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Bengtsson, Fredrik; Svensson, Pär; Hesslow, Germund

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PO Box 117  
221 00 Lund  
+46 46-222 00 00

# Feedback control of Purkinje cell activity by the cerebello-olivary pathway

F. Bengtsson, P. Svensson and G. Hesslow

Department of Physiological Sciences, Section for Neurophysiology, BMC F10, S-221 84 Lund, Sweden

**Keywords:** cerebellum, climbing fibres, deep cerebellar nuclei, inferior olive, nucleo-olivary pathway

## Abstract

The pathway from the deep cerebellar nuclei to the inferior olive, the source of the climbing fibre input to the cerebellum, inhibits olivary transmission. As climbing fibre activity can depress the background firing of the Purkinje cells, it was suggested that nucleo-olivary (N–O) inhibition is a negative feedback mechanism for regulating Purkinje cell excitability. This suggestion was investigated, in a set-up with decerebrate ferrets, both by blocking and by stimulating cerebellar output while recording Purkinje cell activity. Blocking the N–O pathway was followed by an increased climbing fibre activity and a dramatic reduction in simple spike firing. Stimulation of the N–O fibres depressed climbing fibre responses and caused an increase in simple spike firing. These results are taken as support for the feedback hypothesis.

## Introduction

The deep cerebellar nuclei project not only to the cerebral cortex and to various motor centres in the brainstem, but also to the source of the climbing fibre input to the cerebellum, the inferior olive. This nucleo-olivary (N–O) pathway was previously thought to be excitatory but is now known to exert a strong inhibitory control of the inferior olive (Hesslow, 1986; Andersson & Hesslow, 1987a,b; Andersson *et al.*, 1988) and to be GABA-ergic (Nelson & Mugnaini, 1989). In addition, there are also reciprocal olivo-nuclear connections (De Zeeuw *et al.*, 1997). There is no agreement on the function of the N–O projection and different views on this tend to reflect divergent views on the role of the climbing fibre input to the cerebellum. One hypothesis is that the pathway controls electrotonic coupling between olivary neurons and that by modulating this coupling, the N–O pathway could control which groups of Purkinje cells in the cerebellar cortex that would receive coordinated climbing fibre input (Welsh & Llinas, 1997).

A very different suggestion proposed by us (Andersson *et al.*, 1988) is that the N–O inhibition functions as a negative feedback system for controlling cerebellar learning as well as the spontaneous firing level of the Purkinje cells. In a previous investigation (Hesslow & Ivarsson, 1996) we put forward evidence that the N–O pathway indeed has the capability to control learning. However, the suggestion that the N–O inhibition regulates Purkinje cell background activity has not been tested directly.

The suggestion that N–O inhibition could serve as a feedback mechanism for regulating Purkinje cell activity was based on the demonstration that increased activity in climbing fibres from the inferior olive causes a suppression of the background firing rate of Purkinje cells (Rawson & Tilokskulchai, 1981) and, conversely, that silencing the climbing fibres by inactivating the olive causes an increased firing rate in Purkinje cells (Colin *et al.*, 1980; Montarolo *et al.*, 1982; Cerminara & Rawson, 2004). Hence, it is a plausible argument that an increased activity of the Purkinje cells will cause a

suppression of neurons in the deep cerebellar nuclei and, consequently, a reduced inhibition of the inferior olive. This would lead to an increased olivary firing that in turn will depress Purkinje cell activity and release activity in deep nuclear cells.

Although this speculation is consistent with current knowledge, it is far from trivial. For instance, we do not know if physiologically natural variations in the intensity of the N–O inhibition will appreciably change the background firing rate of the olivary neurons to an extent that changes Purkinje cell background firing significantly. This would seem to be a necessary requirement for the feedback hypothesis.

The purpose of the experiments reported here was to determine the effects of blocking and of direct electrical stimulation of the N–O pathway on Purkinje cell activity (Fig. 1A). The effects of both manipulations should reveal if the N–O pathway can control Purkinje cell background activity and thus if one minimum requirement of the feedback hypothesis is met.

## Materials and methods

### *Anaesthesia and surgery*

Data were collected from experiments performed on 10 decerebrate male ferrets (1.05–1.9 kg). Recordings were made from 15 Purkinje cells (nine in the lignocaine injection experiments and six in the N–O stimulation experiments). The animals were deeply anaesthetized with 1.5–2% isoflurane (Abbot Laboratories Ltd, UK) in a mixture of O<sub>2</sub> and N<sub>2</sub>O. They were initially placed in a box into which anaesthetic gas was directed. When deep anaesthesia had been achieved, a tracheotomy was performed and the gas was then channelled directly into a tracheal tube. In four cases the initial gas anaesthesia was switched to intravenously administered propofol (Diprivan 10 mg/mL; Astra Läkemedel AB, Sweden) until the time of decerebration. The propofol was administered continuously and usually 4–6 mL were administered before the decerebration. In all cases the level of anaesthesia was monitored regularly by testing withdrawal reflexes. The level of anaesthesia was kept so that no reflexes were elicited when the animals were pinched with a pair of

Correspondence: Fredrik Bengtsson, as above.

