A \) anodal DC current through each wire for 20 s. The animals were transcardially perfused, and the brains were removed from the skull and sectioned coronally at 50 \( \mu m \) on a freezing microtome. Sections were mounted onto gelatin-coated slides, reacted with ferrocyanide hydrochloride to visualize electrode-recording sites, and then stained with Luxol Fast Blue and Neutral Red. The locations of injection sites as well as electrodes were determined and transferred to a standardized set of 0.5-mm separated coronal sections of the rabbit cerebellum.

**RESULTS**

The effects of DGG and DGG + PTX injections reported herein were observed after injections at sites functionally identified with muscimol. Injection sites were chosen for further tests only when a small injection of 0.5 \( \mu l \) of muscimol (1.75 nmol, 200 ng) at the site abolished the expression of classically conditioned eyeblinks.

**Effects of glutamate antagonists on CR expression**

To determine the optimal amount of DGG required for maximal behavioral effects, we tested the effects of 1-\( \mu l \) injections of different DGG concentrations (from 1 to 98 mM). All of the DGG concentrations had similar effects: they slightly decreased CR incidence and increased CR latency. Even the highest concentration of DGG failed to abolish CRs. Although it is known that 100 mM DGG completely blocks glutamatergic cerebellar cortical input in vitro (Matsushita et al. 2002), we performed an additional functional test to verify the potency of the DGG. Three rabbits were implanted with guide tubes aimed at the HVI folium of the cerebellar cortex. Folium HVI is known to be involved in CR control, and blocking \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors at this region was previously shown to abolish conditioned eyeblinks in the rabbit (Atwell et al. 1999). Rabbirts conditioned in the eyeblink paradigm were injected with 1 \( \mu l \) of 98 mM DGG in HVI (Fig. 1C). Shortly after DGG injections in the cerebellar cortex, CRs became less frequent and eventually were abolished (Fig. 1, A and B). Because the same DGG amount also blocks CRs when infused in the inferior olive (Zbarska et al. 2003), we concluded that the highest tested concentration of DGG (98 mM) should be sufficient to also block the fast glutamate receptors in the IN, and therefore that concentration was used for the main experiment.

DGG injections in the IN had relatively small effects on CR performance (Fig. 2A). The 1st DGG microinjection (49 \( \mu mol \)) increased CR latency, had the tendency to decrease the amplitude of CRs, and slightly decreased CR incidence. The same effect was slightly more pronounced after the 2nd injection of DGG (an additional 49 \( \mu mol \)). At the group level, the latency of CRs before the injection was 224.4 \( \pm \) 5.8 ms (group mean \( \pm \) SE) in a group of 15 animals (Fig. 2C). After the 1st injection, CR latency significantly increased to 249.9 \( \pm \) 5.1 ms. After the 2nd injection of DGG, CR latency further increased to 253.5 \( \pm \) 5.5 ms. This increase, however, was not significant when compared with the 1st injection. Injections of aCSF did not have significant effects on CR latency.

Injections of DGG decreased CR incidence in a dose-dependent manner (Fig. 3). At the group level, CR incidence significantly decreased from the preinjection level of 90.2 \( \pm \) 6.5% to 73.2 \( \pm \) 1.9% after the 1st injection (trials 41–80) and to 63.2 \( \pm \) 3.6% after the 2nd injection (trials 81–200). For the reconstruction of injection sites, see Fig. 1 in our previous report (Aksenov et al. 2004).

**Effects of the DGG + PTX combination on CR performance**

The effects of the DGG + PTX cocktail on CR expression were reminiscent of the previously reported effects of PTX (Aksenov et al. 2004). Injections of DGG + PTX decreased CR incidence in a dose-dependent manner (Fig. 3). At the group level, CR incidence significantly decreased from 91.1 \( \pm \) 1.44% (\( n = 7 \)) CRs before injections to 42.1 \( \pm \) 4.45% after the 1st injection and to 18.4 \( \pm \) 3.65% after the 2nd injection. During the last block of trials in the DGG + PTX experiments, CRs were abolished in most of the animals. In contrast to the effects on CR incidence, CR latency was not significantly affected by the DGG + PTX combination.

