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CLIMBING FIBRE INDUCED DEPRESSION OF BOTH MOSSY FIBRE RESPONSIVENESS AND GLUTAMATE SENSITIVITY OF CEREBELLAR PURKINJE CELLS

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SUMMARY

1. In high decerebrate rabbits, cells were sampled extracellularly from the rostral flocculus. Purkinje cells were identified by their characteristic responses to stimulation of the contralateral inferior olive. Identification of basket cells was based on the absence of olivary responses and also on their location in the molecular layer adjacent to identified Purkinje cells. Mass field potentials in the flocculus were also studied.

2. Single pulse stimulation of a vestibular nerve, either ipsilateral or contralateral, at a rate of 2/sec excited Purkinje cells with a latency of 3–6 msec. This early excitation represents activation through vestibular mossy fibres, granule cells and their axons (parallel fibres). Similar early excitation also occurred in putative basket cells.

3. Conjunctive stimulation of a vestibular nerve at 20/sec and the inferior olive at 4/sec, for 25 sec per trial, effectively depressed the early excitation of Purkinje cells by that nerve, without an associated change in spontaneous discharge. The depression recovered in about ten minutes. This recovery was followed by the onset of a slow depression lasting for an hour.

4. Conjunctive vestibular–olivary stimulation produced no such depression in the following responses: early excitation in Purkinje cells induced from the vestibular nerve not involved in the conjunctive stimulation; early excitation in putative basket cells from either vestibular nerve; inhibition or rebound facilitation in Purkinje cells following the early excitation; vestibular-evoked field potentials in the granular layer and white matter of the flocculus. These observations lead to the conclusion that the depression occurs specifically at parallel fibre–Purkinje cell synapses involved in conjunctive stimulation.

5. Ionophoretic application of glutamate to Purkinje cells in conjunction with 4/sec olivary stimulation depressed the glutamate sensitivity of Purkinje cells; aspartate sensitivity was depressed to a much lesser degree. The depression diminished in about 10 min, but this recovery was succeeded by a slow depression lasting

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for an hour. The depression was seen only when glutamate sensitivity was relatively high, suggesting that the micro-electrode was impinging onto Purkinje cell dendrites. These observations suggest that subsynaptic chemosensitivity of Purkinje cells to the putative neurotransmitter of parallel fibres is involved in the depression observed after conjunctive stimulation of a vestibular nerve and the inferior olive.

6. The present results are consistent with the Marr-Albus assumption concerning plasticity of cerebellar neuronal networks.

INTRODUCTION

The cerebellar cortex receives two distinctively different afferent fibre inputs. Mossy fibres make synaptic contact with cortical granule cells, while climbing fibres supply synapses to dendrites of Purkinje cells (Cajal, 1911; Eccles, Ito & Szentágothai, 1967*a*; Palay & Chan-Palay, 1974). Mossy fibres arise widely from the brain stem, spinal cord and even peripheral ganglia, while climbing fibres originate, presumably solely, from the inferior olive (Szentágothai & Rajkovits, 1959; Desclin, 1974). Mossy fibres excite Purkinje cells and other cortical cells polysynaptically through granule cells and their axons (parallel fibres), while climbing fibres exert a powerful excitatory action directly on Purkinje cells (Eccles, Llinás & Sasaki, 1966*a*, 1967*b*). In spite of this powerful excitatory action, climbing fibres appear to have a minor contribution to output signals from Purkinje cells, as climbing fibre impulses discharge at a low irregular rate (1–3/sec, Thach, 1968).

In 1969, Marr proposed an ingenious hypothesis that these two types of cerebellar afferents play essentially different functional roles. Mossy fibres provide major inputs to the cerebellar cortex which are eventually converted to Purkinje cell outputs, while climbing fibres carry 'instruction' signals for re-organizing the relationship between mossy fibre inputs and Purkinje cell outputs. The hypothesis is based on a plasticity assumption: the transmission efficacy of a parallel fibre synapse on a Purkinje cell dendrite is modified when parallel fibre and climbing fibre impulses converge simultaneously on the same Purkinje cell dendrite. Support for this assumption has been derived from observations of Purkinje cell responses under two experimental situations where the cerebellar cortical network is supposed to undergo a re-organization: (1) in lobules V and VI during adaptive alteration of monkey's hand movements to a suddenly imposed load change (Gilbert & Thach, 1977), and (2) in the flocculus during adaptive modification of rabbit's vestibulo–ocular reflex to sustained vestibular–visual interaction (Ito, 1977; Dufossé, Ito, Jastreboff & Miyashita, 1978). However, since responses of Purkinje cells to natural stimuli in alert animals involve a number of complicating factors, it is desirable to have a more direct test of the plasticity assumption under simpler experimental conditions.

This article describes such a test performed with electric stimulation of mossy fibre and climbing fibre afferents to the flocculus in decerebrate rabbits. An advantage of using the flocculus is that one source of mossy fibres, the vestibular nerves, can be activated electrically without stimulating central nervous system structures. One complication requiring control is that vestibular mossy fibres contain both primary and secondary projections (Brodal & Høivik, 1964; Shinoda & Yoshida, 1975). This article also describes a test performed with electric stimulation of climbing fibres and

ionophoretic application of glutamic acid, the putative neurotransmitter of parallel fibres (see Sandoval & Cotman, 1978; Hacket, Hou & Cochran, 1979), to Purkinje cells. Results of these tests support the plasticity assumption and suggest involvement of subsynaptic chemosensitivity of Purkinje cells.

Preliminary reports have been published (Ito, Sakurai & Tongroach, 1981*a*, *b*).

METHODS

Twenty-eight adult albino rabbits (2·5–3·5 kg) were used. Under anaesthesia by Thiopental (I.V., initial dose 30 mg/kg with supplementation as required), the dorsal surface of the cerebrum was exposed by craniotomy. The entire cerebrum and the rostral part of the diencephalon were aspirated, leaving intact the brain stem caudal to an oblique plane passing rostral to the lateral geniculate body and the optic chiasm. Middle ears were exposed by a lateral approach. The atlanto–occipital membrane was dissected to expose the lower medulla. The bone covering the left paraflocculus was removed. Edges of skin flaps and exposed muscles and bone tissues were covered with jelly containing 2 % xylocaine. The animal was mounted on a metal frame (Highstein, Ito & Tsuchiya, 1971). Anaesthesia was then terminated, and the animal was paralysed with Flaxedil and respired artificially. The exposed surface of the paraflocculus was covered with a mixture of vaseline and mineral oil. Micro-electrode recording started at least 2 hr after decerebration and continued for 20–40 hr.

Needle electrodes made of platinum–iridium wire (0·2 mm in diameter) were lacquered except for the pointed tips. Two needles were inserted into each oval window for bipolar stimulation of vestibular nerves (S_1, S_2 in Fig. 1*A*). Another two needles paired along the neuraxis (S_3) were inserted dorsally into the medulla aiming at the dorsal cap of the inferior olive (source of climbing fibre afferents to the flocculus, Maekawa & Simpson, 1973), at the level of the obex and 0·5 mm right to the mid line. A single needle electrode for recording was placed on the dorsal surface of the C_1 segment of the spinal cord (R in Fig. 1*A*). The electrode, however, was often replaced by one of the two needles of S_3.

Single-barrelled glass micro-electrodes were filled with 2M-NaCl solution. When multi-barrelled glass micro-electrodes were used, the central barrel was filled with 4M-NaCl solution and used for recording neuronal spike potentials, while each of the peripheral barrels was filled with either of the following solutions adjusted to pH 7·5 for ionophoresis: 0·5 M-Na-L-glutamate, 0·5 M-Na-L-aspartate, or 0·05 M-Na-*N*-methyl-aspartate in 0·165 M-NaCl. *N*-methyl-aspartate is a more specific agonist of aspartate (Davis & Watkins, 1979). These electrodes were either bevelled or bumped against a brass rod so that the over-all tip diameter was 2–6 μm. Multi-barrelled electrodes were connected to constant current injectors (Neurophore BH-2, Medical System Corp) for ionophoresis. Retaining currents of 2 nA were usually passed through the pipettes in order to reduce the diffusion of amino acids. To minimize the direct effects of current flow on the neurones under study, neutralizing currents were passed through one barrel containing 2 M-NaCl, so as to nullify the total currents flowing through micro-electrodes.

Micro-electrodes were inserted into the flocculus posteriorly (Ghelarducci, Ito & Yagi, 1975). Spike potentials were selected through a window slicer device and instantaneous rates of their occurrence were measured by an electronic counter. A data processing computer (ATAC 501–10, Nihon Koden) was used for constructing peristimulus time histograms. In a few experiments, micro-electrodes were also inserted into vestibular nuclei dorsally through the cerebellum (Akaike, Fanardjian, Ito, Kumada & Nakajima, 1973*a*).