E-mail: fredrik.bengtsson@mphy.lu.se

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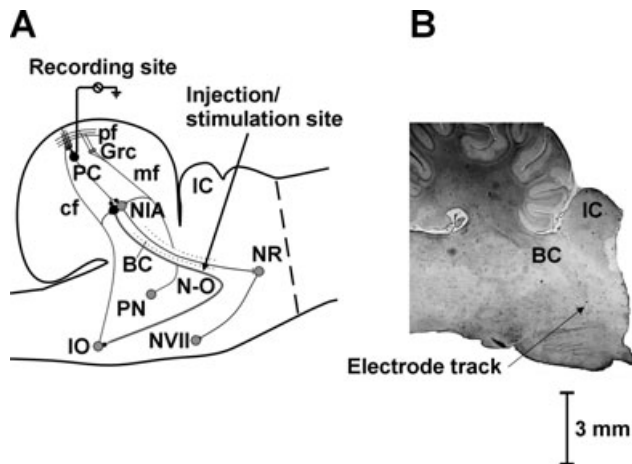


Fig. 1. Experimental set-up and sample histological section. (A) Experimental set-up and wiring diagram of the cerebellar cortex, deep cerebellar nuclei and the inferior olive. Purkinje cell (PC), climbing fibre (cf), granule cell (Grc), mossy fibre (mf), parallel fibre (pf), anterior interpositus nucleus (NIA), inferior olive (IO), nucleo-olivary fibres (N-O), nucleus ruber (NR), facial nucleus (NVII), brachium conjunctivum (BC), pontine nuclei (PN). Site of recording, stimulation and lignocaine injection indicated in the figure. (B) Sample histological section (low decerebration, see methods) showing the brainstem and cerebellum. Electrode track for stimulation of the N-O fibres indicated by arrow. Abbreviations as defined in A.

forceps in the plantar side of the paws. In order to prevent oedema of the cerebellum and disseminated coagulation, 0.5 mL betamethasone *i.v.* (Betapred, 4 mg/mL; Glaxo Operations Ltd, Greenford, UK) and 0.5 mL doctacillin *i.m.* (250 mg/mL; Astra, Södertälje, Sweden) were given at the start of the experiments. During the whole experiment infusion was given intravenously [glucose 50 mg/mL, isotonic Acetate Ringer, Albumin fraction V (from bovine serum) Merck Eurolab 0.004 mg/mL; proportion 1 : 1; 3 mL/kg/h]. The albumin was added to prevent slowly developing oedema of the cerebellum.

After the head had been fixed to a stereotaxic frame, the skull was opened on the left side and substantial portions of the cerebral hemispheres and the thalamus were removed by aspiration. This exposed the left half of the cerebellum and the superior and the inferior colliculi of the brainstem. When surgery was complete, the animals were decerebrated by a section with a blunt spatula through the brainstem  $\approx 1$  mm rostral to the superior colliculus and the red nucleus (high decerebration). In two cases the brainstem was sectioned just rostral to the inferior colliculus (low decerebration, Fig. 1B). The anaesthesia was then terminated. The completeness of the brainstem section was always checked by post mortem examination. In a previous investigation (Ivarsson *et al.*, 1997) bilateral electroencephalogram (EEG) activity was recorded from several sites on the remaining cortex in a similar preparation. These recordings showed no activity several hours after the decerebration. A pool of cotton-reinforced agar was constructed and the exposed brain was covered with warm high-density insulating carbon-fluor liquid (FC40, 3M Company, Belgium). The animals were kept on artificial respiration throughout the experiment. The end-expiratory  $\text{CO}_2$  concentration, arterial blood pressure and rectal temperature were monitored continuously and kept within physiological limits.

#### Mesencephalic lignocaine injections

After an hour of recovery, a tungsten electrode (30  $\mu\text{m}$  diameter, deinsulated tip 75  $\mu\text{m}$ ) was lowered into the medulla in a series of

consecutive 250  $\mu\text{m}$  lateral-to-medial tracks, just caudal to the inferior colliculus. Trains of stimuli (three square pulses, 0.5 ms, 333 Hz, 100  $\mu\text{A}$ ) were given at different depths, in 250- $\mu\text{m}$  steps, in each track in order to locate the brachium conjunctivum. The electrode was assumed to be in the brachium conjunctivum when a site for eliciting short latency (5–8 ms), low threshold (14–55  $\mu\text{A}$ ) electromyograph (EMG) activity in the ipsilateral orbicularis oculi muscle was found. This site was usually found 3–3.5 mm lateral to the midline at a depth of 3.25–3.75 mm. Subsequently the electrode was replaced by a lignocaine (Xylocain 40 mg/mL; Astra) filled pipette (tip diameter 50–100  $\mu\text{m}$ ) attached to a Hamilton syringe. Because it is known that the N-O fibres run between 0.75 and 1 mm ventral to brachium conjunctivum in the cat (Legendre & Courville, 1987), the lignocaine-filled micropipette was placed 1–2 mm dorsal to the brachium conjunctivum. Just before the injection the pipette was lowered to the position of the N-O fibres and 2  $\mu\text{L}$  lignocaine was injected manually over a time period of 15–30 s. In three cases where a second injection of lignocaine was made in the same animal, the second injection was not administered until at least 1 h after the first.