**Effects of DGG on IN neuronal activity**

During DGG experiments, 100 cells expressing significant responses to the CS and/or US were recorded in the IN. The

![FIG. 1. Example of \( \gamma \)-d-glutamylglycine (DGG) effects when injected in the cerebellar cortex on the performance of classically conditioned eyeblinks. A: stack plot of eyeblink mechanograms from an experiment with an injection of 1 \( \mu l \) DGG. Experiment starts at the top and each plot represents one trial. Note that conditioned responses (CRs) [upward deflections between the conditioned stimulus (CS) and unconditioned stimulus (US) markers] were abolished shortly after the injection. B: CR incidence (means \( \pm \) SE) in a group of 3 animals injected in the cerebellar cortex. Injection of DGG severely reduced and eventually abolished CR incidence. C: location of injection sites (dots) for this group. All 3 injection sites were in the area of cerebellar lobule HVI. CS, onset of the conditioned stimulus; US, onset of the unconditioned stimulus.](http://jn.physiology.org/DownloadedFrom/10.1152/jn.00857.2004)
In general, 2 types of DGG effects on IN cell firing could be distinguished: the effect on spontaneous activity and the effect on neuronal responses. DGG slightly but significantly decreased the spontaneous discharge rate in MFC. The MFC baseline firing frequencies of about 18–30 Hz and exhibited large excitatory responses (frequently >100 Hz) in the CS–US interval. This excitatory response was often followed by an injection that started about 50 ms after the onset of the US. The 2nd largest cell group consisted of low-frequency cells that exhibited mostly excitatory responses (LFC, n = 28, Fig. 4D). LFC cells had low baseline firing rates (2–10 Hz), and their peak excitatory responses could reach ≥60 Hz. The third group of neurons consisted of high-frequency cells with inhibitory responses (HFC, n = 28, Fig. 4G). HFC typically had a baseline activity of 30–55 Hz and exhibited short-latency excitatory responses to the CS and a large inhibitory response to the US.

In general, 2 types of DGG effects on IN cell firing could be distinguished: the effect on spontaneous activity and the effect on neuronal responses. DGG slightly but significantly decreased the spontaneous discharge rate in MFC. The MFC baseline rate before DGG injections was 25.1 ± 1.6 Hz, and decreased to 22.1 ± 1.6 Hz after the 1st injection and to 18.9 ± 1.5 Hz after the 2nd DGG injection. The spontaneous firing rates of HFC and LFC were not affected by DGG injections. The mean spontaneous frequencies in the corresponding time periods were 3.4 ± 0.3, 3.1 ± 0.4, and 2.6 ± 0.3 Hz in the LFC cell subpopulation and 46.4 ± 1.7, 47.6 ± 2.7, and 42.4 ± 2.6 Hz in HFC. With all 3 cell types pooled together (n = 100), the DGG effect on the spontaneous firing rate was not significant. Similarly, injections of aCSF in control experiments did not affect the spontaneous firing rate of IN cells.

The responses to the CS (in the interval between the onsets of the CS and US) were not abolished by DGG injections (Fig. 4, C, F, and I). The amplitude of the neuronal excitatory modulation visibly decreased in all 3 cell types after both DGG injections (compare Fig. 4, A vs. C, D vs. F, and G vs. I). A decrease of modulation amplitude was observed during control injections of vehicle (Fig. 4, M–O). ANOVA performed on normalized data (the baseline was subtracted from population histograms and the response amplitudes were expressed as a percentage of the mean amplitude before drug injection) yielded a significant effect of drug injection for all cell types and also for the control experiment. A separate ANOVA performed on normalized population histograms from DGG and control experiments (all cell types pooled) yielded a significant effect of injection number, but no effect of the type of drug. This finding seems to indicate that the involvement of fast glutamate receptors in the depth of modulation of IN neuronal activity is relatively small, if any. A gradual decrease of cell responses does not seem to be unique to injection experiments: we observed this phenomenon in occasional experiments that did not involve any injections. It is likely that at least part of the excitatory modulation decrease in DGG injection experiments was nonspecific, perhaps related to the fatigue of underlying neuronal substrates. Perhaps the most dramatic effect of the DGG injections was on inhibitory responses to the US. These inhibitory responses were abolished after the 2nd DGG injection (Fig. 4, A–C and G–I).