RESULTS

Positive–negative or negative–positive diphasic unitary spikes (Fig. 1*B* and *C*) were recorded in the rostral flocculus (at depths of 8–10 mm from the surface of the paraflocculus). Purkinje cells were identified by their response to stimulation of the contralateral inferior olive. The response occurred with a fixed latency of about 4 msec and was superimposed on the prominent field potentials representing activation of

Purkinje cells by climbing fibre impulses (Eccles *et al.* 1966*a*; Maekawa & Simpson, 1973). The response usually consisted of several spikes in the form of *complex* spikes (Thach, 1968; Fig. 1 *D*). The intensity of olivary stimulation (duration 0·1 msec) was adjusted to suprathreshold for activating Purkinje cells under observation. The threshold was as low as 20 μA when the electrode was optimally positioned. Purkinje

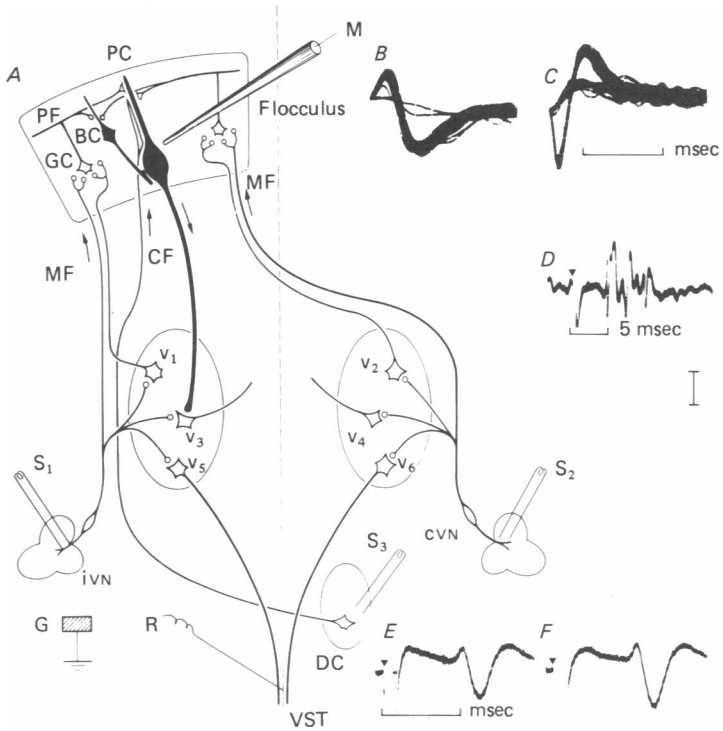

Fig. 1. Experimental arrangement for recording and stimulation. *A*, schematic diagram showing neuronal connexions of the flocculus and medulla relevant to the present experiment. PC, Purkinje cell; GC, granule cell; MF, mossy fibres; CF, climbing fibre; iVN and cVN, ipsilateral and contralateral vestibular nerves; DC, dorsal cap of the inferior olive. Ellipsoids represent vestibular nuclei. v_1 and v_2, vestibulo–cerebellar relay cells; v_3 and v_4, vestibulo–ocular relay cells; v_5 and v_6, vesibulospinal relay cells; S_1, S_2, S_3, stimulating electrodes; R, needle electrode for recording; G, indifferent electrode; VST, vestibulospinal tracts. *B* and *C*, records of unitary spikes recorded from the flocculus. Sweeps were triggered by the spikes. Trigger levels were adjusted so as to yield some baseline traces. *D*, single sweep record of spike responses to stimulation of DC. *E* and *F*, secondary vestibulospinal volleys recorded with *R*, in response to stimulation of the ipsi- (*E*) and contra- (*F*) lateral vestibular nerve. In *D*–*F*, times of stimulation are indicated by triangles. Voltage scale, 0·2 mV. Positivity is represented by upward deflections. Low cut time constant for a.c. recording in *B*–*F* was 1 msec for *B*–*D* and 10 msec for *E*–*F*.

cells discharged ordinary spikes (simple spikes, Thach, 1968) spontaneously at a rate around 50/sec (range 5–147/sec, mean $\pm$S.D., 49$\pm$28/sec, $n = 50$), comparable with those in alert rabbits (Ghelarducci *et al.* 1975).

Unitary potentials recorded from the flocculus that did not respond to olivary stimulation were assumed to be from cells other than Purkinje cells. Two types of

non-Purkinje units were distinguished. One type was characterized by extremely regular spontaneous discharge and was sampled in the granular layer, as judged from the configuration of olivary-evoked field potentials (Eccles *et al.* 1966*a*). These cells are presumed to be Golgi cells (Miles, Fuller, Braitman & Dow, 1980). They were discarded in this study. Another type of unit exhibited spontaneous discharges very similar to Purkinje cells (range 9–109/sec, mean ±S.D., 48±22/sec, $n = 30$). They were located in the molecular layer, as judged from olivary-evoked field potentials, and were a few hundred micrometres from identified Purkinje cells. They are presumed to be basket cells. In four rabbits, using the very superficial folia of the dorsal paraflocculus, it was confirmed that such putative basket cells could be sampled very frequently from molecular layers just superficial to Purkinje cell layers.

Test vestibular nerve stimulation

Test mossy fibre inputs were provided by electrical stimulation of the vestibular nerve. The intensity of pulses (0·1 msec duration) was adjusted to be supramaximal (usually 2 × maximal) for evoking secondary volleys in the descending vestibulospinal tract (Fig. 1*E* and *F*). This level of stimulation presumably activates all primary vestibular fibres (Akaike, Fanardjian, Ito & Ohno, 1973*b*). This section describes vestibular–evoked responses in the flocculus.

Responses of Purkinje cells

A major response of flocculus Purkinje cells to vestibular nerve stimulation was early excitation, i.e. initiation of simple spikes with a latency of 3–6 msec. As expected for activation of a Purkinje cell by mossy fibre impulses (Eccles *et al.* 1967*b*), the latency of this excitation fluctuated from trial to trial, and spike initiation occasionally failed (Fig. 2*A* and *B*). Fig. 2*C* shows a peristimulus histogram from a hundred presentations of 2/sec stimulation and illustrates the time course of the early excitation. The early excitation was often followed by an inhibition lasting for 10–20 msec.

The magnitude of the early excitation was quantified by calculating the firing index: [100 % × (total number of spikes during the period of 2–5 msec covering the early excitation − number of spikes attributable to spontaneous discharge according to measurement in 10 msec prior to stimulation)/number of sweeps]. The number of sweeps varied from 100 to 500, repeated at 2/sec. With stimulation of the ipsilateral vestibular nerve, values of the firing index in Purkinje cells varied from 8 to 100 %, the average being 40 % (±22 % S.D., $n = 27$). In twenty Purkinje cells selected for a relatively large firing index (greater than 25 %), the latency of onset of the early excitation was 4·3 msec (±1·4 msec S.D.), and the peak time 4·9 msec (±1·3 msec S.D.). With stimulation of the contralateral vestibular nerve, the evoked early excitation was of smaller magnitude and longer latency than ipsilateral responses (compare Fig. 6*A* and *D*). Mean values of the firing index were 52 % (±12 % S.D.) for ipsilateral and 37 % (±19 % S.D.) for contralateral responses in eight Purkinje cells tested with both vestibular nerves ($P < 0·05$, Student's *t* test). Both the onset latency (±0·6 msec S.D.) and peak time (±0·4 msec S.D.) for contralateral responses were 0·9 msec longer than for ipsilateral responses ($P < 0·01$).