#### Stimulation of the N-O pathway

The same procedure as in the injection experiments was used for localizing brachium conjunctivum in the high decerebrate preparations. Subsequent to the identification of the brachium conjunctivum, the tungsten electrode was lowered in the same track in 250- $\mu\text{m}$  steps and trains of stimuli were given (five square pulses, 0.5 ms, 200 Hz, 100  $\mu\text{A}$ ). At the same time the amplitude of a climbing fibre response, elicited by periorbital stimulation (for details see below), was determined by recording from the surface of the cerebellar cortex in the C3 zone with a pair of monopolar silver balls ( $\phi \approx 250 \mu\text{m}$ ) electrodes. Onset of the mesencephalic stimulation was time-locked to the onset of the periorbital stimulation which it preceded by 45 ms; this time was chosen as it is known that the latency for peak inhibition is between 35 and 50 ms in the cat (Hesslow, 1986; unpublished data reveal similar latencies in ferrets). The site, at which the mesencephalic stimulus caused a more than 50% reduction of the climbing fibre response amplitude with a stimulus strength of 20  $\mu\text{A}$ , was considered to be sufficiently close to the N-O fibres. Usually this site was found 4–4.75 mm ventral to the surface of the colliculus. The electrode was then left at this point.

In the low decerebrate animals, stimulation of brachium conjunctivum did not elicit eye blinks. In these animals we therefore had to rely on the positions of previous identifications of the N-O fibres. The same tracking procedure as described above for the identification of the N-O fibres was used.

In the test situation, during the Purkinje cell recordings, the stimulus frequency was lowered and a range of frequencies was tested (1–10 Hz). The stimulation lasted for 5.2 min and consisted of 0.5 ms single pulses delivered at the frequencies described above. Two stimulus strengths (20/60  $\mu\text{A}$ ) were tested. Stimulus strengths were chosen so that the stimulation caused a net inhibition of the complex spike frequency.

#### Periorbital stimulation

Peripheral electrical stimulation (one square pulse, 0.5 ms, 0.5 Hz, 3 mA) was applied through two stainless steel electrodes (insulated insect pins, deinsulated tip  $\approx 100 \mu\text{m}$ ), the tips of which were inserted approximately 5 mm apart into the skin of the medial part of the

periorbital area. This stimulus intensity reliably elicited climbing fibre responses in the C3 zone of the cerebellar cortex.

### Recordings

The cerebellar blink area (Hesslow, 1994) in the C3 zone of lobule HVI was localized by stimulation of the periorbital skin (as described above) while recording the evoked climbing fibre response from the cerebellar surface with a pair of monopolar silver ball electrodes (diameter  $\approx 250 \mu\text{m}$ ). Single unit recordings were then performed in the blink area either with self-made glass-coated tungsten electrodes (exposed tips 6–15  $\mu\text{m}$ ) or with commercially available glass-coated quartz-platinum/tungsten electrodes (tip diameter = 30–40  $\mu\text{m}$ ; Thomas Recording GmbH, Germany). During the recordings the animals were curarized (Norcuron, 10 mg/mL; Organon Teknika bv, Bostel, The Netherlands). The recordings were put through an A/D converter, CED micro 1401 (CED Ltd, Cambridge, UK) and the results were processed in the CED program Spike2.

### Histology

After the experiments, the animals were perfused with sodium chloride followed by 10% (w/v) formaldehyde (Merck, Germany) in phosphate buffer (0.2 M, pH 7.7) solution. The cerebellum was removed from the skulls, stored in 10% formaldehyde for at least 3 weeks, frozen and sectioned transversally or sagittally in 50- $\mu\text{m}$  slices in a cryostat. The slices were mounted and stained with cresyl violet and then examined under a light microscope. A sample histological section (low decerebration, as described earlier) is shown in Fig. 1B.

### Analysis

Criteria for identifying extracellularly recorded climbing fibre responses, 'complex spikes', follow those of Thach (1968) and Eccles *et al.*, (1967) (see Fig. 2 for sample records). Simple and complex spikes were discriminated using the CED program Spike2. In cases of

uncertainty, data were also analysed manually. Recordings that did not contain any complex spikes were not included in the study. In two cases where computer discrimination between simple and complex spikes was inadequate we chose to only analyse the effect on simple spikes.

To quantify the effects of blocking and stimulation of the N–O fibres we measured the frequencies of complex and simple spikes before, during and after manipulations. For statistical analysis of the injection experiments we compared three time periods. The preinjection time period started 5 min before injection and lasted to the time of injection (–5–0 min). The postinjection time period was divided into two periods. The first started at the time of injection and lasted 5 min postinjection (0–5 min). The second postinjection period started 5 min postinjection and lasted 5 min (5–10 min).