The effects of DGG and vehicle on the relative incidence of significant neuronal responses at the population level are illustrated in Fig. 5. Both injections of DGG decreased the incidence of bins with excitatory responses. The mean percentage of cells exhibiting excitatory responses per bin in the CS–US period declined from 32.5% (before injection) to 18.5% (after DGG).
the 2nd DGG injection). This change was statistically significant. The incidence of inhibitory responses (occurring predominantly after the onset of the US) dramatically declined after the 1st DGG injection, and these responses were practically abolished after the 2nd injection of the drug. Changes in the frequency of both excitatory and inhibitory responses in control experiments did not reach statistical significance.

**Effects of DGG + PTX on IN neuronal activity**

Overall, 43 modulated IN cells were recorded in experiments involving combined DGG and PTX injections. Because the number of recorded cells was not large enough to warrant separating them into subpopulations, the data from all cells in this part of the study were analyzed as a single group. Injections of DGG + PTX in the IN affected IN cell baseline firing rate as well as the responses to stimuli in a dose-dependent manner (Fig. 4, J–L). Similar to the effect of PTX alone (Aksenov et al. 2004), the spontaneous firing rate of recorded cells significantly increased (more than doubled) from 22.2 ± 2.4 to 50.6 ± 4.9 Hz after the 1st injection, and to 56.7 ± 6.3 Hz after the 2nd injection of the DGG + PTX cocktail. This observation contrasted with the aCSF and DGG alone injections, in which either no or small changes of spontaneous IN neuronal activity were seen. In parallel to changes in baseline firing rate, DGG + PTX injections greatly reduced and then virtually abolished all types of stimuli-related firing modulation. This effect could be seen both in the average population histograms (Fig. 4, J–L) as well as in frequency plots of significant neuronal modulation (Fig. 5).

**DISCUSSION**

The present study demonstrated that blocking fast glutamate neurotransmission in the cerebellar interposed nuclei has an unexpectedly small effect on the expression of classically conditioned eyeblinks and on the activity of IN cells. Injections of DGG decreased CR incidence but did not abolish CRs and had relatively small effects on the task-related modulation of IN cells. Combined injections of DGG + PTX abolished CRs as well as the CS- and US-related activity of IN neurons.

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**FIG. 4.** Effects of DGG, DGG + PTX, and aCSF injections on the recorded populations of interposed nuclei (IN) cells. Averaged peristimulus population histograms are arranged in rows corresponding to subpopulations of cells and injected drugs and in columns corresponding to the activity before injections, after the 1st injection, and after the 2nd injection. **A–C:** medium-frequency cells (MFC) in DGG experiments. **D–F:** low-frequency cells (LFC) in DGG experiments. **G–I:** high-frequency cells (HFC) in DGG experiments. **J–L:** all cells recorded during DGG + PTX injections. **M–O:** all cells recorded during control experiments. Note that DGG (A–I) did not affect baseline activity (before the CS marker), but reduced the depth of the excitatory modulation of cell activity. Note the disappearance of US-related inhibitory modulation of HFC cells. DGG + PTX increased baseline activity of the cell population and the modulation of cell activity was first diminished and then virtually abolished after the 2nd injection. No changes of baseline activity and a slight decrease of cell modulation were observed in aCSF experiments. Bin width = 10 ms. Horizontal lines in each histogram represent tolerance limits. CS, onset of conditioned stimulus; US, onset of unconditioned stimulus.
**Behavioral effects of DGG and DGG + PTX**