The early excitation, however, was seen only in a relatively small proportion of

floccular Purkinje cells. It occurred only in three out of fifteen Purkinje cells sampled randomly from flocculi of two rabbits. The remaining majority (ten cells; two cells were unresponsive) exhibited an inhibition, as illustrated in Fig. 2*D* and *E*. In seventeen Purkinje cells in which the onset of the inhibition could be determined sharply (as in Fig. 2*D* and *E*), the mean latency of the ipsilaterally evoked inhibition was 5·7 msec (range 3–6 msec, ±1·1 msec S.D.). The onset of contralaterally evoked

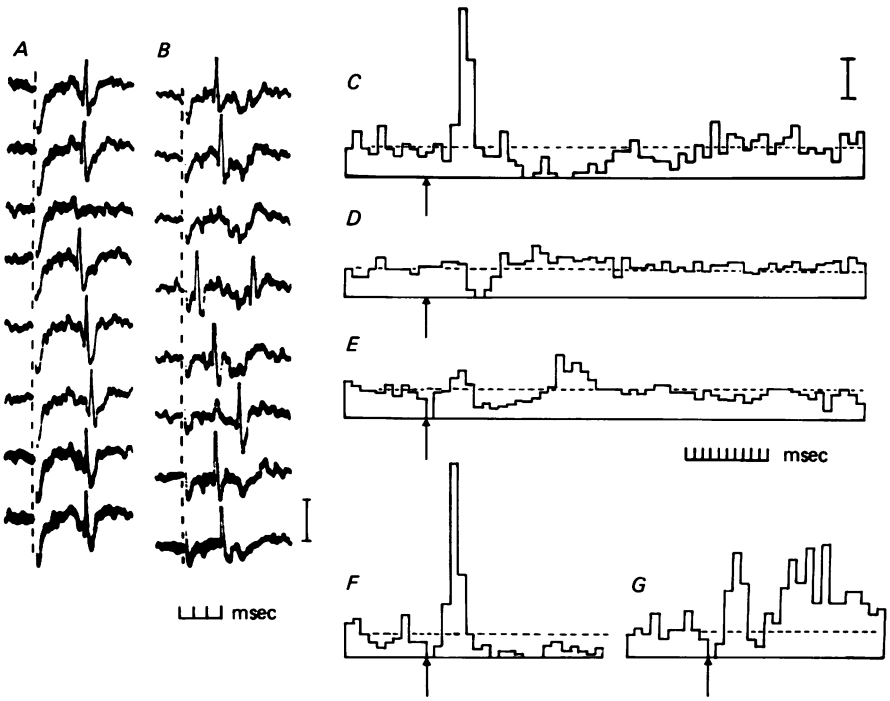

Fig. 2. Responses of Purkinje cells and putative basket cells to stimulation of vestibular nerves. *A* and *B*, single sweep records showing initiation of *simple* spikes in a Purkinje cell following stimulation of the ipsi-(*A*) and contra- (*B*) lateral vestibular nerves. Vertical dashed lines indicate times of stimulation. Upward deflexions represent negativity. Voltage scale, 0·3 mV. Sweeps were repeated every 2 sec and taken successively from the top to the bottom. *C–G*, peristimulus time histograms generated by repeating 100 (*C*, *F* and *G*) or 500 (*D* and *E*) sweeps. *C–E*, responses of three different Purkinje cells to stimulation of the ipsilateral vestibular nerve. *F* and *G*, responses of a putative basket cell to stimulation of the ipsi- (*F*) and contra- (*G*) lateral nerves. Upward arrows mark times of stimulation. In *E–G* as well as in succeeding figures, it is noted that stimulus artifacts obstructed spike count in the bin immediately following stimulation. Calibration for *C–G*, 10 impulses/bin/100 sweeps.

inhibition in the same cells was usually delayed by an average of 0·7 msec (±0·6 msec S.D., $n = 6$) as compared with the ipsilaterally evoked inhibition ($P < 0·01$). The duration of the inhibition varied widely from 4 to 40 msec, the average being 13 msec (±10 msec S.D., $n = 16$, calculated for the ipsilaterally-evoked inhibition). The inhibition was often followed by a rebound facilitation (Fig. 2*E*). The latency of the facilitation was 16 msec (±15 msec S.D., $n = 9$) and the duration was 18 msec (±9 msec S.D., calculated for either ipsi- or contralateral responses).

Responses of putative basket cells

Putative basket cells regularly exhibited an early excitation in response to stimulation of either vestibular nerve (Fig. 2*F* and *G*). The firing index for ipsilaterally-evoked early excitation varied from 25 to 85 %, the average being 47 % ($\pm$15 % S.D., $n = 26$). The mean latency for the onset of the ipsilaterally-evoked early excitation was 4·0 msec (1·0 msec S.D.) and the peak time was 4·7 msec ($\pm$1·1 msec S.D., $n = 28$). In fifteen cells where responses to stimulation of both vestibular nerves were tested, contralateral responses were usually smaller (firing index, 41$\pm$20 % *vs.* 52$\pm$13 %, $P < 0{\cdot}05$) and more delayed (by 1·1$\pm$0·5 msec for the onset and 0·9$\pm$0·7 msec for the peak, $P < 0{\cdot}01$) than ipsilateral responses. The early excitation was usually followed by an inhibition (Fig. 2*F*), but sometimes there was a delayed facilitation instead of inhibition (Fig. 2*G*). The time course of these late responses was not studied in detail.

Afferent volleys from the ipsilateral vestibular nerve

In the white matter of the flocculus, stimulation of the ipsilateral vestibular nerve induced double-peaked field potentials, as shown in Fig. 3*B*. In Fig. 3*B* the initial spiky potential has a brief latency, 0·30 msec for the initial positivity (P) and 0·44 msec for the succeeding negativity (N), so that it may represent afferent volleys of primary vestibular fibres. Volleys recorded from the medulla had briefer latencies: 0·14 msec for the P potential and 0·22 msec for the N potential in a region lateral to the vestibular nuclei (Fig. 3*C*), and 0·18 msec for the P potential and 0·32 msec for the N potential in the vestibular nuclei (Fig. 3*D*). Taking the peaks of the P potentials as representing the arrival of propagating primary vestibular volleys, one can calculate that, after entrance to the medulla, primary vestibular volleys travel to the flocculus in 0·16 msec over a distance of several millimetres, while they reach vestibular nuclei in 0·04 msec (a distance of a millimetre).

The second spiky component of the field potentials evoked in the flocculus (P_1–N_1, Fig. 3*B*) had a latency of 0·60 msec for the initial positivity (P_1) and 0·67 msec for the succeeding negativity (N_1). These values are longer than the onset latency of the N_1 potential in vestibular nuclei (0·50 msec, Fig. 3*D*) which represents post-synaptic activity of vestibular nuclei cells (Precht & Shimazu, 1965; Akaike *et al.* 1973*b*), and are shorter than those of secondary volleys recorded from the C_1 segment (Fig. 3*E*, 0·79 msec for P_1 and 0·90 msec for N_1 potentials). It is likely that P_1–N_1 potentials in the flocculus represent secondary volleys relayed by vestibular nuclei cells (v_1 in Fig. 3*A*). These values suggest that secondary volleys initiated from vestibular nuclei reach the flocculus in about 0·1 msec, while they pass down to the C_1 segment in 0·2 msec (a distance of about 10 mm).

The distinction between P–N and P_1–N_1 potentials in the flocculus as representing primary and secondary vestibular volleys, respectively, is supported by their differential susceptibility to high-frequency, repetitive stimulation. During stimulation at 333 Hz for 30 sec, primary volleys remained with a reduction of less than 20 %, even though their time course became appreciably slower (compare Fig. 3*F–H* with *B–D*). By contrast, P_1–N_1 potentials in the flocculus (*F*), the post-synaptic activity in vestibular nuclei (*H*) and secondary volleys at C_1 (I) were all markedly depressed.

Afferent volleys from the contralateral vestibular nerve

Stimulation of the contralateral vestibular nerve also evoked field potentials in the white matter of the flocculus which consisted of two prominent spiky components (P_1 and P_1' in Fig. 4*A*). The peak latencies were 1·1 (P_1) and 1·5 msec (P_1'), respectively. P_1 potential was sometimes very small (Fig. 4*B*), indicating separate origins of P_1