In the stimulation experiments the analysis was also divided into three time periods. Here the preinjection period started 2 min before the onset of the stimulation and lasted to the onset of the stimulation (–2–0 min). The first stimulation time period starts at the onset of the stimulation and lasts 2 min (0–2 min). The second stimulation time period starts 2 min after the stimulus onset and lasts 2 min (2–4 min). In the analysis of both experiments we used an unpaired *t*-test to evaluate the significance ( $P < 0.05$ ) of the post manipulation changes in complex and simple spike frequencies compared to pre manipulation levels. Individual *P*-values are indicated in the results. Corrections were made, where appropriate, for significant differences in variance.

## Results

### Lignocaine injections

An hour after recovery from surgery, a tungsten electrode was lowered into the medulla in a series of consecutive tracks at the border between the cerebellum and the inferior colliculus. Trains of stimuli were given at different depths in each track while recording EMG from the orbicularis oculi muscle in order to locate the brachium conjunctivum. After the brachium conjunctivum had been localized the electrode was replaced by a lignocaine-filled pipette. Next, a microelectrode was inserted into the C3 zone blink area (Hesslow, 1994) in lobulus HVI. After a stable Purkinje cell recording had been obtained, the lignocaine pipette was placed 0.75–1 mm ventral to the brachium conjunctivum where the N–O fibres are known to be located (Legendre & Courville, 1987) and 2  $\mu\text{L}$  lignocaine was injected to block the N–O inhibition (Fig. 1). Previous experiments (Tehovnik & Sommer, 1997) have shown that infusions (4  $\mu\text{L}/\text{min}$ ) of 2  $\mu\text{L}$  2% lignocaine, in the cerebral cortex of monkeys, had negligible effects on neuronal background activity 1 mm away from the injection site.

The effects of the injections were usually quite dramatic. In the sample records shown in Fig. 2, the complex spike frequency more than doubled after the injection, while the simple spikes disappeared completely. This effect was obtained within seconds after the injection suggesting that the pipette was placed accurately, close to the N–O fibres.

The effect of the injections are illustrated for all cells in Fig. 3A. In the graphs different symbols represents the level of activity (Hz) in individual cells based on averaged numbers of spikes in 5-min intervals pre- and postinjection. The same symbols are used in both the complex (upper) and the simple spike (lower) graphs. The figure showing the effect on simple spike activity has two more graphs than the complex spike frequency figure. This is because the computer discrimination between simple and complex spikes was inadequate in these cases and we therefore chose to analyse only the effect on simple

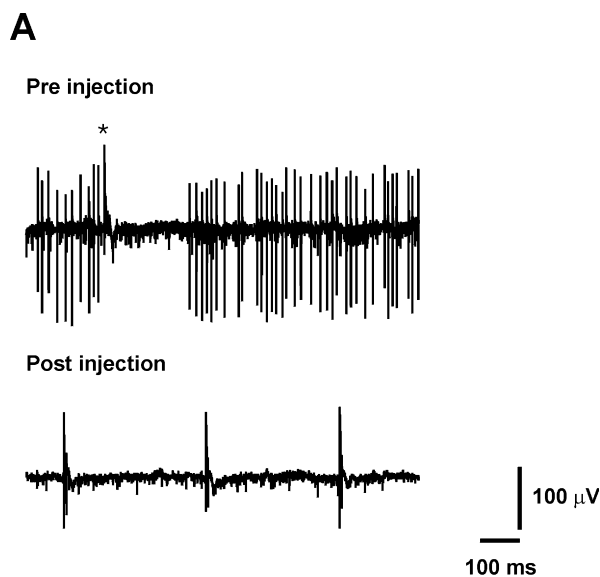


FIG. 2. Purkinje cell activity before and after blockade of the nucleo-olivary fibres. Sample records of Purkinje cell activity pre- and post-lignocaine injection (2  $\mu\text{L}$ ). Complex spike indicated with an asterisk.