Several concepts of cerebellar involvement in eyeblink conditioning and CR expression postulate a major role for the direct input of mossy and climbing fiber collaterals in the IN (Christian and Thompson 2003; Raymond et al. 1996). These notions are primarily based on 2 observations: 1) cells in the IN express neuronal correlates of conditioned eyeblinks (Aksenov et al. 2004; Berthier and Moore 1990; Jimenez-Diaz et al. 2002) and 2) although lesions of the IN abolish CRs, several studies have reported preserved CRs after large lesions of the cerebellar cortex (McCormick and Thompson 1984; Steinmetz et al. 1992). Assuming that modulation of IN neurons is required for CR expression, the ability of rabbits to produce CRs in the absence of the cerebellar cortex implies that IN neuronal responses are driven by direct inputs of cerebellar afferents in the IN. In spite of the pivotal significance of this concept, the function of direct inputs has never been confirmed. Over the last several years, we have conducted a series of pilot neuropharmacological experiments with the objective of selectively blocking the input of mossy and climbing fiber collaterals, which are assumed to carry the information about the CS (mossy fibers of pontine origin) and the US (climbing fibers originating in the inferior olive). Because these projections to the cerebellum are presumably glutamatergic and because it is known that cerebellar nuclear cells possess functioning N-methyl-d-aspartate (NMDA) and AMPA receptors (Audinat et al. 1990, 1992), we tested a number of fast glutamate receptor antagonists (CNQX, DNQX, AP-5, kynurenic acid, DGG) and their combinations to block these IN inputs in rabbits expressing previously learned responses. Although some of these drugs had limited effects on CR expression (e.g., AP-5), we failed to abolish the expression of previously acquired CRs in all of these studies. On a comparative scale, the most notable results are from the DGG injections reported in this study. DGG is an antagonist of both AMPA/kainate and NMDA glutamate receptors (Wadiche and Jahr 2001; Watkins et al. 1990). To ensure the maximum block of fast glutamate neurotransmission in the functionally relevant parts of the IN, we took several precautions. First, the drug dose exceeded that which is known to block excitatory postsynaptic potentials in Purkinje cells in response to climbing fiber input (Matsushita et al. 2002). To confirm the potency of the drug, we verified that the same concentration, amount, and batch of DGG block the expression of CRs when applied to the ipsilateral intermediate cerebellar cortex, as expected based on previous reports (Attwell et al. 1999). Finally, to deliver the drug to relevant parts of the IN, all animals were injected with DGG only at sites which small microinjections of muscimol (0.5 µl, 200 ng) abolished the expression of CRs (Bracha et al. 1994). DGG injections reduced CR incidence in a dose-dependent manner and slightly increased CR latency. These effects were small when compared with the effects of inactivating the IN with muscimol (Bracha et al. 1994; this study) or overactivating the same area with PTX (Aksenov et al. 2004). This finding is in agreement with a previous report that demonstrated a small decrease of CR frequency after blocking cerebellar nuclear NMDA receptors (Chen and Steinmetz 2000). On the other hand, our unpublished pilot experiments as well as data from another laboratory (Attwell et al. 2002) demonstrated that selectively blocking only AMPA/kainate receptors in the IN

**FIG. 5.** Population histograms of the relative incidence of significant excitatory (black line) and inhibitory (gray line) responses in the population of the 100 cells recorded in the DGG experiments (A–C), the 43 cells in the DGG + PTX (D–F) experiments, and the 48 cells in the aCSF control experiments (G–I). For comparison purposes, previously published (Aksenov et al. 2004) effects of PTX injections (2 0.5-µl injections, 1.25 nmol of PTX each) are provided in the 3rd row of the figure (G–I). Columns correspond to the time periods before, after the 1st injection, and after the 2nd injection. Note that DGG reduced but did not abolish the incidence of excitatory responses. On the other hand, inhibitory responses to the US were practically abolished after the 2nd DGG injection. DGG + PTX virtually abolished both the excitatory and inhibitory response. A similar, but slightly less pronounced effect was observed in the previous PTX injection experiments (G–I). In control (aCSF) experiments, the incidence of excitatory responses in the CS–US interval slightly declined.
has no effect on CR expression. These findings collectively suggest that most of the DGG effects observed in the present study are related to the action of this drug on NMDA receptors. In summary, even though fast glutamate receptor-mediated neurotransmission seems to have an accessory role in the expression of previously learned eyelink CRs, it is not essential because rabbits are able to produce CRs even when both AMPA/kainate and NMDA receptors are blocked. This finding fails to support the concepts postulating the critical role of glutamatergic inputs from collaterals of mossy and climbing fibers during the execution of conditioned eyelinks.

Simultaneously blocking both GABA and fast glutamate neurotransmission using the cocktail of DGG and PTX decreased CR incidence and eventually abolished conditioned eyelinks. This finding resembles the results of previous studies that demonstrated that PTX injections alone can block the expression of CRs (Aksenov et al. 2004; Attwell et al. 2002; Mamounas et al. 1987). The effect of PTX on CR incidence was previously attributed to excessive activation of IN cells (Aksenov et al. 2004; also see the discussion of drug effects on IN activity below). Besides effects on CR incidence, previous studies also reported either dramatic (Garcia and Mauk 1998) or modest (Aksenov et al. 2004) shortening of CR latencies after PTX injections into the IN. Interestingly, the DGG + PTX combination in this study did not alter CR latency. It could be that the degree and/or the presence of CR latency changes reflect nuances in the precise location of injections in the respective studies. However, because the injection sites in the present study were identical to our previous report (Aksenov et al. 2004), it is more likely that DGG somehow counterweighed the PTX effects on CR latency.