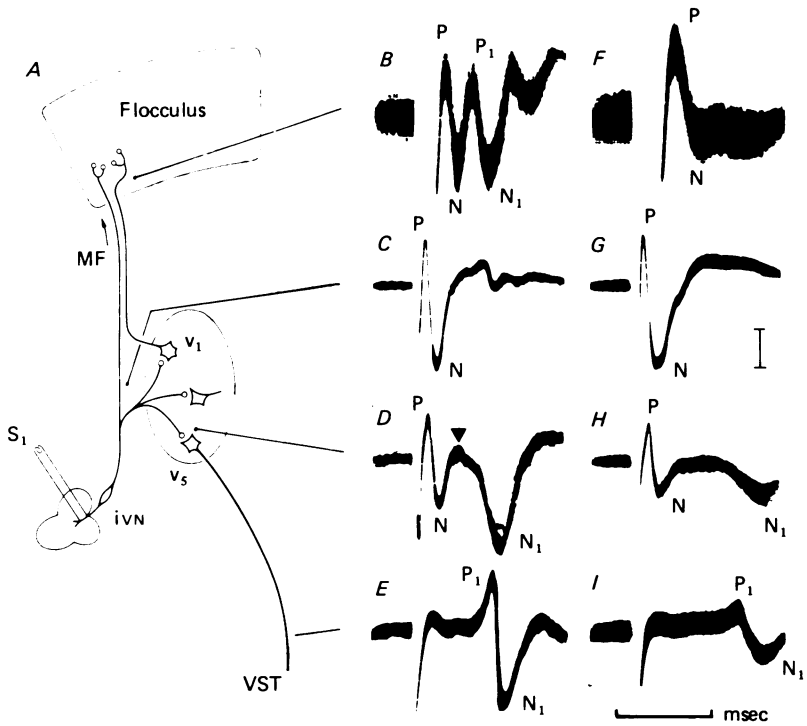

Fig. 3. Vestibular afferent volleys evoked by stimulation of the ipsilateral vestibular nerve. *A* shows diagrammatically the relationship between the four recording sites and the courses of primary and secondary vestibular projections. Symbols are defined in Fig. 1*A*. The ipsilateral vestibular nerve was stimulated supramaximally at frequencies of 2/sec in *B–E* and 333/sec in *F–I*. *B* and *F*, recorded in the white matter of the flocculus, *C* and *G*, in a region lateral to vestibular nuclei. *D* and *H*, in vestibular nuclei; *E* and *I*, at C_1 segment of the spinal cord. In each of these records, ten sweeps were superimposed. P and N specify the positivity and negativity, representing primary vestibular volleys. P_1 and N_1 in *B*, *E*, *F* and *I* specify those representing secondary volleys. N_1 in *D* and *H* denote post-synaptic acitivity of vestibular neurones. Triangle in *D* marks the onset of N_1 potential. Voltage scale, 0·1 mV for *B* and *F*, 1 mV for *C*, *D*, *G* and *H*, and 0·05 mV for *E* and *I*.

and P_1' potentials. Both of these contralaterally evoked potentials were susceptible to high frequency repetitive stimulation (not illustrated). Moreover, both of these potentials were effectively depressed by conditioning stimulation of the ipsilateral vestibular nerve, as shown in Fig. 4*C–F*. The depression followed the conditioning stimulation with a brief latency and lasted for several milliseconds. This depression probably represents a commissural inhibition of vestibular relay cells (Shinoda &

Yoshida, 1975), indicating the secondary origin of the contralaterally evoked spiky field potentials in the flocculus. Earlier field potentials might involve contralateral primary volleys, but these were too small for analysis.

Field potentials in the granular layer

When the micro-electrode was placed in the granular layer of the flocculus, as judged from the configuration of olivary-evoked responses and the spatial relationship

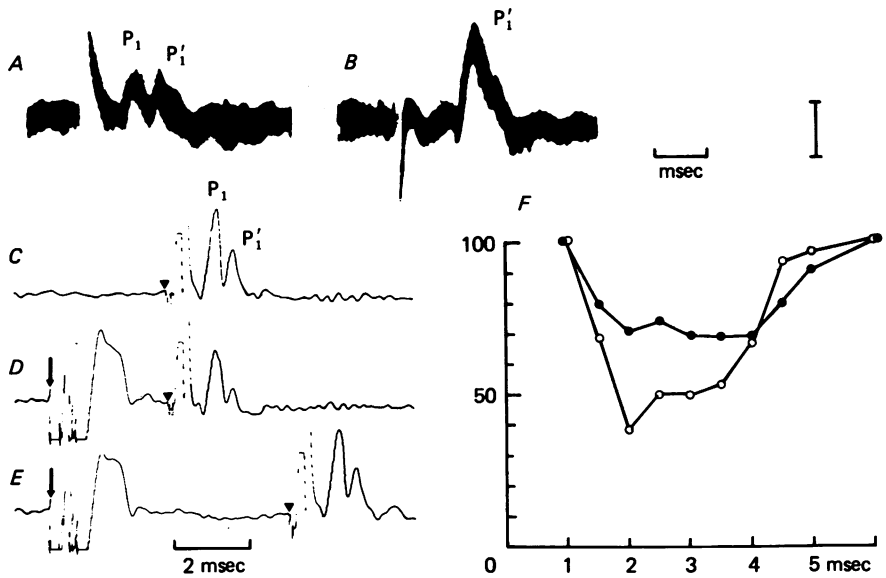

Fig. 4. Vestibular afferent volleys evoked by stimulation of the contralateral vestibular nerve. *A–E*, field potentials recorded from the white matter of the flocculus during supramaximal stimulation of the contralateral vestibular nerve. *A* and *B*, specimen records obtained from two rabbits by superimposing 10 sweeps at 2/sec. P_1 and P_1' specify two spiky positive components of the field potentials. Note in *B* that P_1 is virtually absent. *C*, same as in *A*, but averaged during 20 sweeps and recorded on a strip chart. *D* and *E*, similar to *C*, but with conditioning stimuli to the ipsilateral vestibular nerve at the moments indicated by downward arrows. Triangles mark the moments of test stimulation. Time scale of msec is common to *A* and *B*, and that of 2 msec to *C–E*. Voltage scale of 0·1 mV is for *A* and *B*. *F* plots amplitudes of P_1 and P_1' potentials as function of the time interval between the ipsilateral conditioning stimuli and contralateral test stimuli, for a series partly illustrated in *C–E*. Note that amplitudes of P_1 and P_1' potentials are normalized by control values without conditioning in *C*.

with identified Purkinje cells, incoming vestibular volleys were followed by a negativity (N_2 in Fig. 6*K* and *L*). This represents activation of granule cells (Precht & Llinás, 1969; Shinoda & Yoshida, 1975). Other components of the granular layer field potentials, i.e. P_2 for impulse propagation to parallel fibres and N_4 for excitation of Purkinje cells (Eccles *et al.* 1967*b*) were also studied (not illustrated).

Conjunctive vestibular–olivary stimulation

The effects of olivary stimulation alone were tested in some Purkinje cells using various stimulus frequencies. During stimulation at higher frequencies (10 to 200/sec), the spontaneous discharges of Purkinje cells disappeared, but they recovered a few minutes after cessation of stimulation. At lower frequencies (below 5/sec), no such depression was seen. Given this result and the observation that the highest

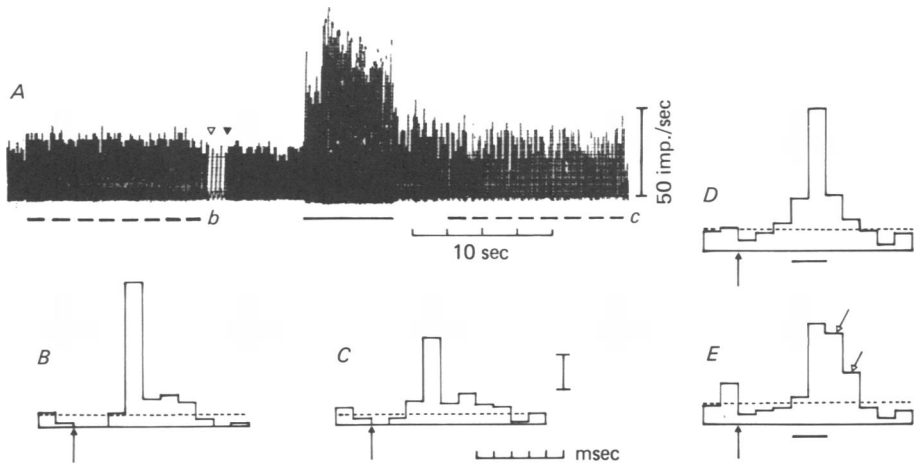

Fig. 5. Effects of conjunctive stimulation of a vestibular nerve and the inferior olive. *A*, strip chart record of *simple* spike discharge from a Purkinje cell. Bin width, 0·5 sec. The chart runs from the left to the right. Between the open and filled triangles was a gap for 3 min. Horizontal dashed lines, period of 2/sec stimulation of the ipsilateral vestibular nerve. Horizontal continuous line, period of conjunctive 4/sec stimulation of the inferior olive and 20/sec stimulation of the ipsilateral vestibular nerve. *B* and *C*, peristimulus histograms constructed during the periods *b* and *c* in *A*. *D* and *E*, peristimulus histograms before and after conjunctive stimulaton for another Purkinje cell. Upward arrows mark times of stimulation. Oblique arrows in *E* indicate the later part of the early excitation which was enhanced after conjunctive stimulation. Horizontal bars below *D* and *E* mark the bins for which the firing index was calculated. Calibration for *B–E*, 10 impulses/bin/100 sweeps.

discharge rate of complex spikes obtained in alert rabbits is about 4/sec (Ghelarducci *et al.* 1975), a rate of 4/sec was adopted for conjunctive stimulation. In conjunction with olivary stimulation, a vestibular nerve was stimulated at a rate of 20/sec, which is a relatively low discharge rate for vestibular nerve fibres observed during sinusoidal head rotation of alert monkeys (Fernandez & Goldberg, 1971). One trial of conjunctive stimulation consisted of simultaneous 4/sec olivary stimulation and 20/sec vestibular nerve stimulation for 25 sec, resulting in 100 olivary and 500 vestibular volleys.