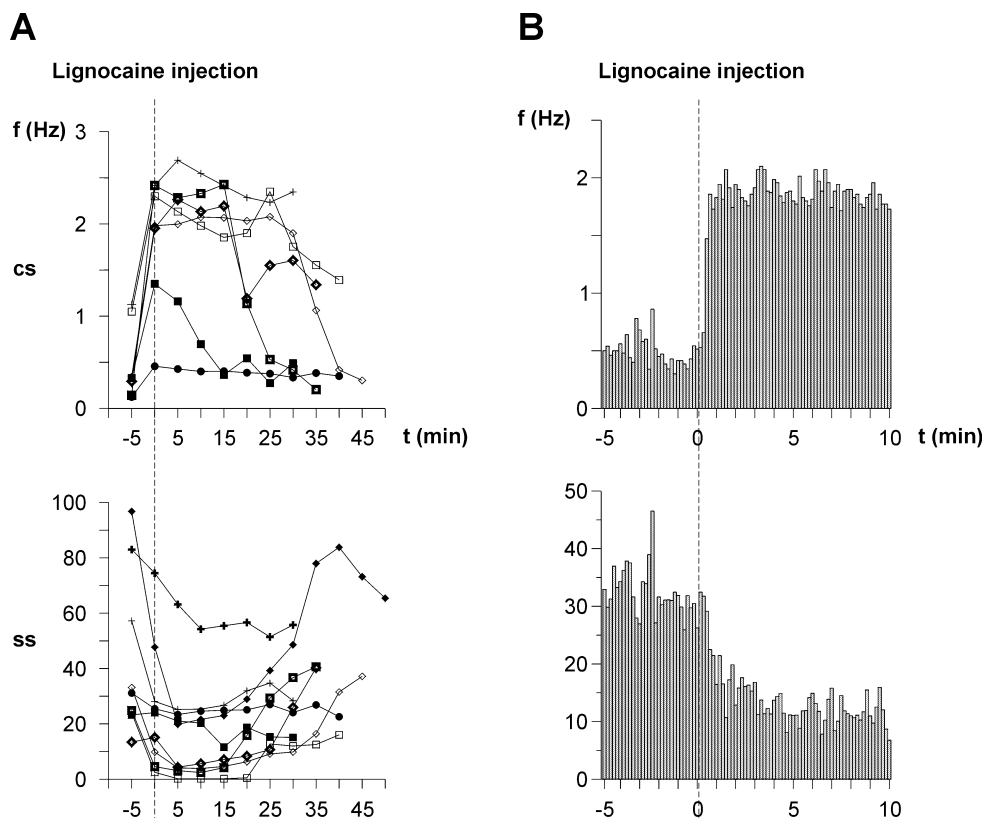


FIG. 3. Purkinje cell activity after reversibly blocking activity in the nucleo-olivary fibres. (A) The effect of injecting 2  $\mu$ L lignocaine into the nucleo-olivary fibres based on 5-min averages of complex (cs) and simple spike (ss) frequencies recorded pre- and postinjection. Symbols in the cs graphs correspond to symbols in the ss graphs and represents the activity in single Purkinje cells before and after blocking. For technical reasons (see methods) there are two more ss graphs ( $n = 9$ ) than cs graphs ( $n = 7$ ). (B) A histogram (bin width 10 s) of the mean complex and simple spike frequencies before and after blocking the nucleo-olivary fibres. For reasons of comparison only data from recordings ( $n = 7$ ) in which both simple and complex spike activity could be measured are included.

spikes. Figure 3B shows the mean effect of the injections on complex and simple spike frequencies (Hz) in 10-s bins 5 min before and 10 min after injection. For reasons of comparison only data from recordings in which both simple and complex spikes could be discriminated ( $n = 7$ ) were included in the histogram in Fig. 3B.

Despite differences in the magnitude of the effect, the frequency of complex spikes increased in all cases (7/7) after the injections. Following the complex spike increase there was a distinct decrease in simple spike frequency in all cases (9/9). As described in methods the analysis was divided into three time periods one before and two after manipulation. Frequencies before and after were then compared for significant changes (unpaired students *t*-test). Before injection the mean complex spike frequency in seven cells was  $0.48 \pm 0.04$  Hz (mean  $\pm$  SEM; range 0–2.2) and directly following the injection the mean complex spike frequency increased to  $1.79 \pm 0.06$  Hz (range 0–3.7). In the second postinjection time period the mean frequency increased to  $1.85 \pm 0.05$  Hz (range 0.2–3.9;  $P < 0.0001$  in both postinjection time periods).

Analysis of the same time periods for the mean simple spike activity in nine cells showed a preinjection level of simple spike activity of  $38.91 \pm 1.64$  Hz (range 9.5–111.5) and a first postinjection time period mean activity of  $26.89 \pm 1.58$  Hz (range 0–109). During the second postinjection period mean simple spike activity decreased to  $18.28 \pm 1.23$  Hz (range 0–96.2;  $n = 9$ ;  $P < 0.0001$  in both postinjection time periods).

In one case (not shown) where brachium conjunctivum could not be properly identified we made nonspecific 2- $\mu$ L injections of lignocaine at different depths in the same track (2.75 mm lateral, 6–3.25 mm

depth) going from ventral to dorsal. In this case no direct effects on complex or simple spike activity could be observed after the injections.

#### Activation of the N–O fibres

In these experiments, the N–O fibres were stimulated electrically while recordings were made from Purkinje cells. The initial procedure for localizing brachium conjunctivum was the same as described earlier for the injection experiments. In addition, here we also stimulated the N–O fibres directly and monitored the inhibitory effect on an evoked climbing fibre response recorded on the surface of the cerebellar cortex. After a stable Purkinje cell recording had been obtained stimulation of the N–O fibres started.