Effects of DGG and DGG + PTX on IN neuronal activity

Cells in the deep cerebellar nuclei exhibit spontaneous activity that is modulated during eyelink conditioning in response to the CS and US and also during the execution of conditioned and unconditioned blinks (Aksenov et al. 2004; Berthier and Moore 1990; Jimenez-Diaz et al. 2002). A number of in vitro studies demonstrated that the spontaneous activity of cerebellar nuclear cells is caused by a combination of their pacemaker-like properties and of the tonic effects of GABA_A (Mouginot and Gahwiler 1996) and glutamate (Anchisi et al. 2001) inputs. The modulated responses (predominantly excitatory during most behaviors) of nuclear cells could be induced either by a transient decrease of inhibitory input from cortical Purkinje cells (Aizenman and Linden 1999; Llinas and Muhlethaler 1988), or by an increased excitatory glutamate drive from collaterals of climbing and mossy fibers (Anchisi et al. 2001; Gauck and Jaeger 2003), or by a combination of both GABA and glutamate inputs. Which of these processes determines the activity of IN cells in rabbits expressing previously learned conditioned eyelinks?

In our previous study, we demonstrated that blocking chloride channels using microinjections of PTX affects both the spontaneous activity and the phasic responses of IN cells (Aksenov et al. 2004). Because PTX interferes with the mechanisms by which Purkinje cells regulate IN activity, the application of this drug dramatically increased the tonic activity of IN cells and also reduced their modulation during conditioning trials. It was not clear whether the high spontaneous firing rate after PTX reflected intrinsic neuronal activity and/or unopposed glutamate-mediated excitation from collaterals of mossy and climbing fibers. The present study was designed to further illuminate this issue by blocking the fast glutamatergic neurotransmission using DGG. Consistent with the relatively small effect of DGG on CR expression, recordings of single-unit activity close to injection sites demonstrated that blocking fast glutamate receptors had surprisingly small effects on IN neurons. At the population level, DGG did not affect IN cell baseline firing activity, with the exception of MFC, which significantly decreased their firing rate after the 2nd DGG injection. It should be noted, however, that even in MFC, DGG did not abolish spontaneous activity. This finding is consistent with the notion that the spontaneous activity of IN cells is driven by their intrinsic properties and that in MFC (but not others) the spontaneous rate can be slightly enhanced by the background activity of glutamatergic inputs. This notion was further supported by the results of the combined DGG + PTX injections that dramatically increased the spontaneous activity of IN cells. Because blocking fast glutamate receptors did not prevent PTX-induced upregulation of IN spontaneous rate, it can be concluded that in the rabbit, the spontaneous firing of IN cells is primarily driven by a pacemaker-like process that is, in the normal state, under strong tonic inhibition by Purkinje cells and is moderately facilitated by glutamate inputs.

Fast glutamate neurotransmission seems to contribute to the modulation of IN neuronal responses to the CS and US. This was evidenced by the decrease of the mean frequency of significant excitatory responses per bin to the CS and by the abolition of inhibitory responses to the US. With respect to this effect, it should be emphasized that similar to the behavioral effects of DGG, the responses to the CS had a lower frequency, but were not abolished. Consequently, glutamate input from the collaterals of mossy and climbing fibers seems to play only an accessory role in producing IN neuronal responses. On the other hand, blocking both GABA_A and fast glutamate neurotransmission at the same time virtually abolished both excitatory and inhibitory modulation of IN firing. This indicates that GABA_A input significantly contributes to the generation of IN excitatory responses. Interestingly, the frequency of excitatory responses after DGG + PTX injections seems to be slightly lower than that after PTX alone (Aksenov et al. 2004; for a direct comparison see Fig. 5, D–F and G–I). These findings collectively suggest that the modulation of IN neuronal activity is a combined product of glutamatergic and GABAergic inputs. On a comparative scale, GABAergic input seems to be dominant and in addition to its involvement in the modulation of IN neuronal activity, it exerts a strong influence over the spontaneous activity of IN cells.