Effects on Purkinje cells

In Purkinje cells selected for prominent early excitation from a vestibular nerve, conjunctive stimulation induced a marked enhancement in simple spike discharges which subsided shortly after termination of conjunctive stimulation (Fig. 5*A*).

Although spontaneous discharges were more irregular than control for a short time (Fig. 5*A*), they became regular within a few minutes. Fig. 5*B* and *C* illustrates peristimulus time histograms constructed during one trial of conjunctive stimulation. Early excitation from the ipsilateral vestibular nerve (Fig. 5*B*) was appreciably depressed shortly after the conjunctive stimulation of the ipsilateral vestibular nerve and the contralateral inferior olive (Fig. 5*C*). Fig. 5*D* and *E* are similar histograms obtained from another cell, and show that the depression accompanies a change in time course of the early excitation; the later part of the early excitation is enhanced, while the earlier part is depressed. The extent of the depression was quantified by calculating the corresponding reduction in the firing index. In cases such as Fig. 5*D* and *E*, late components of the early excitation were omitted from calculations of the firing index. Ten Purkinje cells with firing index greater than 30 % were subjected to conjunctive stimulation. Each of eleven trials reduced the firing index to 6–83 % (mean and S.D., 47 ± 21 %) of corresponding control values. This depression also seems to be cumulative. When two to three trials of conjunctive stimulation were repeated in succession (with intervals of 2–3 min), the depression was larger for successive presentations; for example, the depression increased from 6 % after the first to 12 % after the second and 38 % after the third trial of conjunctive stimulation (another example is in Fig. 7).

In five Purkinje cells, early excitations from both vestibular nerves were tested following conjunctive stimulation involving only one nerve. The early excitation from the conditioned nerve alone was regularly depressed (Fig. 6*A* and *B*), while early excitation from the other nerve remained unaffected (Fig. 6*D* and *E*). Therefore the effect of conjunctive stimulation is specific to the vestibular nerve conditioned by conjunctive stimulation. Sustained application of 4/sec olivary stimulation or 20/sec vestibular nerve stimulation in isolation (up to two minutes) was also tried in five Purkinje cells, and this induced no appreciable change in the early excitation.

Effects of conjunctive stimulation were also tested on the inhibition and rebound facilitation evoked in Purkinje cells from a vestibular nerve (Fig. 2*D* and *E*). No appreciable changes were detected in these responses in twenty trials on ten Purkinje cells.

Effects on putative basket cells

Conjunctive stimulation was presented to putative basket cells with supramaximal olivary stimulation for adjacent Purkinje cells. In seventeen putative basket cells, single trials of conjunctive stimulation produced no consistent changes in the early excitation (Fig. 6*G* and *H*). The mean change in the firing index was −3·1 % (minus means decrease, ±9·4 % S.D.) which is of no statistical significance ($P > 0{\cdot}1$). In one putative basket cell, three successive trials of conjunctive stimulation did not appreciably change the firing index (+3 %).

Effects on field potentials

In the white matter of the flocculus, primary and secondary vestibular volleys from the ipsilateral vestibular nerve were tested repeatedly (up to seven trials of conjunctive stimulation in succession), but there were no detectable changes in these potentials (Fig. 6*I*). Similarly, contralateral secondary vestibular volleys were not

influenced by conjunctive stimulation (Fig. 6*J*). In the granular layer of the flocculus, N_2 (Fig. 6*K* and *L*) and P_2 (not illustrated) potentials were tested repeatedly, but no significant changes were detected. The N_4 potential was also tested repeatedly. Despite the expectation that the N_4 potential reflects early excitation of Purkinje cells (Eccles *et al.* 1976*b*), no clear changes were seen. This negative finding may be

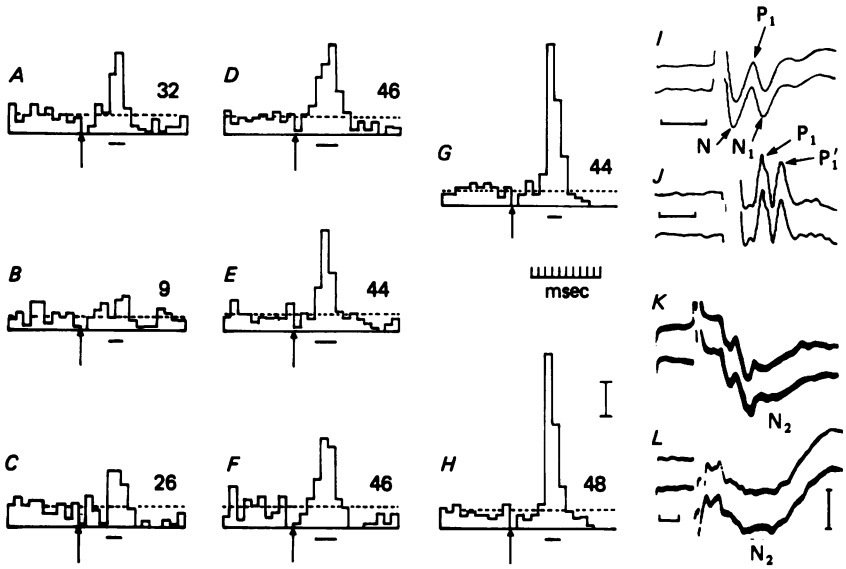

Fig. 6. Specificity of the effects of conjunctive vestibular–olivary stimulation. *A–F*, peristimulus histograms obtained from a Purkinje cells. *A–C*, stimulation of the contralateral vestibular nerve. *D–F*, stimulation of the ipsilateral vestibular nerve. *A* and *D*, control. *B* and *E* were taken within 4 min after conjunctive stimulation of the contralateral vestibular nerve and the inferior olive. *C* and *F*, similar to *B* and *E*, but taken at 10–13 min after conjunctive stimulation. Figures indicate values of the firing index. Horizontal bars mark the bins for which the firing index was calculated. Other conventions are similar to Fig. 5*B–E*. *G* and *H*, peristimulus histograms for a putative basket cell, before and immediately after a trial of vestibular–olivary conjunctive stimulation. Calibration for *A–H*: 10 impulses/bin . 100 sweeps. *I* and *J*, field potentials recorded from the white matter of the flocculus and averaged during 20 sweeps repeated at 2/sec. Upper traces are before and lower traces immediately after a trial of conjunctive stimulation. *I*, with stimulation of the ipsilateral nerve. *J*, with stimulation of the contralateral vestibular nerve. *K* and *L*, field potentials recorded from a granular layer of the flocculus, illustrated similarly to *I* and *J*. Voltage scale, 0·1 mV for *I* and *J*, 0·2 mV for *K* and *L*. Time scales, 1 msec. The time scale for *L* is common to *K*.

related to the fact that only a small fraction of flocculus Purkinje cells exhibited early excitation. By contrast, early excitation was prevalent in putative basket cells. Thus, it is probable that the N_4 potential in the flocculus is not susceptible to conjunctive stimulation because it reflects primarily the early excitation of basket cells, but not Purkinje cells.

Recovery after conjunctive stimulation

Fig. 7 illustrates the time course of recovery for the depressed early excitation during three successive sessions of conjunctive stimulation. Early excitation recovered 5 min after a single trial of conjunctive stimulation in the first session. The recovery took a longer time after repeated conjunctive stimulation: 8 min after two, and 12 min after three successive trials of stimulation. In ten sessions imposed on seven

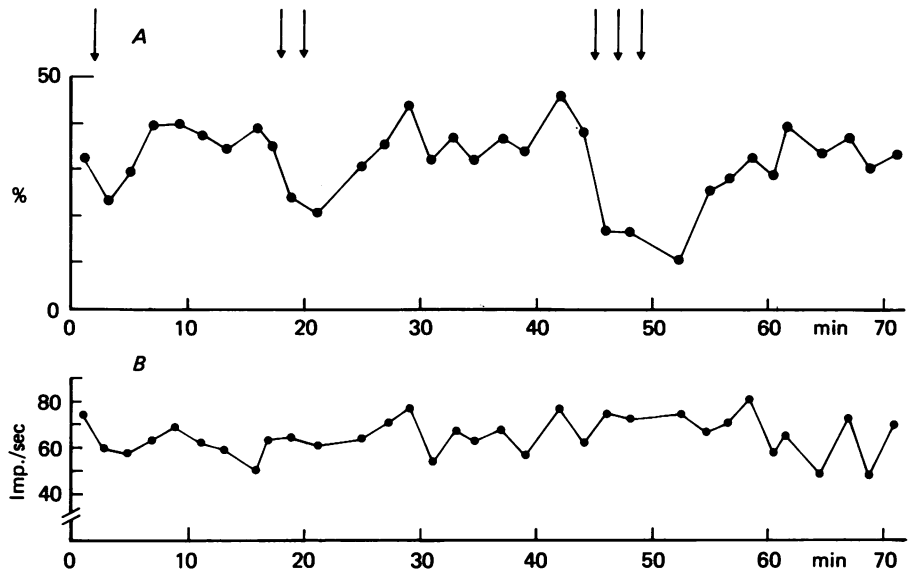

Fig. 7. Time course of depression of the early excitation after trials of conjunctive stimulation. *A*, plot of the firing index for a stimulation of the ipsilateral vestibular nerve in a Purkinje cell. *B*, spontaneous discharge rate measured simultaneously with *A*. Downward arrows mark the times of trials of conjunctive stimulation of the ipsilateral vestibular nerve and the inferior olive.