In a few cases, not included in the study, the stimulation affected blood pressure severely. As this effect seemed to be dependent on stimulus frequency we chose to use as low stimulus frequencies as possible. A range of stimulus frequencies (1–10 Hz) was tested. Frequencies below 10 Hz were poor at suppressing olivary excitability. The stimulation lasted for 5.2 min. Two stimulus strengths, 20  $\mu$ A and 60  $\mu$ A, were tested. In several cases the 60- $\mu$ A stimulation elicited complex spike activity which caused a net inhibitory effect on Purkinje cell activity. We therefore chose to use the 20  $\mu$ A intensity. Stimulation at 20  $\mu$ A (0.5 ms single square pulses, 10 Hz) caused a suppression of complex spike activity and a subsequent increase in simple spike activity, as predicted by the feedback hypothesis. The results of this stimulation is shown in Fig. 4A as the mean complex

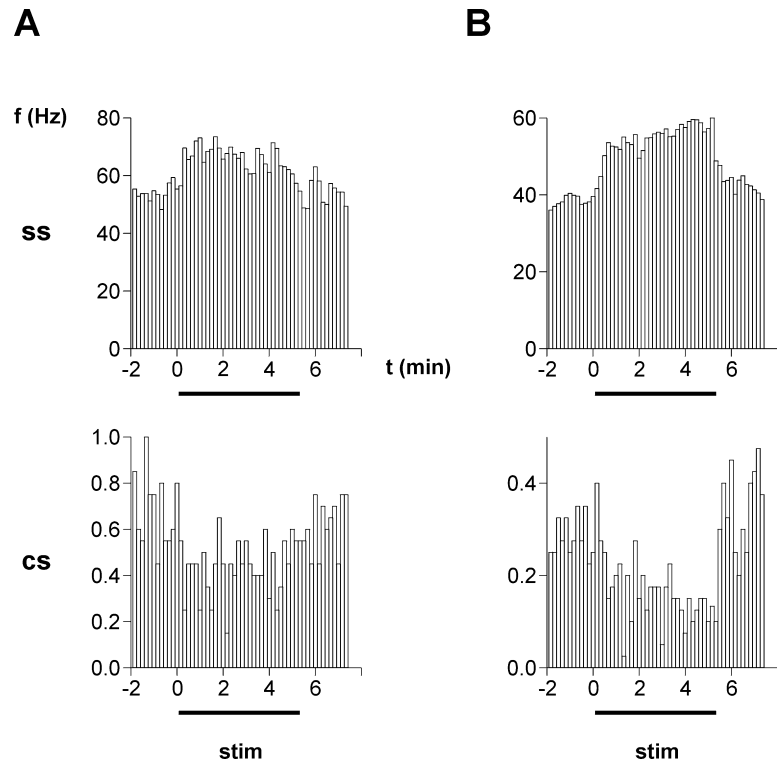


FIG. 4. Effect on Purkinje cell activity of stimulation of the nucleo-olivary fibres. Histograms (bin width 10 s) showing the effect on mean simple (ss) and complex (cs) spike activity (Hz) of stimulating the nucleo-olivary fibres. Time-extent of the stimulation indicated by bars under the histograms. (A) The effect of stimulation (5.2 min, 10 Hz, 20  $\mu$ A,  $n = 2$ ) in the high decerebrate preparation (see methods) and (B) The effect of stimulation (5.2 min, 10 Hz, 60  $\mu$ A,  $n = 4$ ) in the low decerebrate preparation (see methods).

and simple spike frequencies (bin size 10 s) in two cells before, during and after stimulation. Before stimulation the mean complex spike activity was  $0.69 \pm 0.04$  Hz (range 0.4–1.1). The mean then decreased to  $0.42 \pm 0.04$  Hz (range 0.1–0.8;  $P < 0.0001$ ) during the first stimulus time period. During the second stimulation time period the mean complex spike frequency was  $0.43 \pm 0.06$  Hz (range 0–0.9);  $P = 0.0005$ . Before stimulation the mean simple spike activity was  $54.06 \pm 2.97$  Hz (range 36.7–76.8). It then increased during the first stimulus time period to  $67.88 \pm 3.45$  Hz (range 40.2–93.7;  $P = 0.0039$ ). During the second stimulus time period the mean simple spike frequency was  $65.38 \pm 3.33$  Hz (range 44.6–88.7;  $P = 0.015$ ).

#### Lesions of the red nucleus

The previous experiments do not rule out that the inhibitory effect is mediated by the red nucleus, which is known to inhibit olivary excitability (Weiss *et al.*, 1990; Horn *et al.*, 1998). We therefore repeated the stimulation experiments on a group of animals that were decerebrated just rostral to the inferior colliculus. This lower level of decerebration (Fig. 1B) removed both the parvo- and magno-cellular parts of the red nucleus.

Because the output from the cerebellum to the facial nucleus goes via the red nucleus, short latency eyeblink responses could not be elicited by stimulation of the brachium conjunctivum in this preparation. We therefore localized the N–O fibres by monitoring the depression of evoked climbing fibre responses on the cerebellar surface, as described earlier.