Unexpectedly, DGG reduced the incidence of inhibitory responses to the US. This result is paradoxical because glutamate is known to be an excitatory neurotransmitter. Could it be that the observed reduction of IN cell inhibitory modulation was attributable to the spread of the drug to the overlying cerebellar cortex? This seems unlikely because blocking glutamatergic neurotransmission in the cortex would be expected to increase baseline firing of IN cells as a result of the reduction of excitatory drive to inhibitory Purkinje cells. No such correlation between the changes in baseline firing and inhibitory modulation was observed. It is more likely that the abolition of IN inhibitory responses produced by DGG reflects blocking
glutamate input to local IN inhibitory interneurons, or that it is caused even more indirectly by feedback circuits between the cerebellum and other parts of the brain stem.

**Fast glutamate and GABA_\text{A} neurotransmission in the cerebellar nuclei and the control of conditioned eyeblinks**

The cerebellar nuclei receive 2 major inputs: a massive GABAergic input from the Purkinje cells of the cerebellar cortex and a relatively smaller glutamatergic input from collaterals of mossy and climbing fibers. The present study, together with our previous experiments (Aksenov et al. 2004), demonstrates that the 2 major neurotransmitter systems in the IN play a differential role in the control of IN neural activity and in the expression of classically conditioned eyeblinks in the rabbit. Activating GABA_\text{A} receptors with muscimol silences IN cells and abolishes the expression of conditioned eyeblinks (Aksenov et al. 2004). On the other hand, blocking chloride channels with PTX dramatically increases IN spontaneous firing and decreases the incidence of both excitatory and inhibitory modulation of IN cells. This cellular effect of PTX is accompanied first by a slight decrease of CR latency that is followed by a more dramatic event—the disappearance of behavioral CRs. These findings lead to 2 conclusions: 1) the GABA_\text{A} system (represented by both the external projections from the cerebellar cortex and by intrinsic GABAergic interneurons) exerts powerful control over spontaneous firing as well as over the modulation of IN neuronal activity in the rabbit; and 2) the behavioral expression of conditioned eyeblinks is dependent on an optimal level of IN activity because both silencing and excessive activation of IN cells block CR expression.

The present study added an important component to understanding how the intermediate cerebellum contributes to CR control. Specifically, the fast glutamate receptor-mediated input from the collaterals of mossy and climbing fibers plays only an accessory role. It is not essential for the expression of behavioral CRs, and the extent of its effect on IN neuronal activity is smaller when compared with the GABA_\text{A} system. This conclusion is consistent with the suggestion that the involvement of the intermediate cerebellum in conditioned eyelink control critically depends on the normal function of the cerebellar cortex (Attwell et al. 2001, 2002). As evidenced by the presence of CRs in animals injected with DGG, direct input from mossy fiber collaterals into the IN (which receives unobstructed information from the cerebellar cortex) is not required for, and therefore does not primarily drive, behavioral CRs.

These findings have fundamental implications for the interpretation of previous behavioral studies. For instance, earlier experiments demonstrated that lesioning the pontine nuclei (which are a major source of cerebellar mossy fibers) abolishes the expression of conditioned eyeblinks (Steinmetz et al. 1987). In light of the present results, this effect is most likely in large part mediated through mossy fiber input to the cerebellar cortex. Similarly, previous studies indicated that inactivating the inferior olive (the sole source of climbing fibers) either immediately (Welsh and Harvey 1991; Zbarska et al. 2003) or gradually (Medina et al. 2002) abolished behavioral CRs. No such effects were observed in the present study when the direct inferior olive input to the IN was presumably blocked by injections of DGG. This suggests that the behavioral effects of inferior olive manipulations are primarily driven by physiological changes induced outside of the IN, probably in the cerebellar cortex. The present results also have interesting implications for a report that showed that CRs blocked by muscimol infused in the IN can be restored by injecting PTX at the same site (Bao et al. 2002). Because the muscimol/PTX combination completely blocks GABA_\text{A} neurotransmission in the IN, the study by Bao et al. seemed to suggest that some other input to the IN, perhaps the collaterals of mossy fibers, drives the expression of the “restored” eyeblinks. If so, it would be expected that blocking mossy fiber input into the IN should abolish conditioned eyeblinks. This prediction was not confirmed by the present study. This suggests two intriguing possibilities that should be addressed in future experiments. Is it possible that mossy and climbing fibers use some other excitatory neurotransmitter in addition to glutamate? Or, could it be that the expression of behavioral CRs depends more on an optimal level of tonic IN activity than on its modulation?

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**References**