Purkinje cells, the recovery was nearly complete within 10 min after each repetition of conjunctive stimulation. Fig. 7 also illustrates that the spontaneous discharge frequency fluctuated between 50 and 80/sec, but it was not correlated with the depression and recovery of the early excitation.

In five Purkinje cells shown in Fig. 8, the recovery after conjunctive stimulation was followed continuously for 30–70 mins. After the initial recovery, the early excitation was usually depressed below the control level for at least several tens of minutes. At 30–35 min after conjunctive stimulation, the average amount of depression was 36 % (±6 % S.D.). The duration of this slow phase of depression could not be determined in this study because of technical difficulties in maintaining stable recording from the same cells for more than one hour.

Responses to amino acids

When L-glutamic acid, L-aspartic acid, or *N*-methyl-DL-aspartic acid was applied ionophoretically, Purkinje cells responded with increased spontaneous discharges of

simple spikes. Putative basket cells responded similarly. Using 2·5 sec current pulses for ionophoresis, the dose–response relationship for Purkinje cells and putative basket cells was tested. The relationship was approximately linear when discharge frequencies were increased between about 10 and about 100 impulses/sec. Beyond the limit of 100 impulses/sec, inactivation commonly occurred. For each cell and for each amino acid, the intensity of effective ionophoretic currents was chosen so that discharge

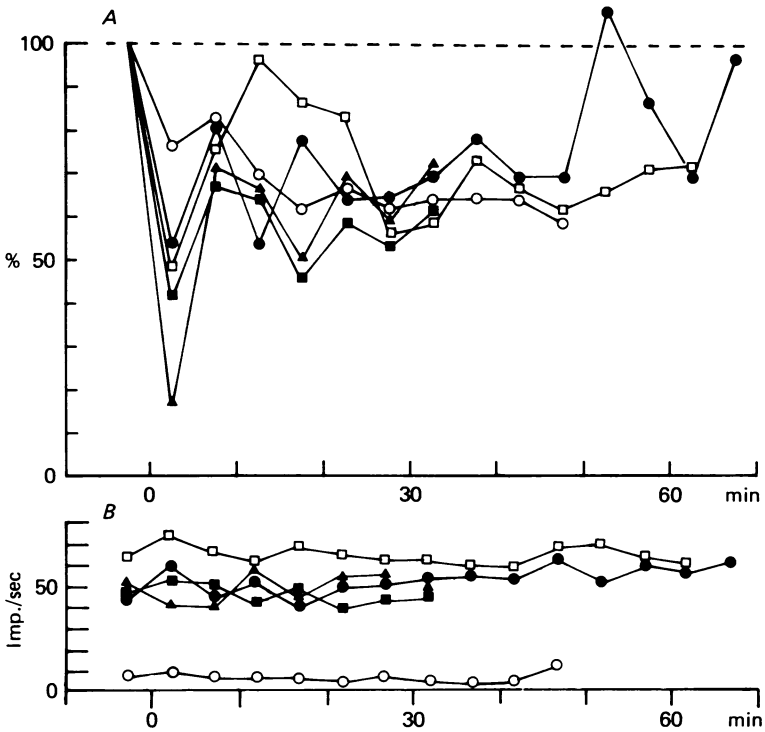

Fig. 8. Long-term recovery after conjunctive vestibular–olivary stimulation. *A*, ordinate: values of the firing index. The average values of two to three trials performed during each 5 min period are plotted. Note that plotted values of the firing index were normalized by control values before conjunctive stimulation. Dashed line is drawn at 100 %. *B*, spontaneous discharge rate, averaged for each 5 min period. Measurements from five different Purkinje cells.

frequencies were increased by 50 to 100 impulses/sec without showing signs of inactivation. For conjunctive stimulation, 4/sec olivary stimuli and ionophoretic currents of the same intensity as those for testing were presented simultaneously for 25–50 sec. Conditioning ionophoresis alone induced a sustained increase in discharges from either Purkinje cells or putative basket cells. But this effect quickly diminished after termination of ionophoretic currents, without leaving any appreciable changes in sensitivity to test amino acids (confirmed in more than ten cells).

The glutamate sensitivity of Purkinje cells was markedly reduced when glutamate was ejected in conjunction with olivary stimulation. In Fig. 9, glutamate–olivary conjunction induced a depression in the spontaneous discharge of simple spikes which

lasted for a few minutes (Fig. 9*A*). However, the absence of a significant decrease in aspartate sensitivity serves as a control for the specificity of the marked reduction in glutamate sensitivity (Fig. 9*C*). The difference between glutamate and aspartate sensitivity was obvious even after spontaneous discharges recovered sufficiently (Fig. 9*D*). Glutamate and aspartate sensitivity were evaluated by determining increases in the mean discharge frequency: mean frequency during test ejection for 2·5 sec − mean frequency during 5 sec prior to testings (f_g and f_a in Fig. 9*D*). Glutamate sensitivity can thus be normalized by aspartate sensitivity (f_g/f_a).

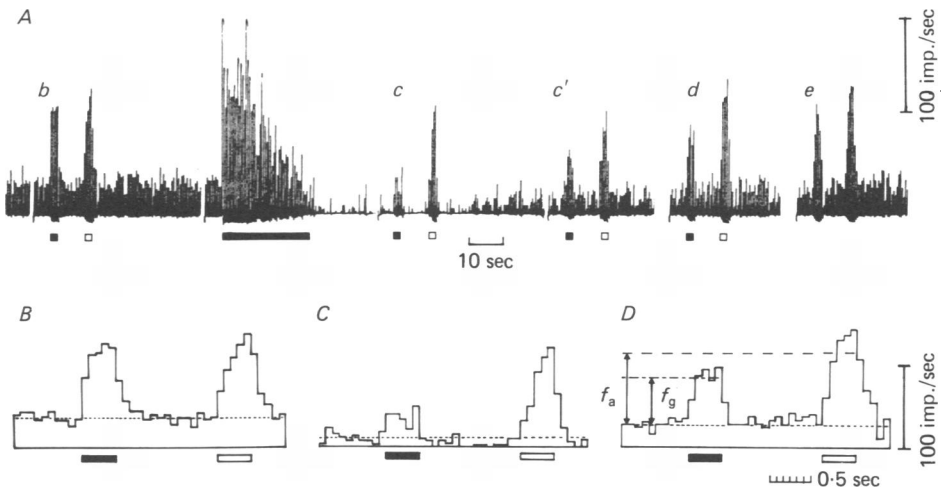

Fig. 9. Responses of a Purkinje cell to amino acids and changes due to conjunctive application of glutamate and stimulation of the inferior olive. *A*, strip chart record showing *simple* spike discharges of a Purkinje cell. Bin width 0·5 sec. Filled squares underneath the records indicate application of glutamate with a 9 nA current. Open squares indicate application of aspartate with 69 nA of current. Filled band: 9 nA glutamate ionophoresis plus stimulation of the inferior olive at 4/sec. Record is interrupted between *c'* and *d*, and *d* and *e*. *d*, 5 min after onset of conjunctive stimulation, *e*, 30 min. *B–D*, spike density histograms expanding records *b*, *c* and *d* in *A*, respectively. The method of measuring f_g and f_a are indicated in *D*. Dotted lines in *B–C* show spontaneous discharge rates calculated from the initial ten bins. Two dashed lines in *D* indicate average discharge rates during application of glutamate and aspartate respectively.