The low decerebrate animals were subjected to the same stimulation paradigm as just described for the high decerebrates. However, in these subjects, 60- $\mu$ A stimulation did not elicit complex spike activity to the same extent. As expected 20- $\mu$ A stimulation was not as efficient in suppressing complex spike activity as 60  $\mu$ A but sufficient to cause changes in complex spike

frequency. As our aim was to show that lesions of the red nucleus did not abolish the inhibitory effect of N–O stimulation on olivary excitability, and consequently the effects on Purkinje cell firing, we chose the more effective 60- $\mu$ A stimulation. The effect of stimulating (10 Hz, 60  $\mu$ A, 5.2 min) the N–O fibres is shown in Fig. 4B as the mean complex and simple spike frequencies (bin size 10 s) in four cells before during and after stimulation. Before stimulation the mean complex spike frequency was  $0.28 \pm 0.02$  Hz (range 0–0.5). During the first stimulation period the mean frequency decreased to  $0.20 \pm 0.02$  Hz (range 0–0.6);  $p = 0.0082$ . In the second stimulation period the mean frequency was  $0.15 \pm 0.01$  Hz (range 0–0.4;  $P < 0.0001$ ). Before stimulation the mean simple spike frequency was  $38.49 \pm 0.50$  Hz (range 33.9–46.9). The mean then increased to  $51.17 \pm 0.95$  Hz (range 35.5–65.4;  $P < 0.0001$ ) during the first stimulation period. During the second stimulation period the mean was  $55.81 \pm 0.78$  Hz (range 44.0–64.6;  $P < 0.0001$ ).

The weaker tendency to elicit complex spikes in this preparation could be a result of removal of the mesodiencephalic junction (Ruigrok & Voogd, 1995), known to have excitatory effects on the inferior olive. Previous investigations (Garifoli *et al.*, 2001) have shown that N–O inhibition is not dependent on a functioning mesodiencephalic junction. Our results confirm this and also show that the inhibition is not dependent on the red nucleus.

#### Timing of changes in simple spike frequency

The feedback hypothesis predicts that the changes in complex spike frequency precede the changes in simple spike firing. This is not easy to determine, because the very low firing frequency of the inferior olive limits the temporal resolution of recorded frequency changes. A gradual simple spike change begins within seconds after just a couple of extra complex spikes. However, the sample graphs in Fig. 5, which show the onset of the effect after injection of 2  $\mu$ L lignocaine and

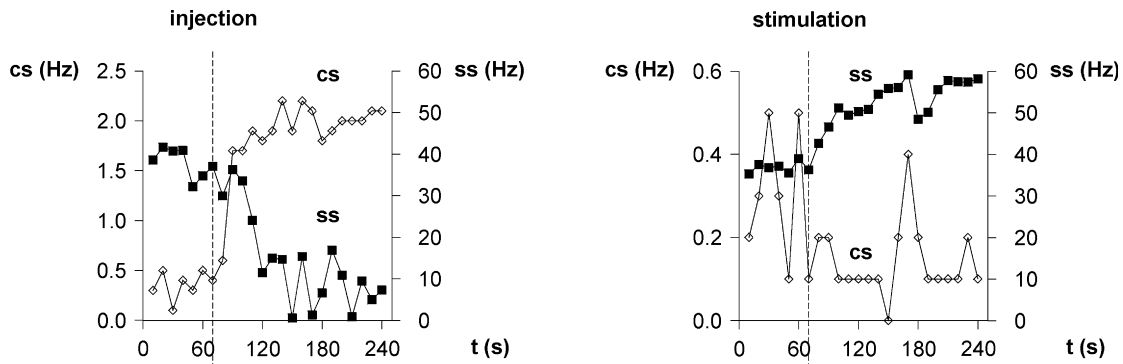


FIG. 5. Timing of changes in simple and complex spike frequencies after manipulation of activity in the nucleo-olivary fibres. Sample graphs illustrating the onset of the effect on Purkinje cell activity (Hz) of stimulation and blockade of the nucleo-olivary pathway. Complex (cs) and simple (ss) spike activity is plotted in the same graphs 1 min before and 3 min during manipulation of activity in the nucleo-olivary fibres. Onset of the injection/stimulation indicated in the graphs. Left graph shows the effect of blocking (injection of 2  $\mu$ L lignocaine into the nucleo-olivary pathway). The right graph shows the effect of stimulating (10 Hz, 60  $\mu$ A, low decerebration) the nucleo-olivary pathway. Complex spike activity on the left y-axis (diamonds) and simple spike activity on the right y-axis (squares).

stimulation (60 mA, 10 Hz, low decerebration), respectively, are reasonably clear. The effects are shown as 10 s averages of complex and simple spike frequencies 1 min before and 3 min after injection/during stimulation. In both cases the effect on the simple spikes starts within seconds. In the injection example (Fig. 5, left graph) it can be seen that the reduction in simple spike frequency is preceded by an increase in complex spike activity.

## Discussion

The feedback hypothesis predicts that blocking N–O fibres should increase olivary firing and suppress spontaneous firing in Purkinje cells, whereas stimulating the N–O fibres should have the opposite effects. Our results confirm both of these predictions.