Marked reduction in f_g/f_a, by more than 50% (Fig. 9*C*) or even by 100% (Fig. 10*C*), was observed in thirteen trials of glutamate–olivary conjunction on seven Purkinje cells. The glutamate sensitivity of these cells in the control state was relatively high; 1–30 nA was sufficient for effectively increasing discharge frequencies. The aspartate sensitivity was reciprocally high; effective currents for aspartate or *N*-methyl-aspartate were two to seventeen times as large as those for glutamate. Glutamate–olivary conjunction failed to influence the glutamate sensitivity in seventy-two trials on thirty-two cells which included fourteen Purkinje cells, two putative basket cells and sixteen unidentified cells. Occurrence of climbing fibre responses was not confirmed by oscilloscopic observation (cf. Fig. 1*D*) for unidentified cells, although the olivary stimulation used was far supramaximal for adjacent

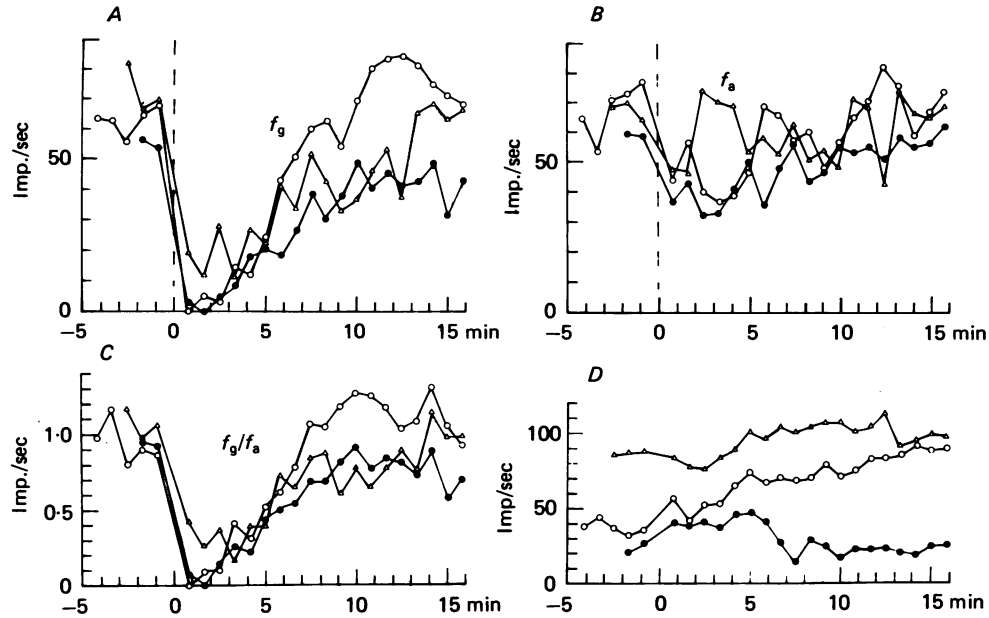

Fig. 10. Time course of the effects of glutamate–olivary conjunction. Three trials for a Purkinje cell are superposed. *A–C*, plots of f_g, f_a and f_g/f_a respectively; *D*, spontaneous discharge rate. ●, 1st trial; ○, 2nd trial; △, 3rd trial. In each trial, glutamate was applied with 2·9 nA for 25 sec, together with 4/sec olivary stimulation. Test ionophoresis; 2·9 nA for glutamate and 99 nA for aspartate. The first trial was succeeded by the second trial without intermission. Records for Fig. 11 were obtained during a one hour gap between the second and third trials.

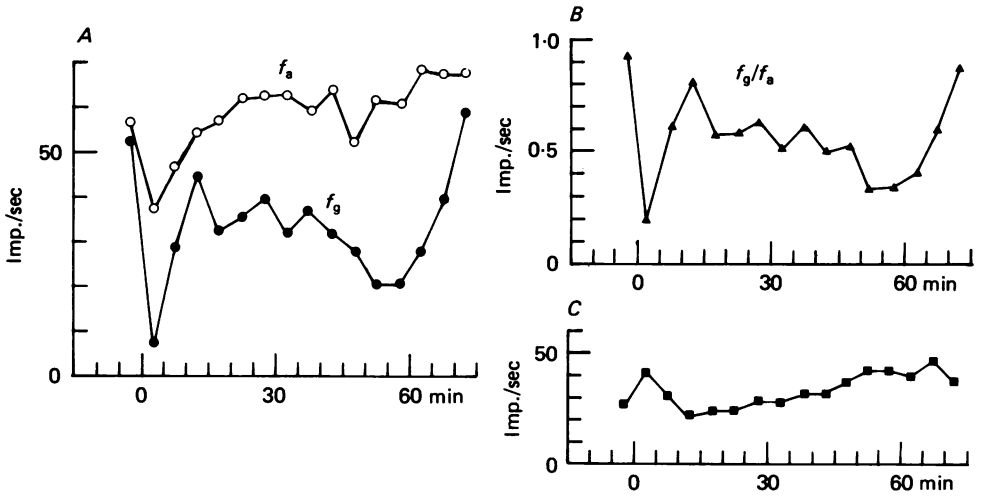

Fig. 11. Long-term recovery after glutamate–olivary conjunction. The entire time course of the second trial of Fig. 10 is illustrated. Each point represents the average value of two to three tests during each 5 min period. *C*, spontaneous discharge rate.

identified Purkinje cells. In these trials with negative results, it was commonly found that effective currents for glutamate were relatively large (larger than 30 nA in twenty-five cells) and that effective currents for aspartate or its *N*-methyl derivative were close to those for glutamate (the former was no more than twice the latter in twenty-two of the twenty-five cells). Therefore, for cells selected for high absolute (effective currents smaller than 30 nA) and relative (effective currents for glutamate are less than half those for aspartate or its *n*-methyl derivative) glutamate sensitivity, glutamate–olivary conjunction frequently depresses glutamate sensitivity (seven out of ten cells).

Fig. 10 illustrates recovery time course of glutamate sensitivity after glutamate–olivary conjunction. Each of three successive trials of glutamate–olivary conjunction produced pronounced depression and succeeding recovery phases which ended about ten minutes after each trial. The ratio f_g/f_a exhibited exponential recovery curves (Fig. 10*C*). Spontaneous discharges varied during prolonged recording, but this variation had no obvious correlation with changes in glutamate sensitivity (Fig. 10*D*). Fig. 11 shows that, after an initial recovery, glutamate sensitivity underwent a later depression which lasted for an hour. In another two cells, a similar slow phase of depression was followed for one half to one hour.

DISCUSSION

Field potentials evoked in the white matter of rabbit flocculus by stimulation of vestibular nerves are very similar to those reported in cat flocculus (Shinoda & Yoshida, 1975). However, the interpretation that ipsilaterally induced P_1–N_1 and contralaterally induced P_1 potentials represent primary vestibular volleys (Shinoda & Yoshida, 1975) does not apply to rabbit flocculus, where these potentials have characteristics of secondary vestibular volleys (Figs. 3 and 4). The early excitation induced by vestibular nerve stimulation in Purkinje cells of rabbit flocculus have features similar to those of cat flocculus Purkinje cells (Shinoda & Yoshida, 1975). The ipsilaterally evoked early excitation has a latency of about 4 msec. Contralaterally evoked excitation shows an additional delay of about 1 msec. Bilateral vestibular inputs converge onto the same cells. Eccles *et al.* (1967*b*) demonstrated that an excitation occurs in vermal Purkinje cells with a latency of 3–4 msec after stimulation of mossy fibres in the deep white matter of the cerebellum or their branches in neighbouring cortical folia. Thus, it seems evident that the early excitation represents activation of floccular Purkinje cells by volleys mediated through primary and/or secondary vestibular mossy fibres and parallel fibres. The inhibition observed in Purkinje cells after vestibular nerve stimulation may represent post-synaptic inhibition exerted by basket cells and superficial stellate cells, or it may reflect post-synaptic inhibition of granule cells through Golgi cells, leading to disfacilitation of Purkinje cells (cf. Eccles *et al.* 1967*a*). The inhibition following the early excitation, observed in Purkinje cells (Fig. 2*C*), may represent such post-synaptic inhibition and disfacilitation and/or refractoriness following the early excitation.

Early excitation of putative basket cells closely resembling that of Purkinje cells can be expected because parallel fibres make excitatory synaptic contact with both Purkinje cells and basket cells (Eccles *et al.* 1967*a*; Palay & Chan-Palay 1974).