### *Deep cerebellar nuclear control of the inferior olive and Purkinje cell activity*

Climbing fibre activity is known to have effects on spontaneous activity in the Purkinje cells (Colin *et al.*, 1980; Rawson & Tilokskulchai, 1981; Montarolo *et al.*, 1982; Demer *et al.*, 1985; Savio & Tempia, 1985; Cerminara & Rawson, 2004) and as a consequence of this also activity in the deep cerebellar nuclei (Benedetti *et al.*, 1983). Therefore, if the N–O pathway can influence olivary firing, it might also be able to influence background firing in the Purkinje cells. As predicted, we found an increase in climbing fibre activity after blocking the N–O pathway that almost immediately suppressed simple spike activity and also that simple spike activity increased after activation of the pathway.

Though the magnitude of the effect on the inferior olive was somewhat variable, removing the N–O inhibition always resulted in an increased spontaneous olivary firing. The increase in complex spike firing was sufficient to significantly reduce simple spike firing in all cases. In terms of actual frequency most of the recordings showed an increase to above 2 Hz. An increase of similar magnitude was reported by Lang *et al.* (1996) after intra-olivary injections of the GABA antagonist picrotoxin and also after lesions of the deep cerebellar nuclei.

In accordance with previous experiments (Hesslow, 1986) we found that continuous direct stimulation of the N–O pathway depressed activity in the inferior olive. Here we also found that this decrease in olivary firing was sufficient to cause an increase in Purkinje cell simple spike firing. As average background firing rates for the deep cerebellar nuclei have been reported to be around 40 Hz, in

decerebrate and intact cats (Eccles *et al.*, 1974; Armstrong & Edgley, 1984), the stimulus frequencies that were used, 10 Hz, here should cause activity in the N–O pathway that is likely to be well within the normal range of firing. This point is important because it supports the contention that physiologically normal variations have significant effects.

### *Mechanism of observed effects*

Can we be certain that the observed effects were really mediated by the N–O fibres? We think this is so for the following reasons. Firstly, we have shown previously that the most effective site for eliciting olivary depression is just ventral to the superior cerebellar peduncle (Hesslow, 1986), in agreement with anatomical localizations of the N–O fibres (Legendre & Courville, 1987). With regard to the lignocaine blocking experiments, the effect of the injections appeared within a few seconds, and it is thus unlikely that the lignocaine would have had time to spread to adjacent structures. Furthermore, in one case where injections were placed only slightly off target, no effects were observed on either complex or simple spikes.

Second, the only plausible alternatives to the N–O fibres, in our decerebrate preparation, are the red nucleus which is known to have inhibitory effects on the inferior olive (Weiss *et al.*, 1990; Horn *et al.*, 1998) and the mesodiencephalic junction, which has been shown to excite the inferior olive (Ruigrok & Voogd, 1995). To rule out these and other possible sources of olivary inhibition, we repeated the stimulation experiments in animals that had been decerebrated just rostral to the inferior colliculus. In these animals, the red nucleus and the mesodiencephalic junction had been removed, but this did not abolish the effects on complex and simple spike activity in the Purkinje cells.

### *Functional implications*

The present results provide very strong evidence that the N–O pathway can control Purkinje cell simple spike firing and it thus supports the suggestion that it works as part of negative feedback system under natural conditions. It should be acknowledged, however, that although we have focused on Purkinje cells in the present investigation, the entire cerebello-olivary loop will be stabilized by the feedback mechanism and it is arbitrary to single out the Purkinje cell activity as the main variable under control. Climbing fibres project to several other types of cerebellar neurons (Ruigrok, 1997; Sugihara

*et al.*, 1999; Jorntell & Ekerot, 2003) and may have regulatory effects on all of them.

Why is the loop necessary rather than a local mechanism? Most neurons are under some sort of negative feedback control, usually in the form of stabilizing ionic conductances and/or recurrent inhibitory interneurons. The loop via nuclear neurons and the inferior olive is unique. A consequence of having the cerebello-olivary loop, rather than relying solely on an intrinsic system, is that the level of background activity will be determined by the nuclear and olivary neurons and not by factors intrinsic to the Purkinje cells. This in turn might allow for continuous changes in the set point depending on the state of other neurons in the loop, in particular activity in the deep cerebellar nuclei, the final output of the cerebellum. This will allow for the regulation of the entire cerebellar circuitry in response to inadequacies in the output of the deep cerebellar nuclei.

The results also have important methodological implications. Current views on cerebellar learning, in particular in classical eyeblink conditioning, rest mainly on various attempts to manipulate parts of the olivo-cerebellar circuitry. As has been shown here such manipulations will have effects on the other components of the olivo-cerebellar circuit. Therefore, as pointed out by Attwell *et al.* (2001), it might be difficult to interpret the effects on behaviour of such manipulations.

Finally, it is interesting to note that N–O inhibition seems to be necessary for normal extinction of a learned blink response (Medina *et al.*, 2002) and that acquisition of the conditioned eyeblink response is very poor in rats before the N–O pathway is fully developed (Nicholson & Freeman, 2003).

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## Abbreviations

EEG, electroencephalogram; EMG, electromyogram; N–O, nucleo-olivary.

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