Putative basket cells exhibited no pure inhibitory responses to vestibular nerve stimulation (Fig. 2*F* and *G*) such as prevailed in Purkinje cells (Fig. 2*D* and *E*). This observation is consistent with the fact that, in basket cells, inhibitory synapses are less common than excitatory synapses supplied from parallel fibres (Palay & Chang-Palay, 1974). These inhibitory synapses may be supplied from Purkinje cells (Hámori & Szentágothai, 1966) or from basket cells and superficial stellate cells (Palay & Chan-Palay, 1974). The depression folowing the early excitation (Fig. 2*F*) may represent an inhibition through these synapses and/or refractoriness following the early excitation.

The present study revealed that conjunctive vestibular–olivary stimulation effectively depressed the early excitation of Purkinje cells evoked through vestibular mossy fibre afferents. The depression was induced by stimulating the inferior olive at a relatively low frequency (4/sec), mimicking climbing fibre activity in alert rabbits (Ghelarducci *et al.* 1975). This was an important factor for successful demonstration of the depression, as olivary stimulation at higher frequencies was sufficient to induce a prolonged depression of Purkinje cells, thus preventing observation of the vestibular-evoked excitation. Olivary stimulation at 10–60/sec for 2–8 sec was reported to produce an eye movement, presumably due to sustained silence of flocculus Purkinje cells (Barmack & Hess 1980).

The possibility that the effect of vestibular–olivary conjunction is merely due to a general depression of Purkinje cell membranes is unlikely for two reasons. First, there was no sign of a general depression in the rates of spontaneous discharge from Purkinje cells (Fig. 5*A*). Secondly, the depression was specific to the early excitation from the vestibular nerve involved in the conjunction; responses from the other nerve were not affected (Fig. 6*A–F*). Therefore, it is concluded that the depression occurs at a limited site or sites in the specific pathway from a vestibular nerve to floccular Purkinje cells. This pathway includes three synaptic sites (Fig. 1*A*): (1) vestibular nuclei which relay a part of vestibular mossy fibres; (2) mossy fibre–granule cell synapses; (3) parallel fibre–Purkinje cell synapses. Sites (1) and (2) can be excluded for the following four reasons. Firstly, conjunctive vestibular–olivary stimulation did not influence the early excitation in putative basket cells (Fig. 6*G* and *H*), which should be evoked through the sites (1) and (2), but not (3). Secondly, vestibular–olivary conjunction did not influence the vestibular-evoked inhibition of Purkinje cells, which should be evoked through sites (1) and (2) and via cortical inhibitory neurones (see above). Thirdly, vestibular–olivary conjunction did not affect secondary vestibular volleys induced either ipsilaterally or contralaterally (Fig. 6*I* and *J*), which should reflect impulse propagation through the site (1). Fourthly, field potentials in the granular layer of the flocculus, which should be relevant to sites (1) and (2), were also unaffected (Fig. 6*K* and *L*). Consequently, it can be concluded that the site (3) is responsible for the depression of the vestibular-evoked early excitation in Purkinje cells.

One may argue that the postulated plasticity of parallel fibre–Purkinje cell synapses should further be tested with direct stimulation of parallel fibres. However, it is not an easy task to control electrical stimulation of unmyelinated parallel fibres packed in the molecular layer. Current may spread from an electrode placed on the surface of a molecular layer to excite mossy fibres or even climbing fibres (Eccles, Sasaki & Strata 1966*b*; Eccles *et al.* 1966*a*, 1967*b*), since these myelinated fibres have a lower

threshold for electric stimulation. In this respect, the well-controlled stimulation of vestibular mossy fibres in peripheral nerves, as adopted in this study, compensates for the indirect reasoning that is required.

The demonstration that the time course of changes in glutamate sensitivity of Purkinje cells evoked by glutamate–olivary conjunction is similar to the depression of vestibular responsiveness following vestibular–olivary conjunction (compare Figs. 7 and 8 with Figs. 10 and 11) provides another line of evidence that parallel fibre–Purkinje cell synapses are the site of the depression. High glutamate sensitivity was a pre-requisite for obtaining this effect. Chujo, Yamada & Yamamoto (1975) showed in slice preparations of the cerebellum that Purkinje cell dendrites have substantially higher glutamate sensitivity than the cell soma. It is likely that the depressant action of glutamate–olivary conjunction is effective only when glutamate impinges on dendrites of Purkinje cells, which are the site of parallel fibre–Purkinje cell synapses. There is accumulating evidence that glutamate is the neurotransmitter of parallel fibres (Young, Oster-Granite, Herndon & Snyder, 1974; McBride, Nadi Altman & Aprison, 1976; Hudson, Valcana, Bean & Timiras, 1976; Herndon & Coyle, 1977; Sandoval & Cotman, 1978; Hacket *et al.* 1979). The present results suggest that the effect of vestibular–olivary conjunction involves the subsynaptic chemosensitivity of Purkinje cell dendrites to the putative neurotransmitter of parallel fibres. A question may arise why such a depression in subsynaptic chemosensitivity causes no consistent changes in spontaneous discharges from a Purkinje cell (Figs. 7*B*, 8*B*, 10*D*, 11*C*) which are presumed to be induced, at least largely, by spontaneous bombardment through parallel fibres. Probably, either the vestibular–olivary or glutamate–olivary conjunction tested here involves only a small fraction of numerous synapses distributed over widely extending dendritic trees of a Purkinje cell, so that it hardly affects the spontaneous discharges determined by integration of the whole synaptic inputs.

How do climbing fibre impulses affect subsynaptic chemosensitivity of Purkinje cells when combined with parallel fibre impulses? Marr (1969) adopted Hebb's idea of a plasticity condition that post-synaptic membrane excitation should coincide with presynaptic activity. According to this hypothesis, climbing fibre impulses act upon parallel fibre–Purkinje cell synapses through membrane excitation in Purkinje cells. However, this is unlikely in the present case for the following three reasons. Firstly, vestibular volleys can induce membrane excitation of floccular Purkinje cells in the form of early excitation and rebound facilitation (Fig. 2*C* and *E*). This means that vestibular stimulation should be sufficient to fulfill the plasticity condition. Yet, sustained vestibular nerve stimulation produces no sign of plastic changes in parallel fibre–Purkinje cell synapses. Secondly, ionophoretic application of glutamate to Purkinje cells induces membrane excitation. Therefore, if glutamate is the neurotransmitter of parallel fibres, sustained application of glutamate also fulfills the plasticity condition. Yet, there is no evidence for this. Thirdly, climbing fibre impulses are effective when delivered at a rate as low as 4/sec. This means that a longer-lasting process than impulse discharges in Purkinje cells following each climbing fibre impulse is essential for the plasticity. Single climbing fibre impulses evoke a membrane depolarization in Purkinje cell dendrites which lasts for as long as 1 sec (Ekerot & Oscarsson, 1981).

What then can be the mechanism for climbing fibre impulses to influence parallel

fibre–Purkinje cell synapses? Since the long-lasting depolarization induced by climbing fibre impulses in Purkinje cell dendrites appears to involve a voltage-dependent increase of calcium permeability of the dendritic membrane (Llinás & Sugimori, 1980), it may be postulated (Ekerot & Oscarsson, 1981) that an increased intradendritic calcium concentration affects subsynaptic receptors of Purkinje cell dendrites, just as intracellular calcium is supposed to desensitize acetylcholine receptors in muscle endplates (Miledi, 1980). An alternative possibility is that climbing fibres liberate a chemical substance(s) which reacts with subsynaptic receptors at parallel fibre–Purkinje cell synapses together with the parallel fibre neurotransmitter, thereby rendering the receptors insensitive to the parallel fibre neurotransmitter. This speculation draws an analogy from the effects of application of thyrotropin-releasing hormone (TRH) to cerebral pyramidal cells; TRH causes a reduction in glutamate sensitivity without affecting aspartate sensitivity (Renaud, Blume, Pittman, Lamour & Tan, 1979).

The present results support the general framework of Marr's plasticity assumptions. However, the modification actually obtained is not a facilitation as originally suggested by Marr (1969), but a depression as postulated later by Albus (1971). Either facilitation or depression has similar theoretical implications, but Albus (1971) preferred depression for some practical reasons. The appropriateness of Albus' assumption has already been indicated by observations of Purkinje cell activity in behaving animals (Gilbert & Thach, 1977; Ito, 1977). An important question still remaining concerns the long-term time course of modification of parallel fibre–Purkinje cell synapses. The present study indicates that the depression at these synapses lasts for at least one hour following conjunctive stimulation for 25 sec (Figs. 8 and 11), but it is not yet clear whether recovery from the depression under the same or some other stimulus conditions involves a still slower phase or a process analogous to a permanent memory. Improved techniques for well-controlled, stable, long-term recording and trials of various conditions of conjunctive stimulation are necessary for answering this question.

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