Floccular Modulation of Vestibuloocular Pathways and Cerebellum-Related Plasticity: An In Vitro Whole Brain Study

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Froccular modulation of vestibuloocular pathways and cerebellum-related plasticity: an in vitro whole brain study. J Neurophysiol 84: 2514–2528, 2000. The isolated whole brain (IWB) preparation of the guinea pig was used to investigate the floccular modulation of vestibular-evoked responses in abducens and oculomotor nerves and abducens nucleus; for identification of flocculus target neurons (FTNs) in the vestibular nuclei and intracellular study of some of their physiological properties; to search for possible flocculus-dependent plasticity at the FTN level by pairing of vestibular nerve and floccular stimulations; and to study the possibility of induction of long-term depression (LTD) in Purkinje cells by paired stimulation of the inferior olive and vestibular nerve. Stimulation of the flocculus had only effects on responses evoked from the ipsilateral (with respect to the stimulated flocculus) vestibular nerve. Floccular stimulation significantly inhibited the vestibular-evoked discharges in oculomotor nerves on both sides and the inhibitory field potential in the ipsilateral abducens nucleus while the excitatory responses in the contralateral abducens nerve and nucleus were free from such inhibition. Eleven second-order vestibular neurons were found to receive a short-latency monosynaptic inhibitory input from the flocculus and were thus characterized as FTNs. Monosynaptic inhibitory postsynaptic potentials from the flocculus were bicuculline sensitive, suggesting a GABA<sub>A</sub>-ergic transmission from Purkinje cells to FTNs. Two of recorded FTNs could be identified as vestibulospinal neurons by their antidromic activation from the cervical segments of the spinal cord. Several pairing paradigms were investigated in which stimulation of the flocculus could precede, coincide with, or follow the vestibular nerve stimulation. None of them led to long-term modification of responses in the abducens nucleus or oculomotor nerve evoked by activation of vestibular afferents. On the other hand, pairing of the inferior olive and vestibular nerve stimulation resulted in approximately a 30% reduction of excitatory postsynaptic potentials evoked in Purkinje cells by the vestibular nerve stimulation. This reduction was pairing-specific and lasted throughout the entire recording time of the neurons. Thus in the IWB preparation, we were able to induce a LTD in Purkinje cells, but we failed to detect traces of flocculus-dependent plasticity at the level of FTNs in vestibular nuclei. Although these data cannot rule out the possibility of synaptic modifications in FTNs and/or at other brain stem sites under different experimental conditions, they are in favor of the hypothesis that the LTD in the flocculus could be the essential mechanism of cellular plasticity in the vestibuloocular pathways.

INTRODUCTION

The cerebellar flocculus exerts a specific inhibitory modulation of both excitatory and inhibitory branches of vestibuloocular pathways to various extraocular muscles (Baker et al. 1972; Fukuda et al. 1972; Highstein 1973; Hirai and Uchino 1984; Ito et al. 1970, 1973, 1977; Kawaguchi 1985; Sato and Kawasaki 1987, 1990; Sato et al. 1988). These actions are mediated through monosynaptic inhibitory projections of floccular Purkinje cells to the vestibular nuclei neurons, known as “flocculus target neurons” (FTNs), at least some of which are relay neurons of vestibuloocular reflex arcs (see for reviews du Lac et al. 1995; Sato and Kawasaki 1991). The flocculus is also a region of convergence of the vestibular afferent signals through mossy fiber inputs from vestibular nuclei and the visual signals coming via climbing fibers of the inferior olive (see for reviews De Zeeuw et al. 1994; Sato and Kawasaki 1991).

The flocculus plays a key role in a specific form of motor learning, the adaptation of the vestibuloocular reflex (VOR). Based on theoretical studies of cerebellar cortical function, M. Ito has proposed that VOR gain changes characteristic of the vestibular adaptation could be based on long-term alterations of the modulatory influence of floccular Purkinje cells on target relay neurons of vestibuloocular pathways (Ito 1972, 1982). Such modifications would result from regulation of a signal flow from labyrinths to the flocculus in a form of long-term changes in efficiency of synaptic transmission from parallel fibers to Purkinje cells. An unusual combination of vestibularly driven parallel fiber inputs and visually driven climbing fiber inputs to Purkinje cells, due to a mismatch between vestibular and visual signals, would trigger these modifications. In accordance with this hypothesis, it was demonstrated both in intact animals and in brain slices that combined activation of parallel and climbing fibers could lead to a long-term depression (LTD) of the parallel fiber synaptic input to Purkinje cells (Ekerot and Kano 1985; Ito and Kano 1982; Ito et al. 1982; Sakurai 1987; see also for reviews Ito 1989; Linden and Connor 1995).

On the other hand, by comparing the latencies of the modifiable component of VOR with related neuronal responses at various sites of the vestibuloocular network and based on extensive modeling, S. Lisberger and colleagues have concluded that the plasticity at the level of cerebellar cortex is not sufficient to account for the VOR adaptation. They have suggested the existence of a brain stem site of learning, in particular at the level of vestibular inputs to FTNs (see for reviews...
Preparation and maintenance of the IWB in vitro

**METHODS**

Some of their functional characteristics. To demonstrate that in this species the VOR gain could be plastically altered by appropriate paradigms of VOR adaptation (Serafin et al. 1997) and their plasticity following unilateral labyrinthectomy (Vibert et al. 1999). The IWB of the guinea pig could be an adequate model to study the cellular mechanisms of VOR adaptation because it has been recently demonstrated that in this species the VOR gain could be plastically altered by appropriate paradigms of VOR adaptation (Serafin et al. 1999). The IWB preparation has also an advantage of much lower levels of background neuronal activity as compared with intact animals. This provides more favorable conditions for efficient, selective stimulation of various pathways and detection of plasticity than experiments in vivo.

Therefore one of the aims of the present study was to test in the IWB preparation the possibility of flocculus-dependent synaptic plasticity in FTNs probably cannot be tested in brain slices. Indeed, preservation of FTNs together with their inputs from the vestibular nerve and the flocculus, identification and selective activation of these projections in the same slice would be an extremely difficult task. These problems could be circumvented by using the in vitro isolated whole brain (IWB) preparation of the guinea pig that allowed us to study the neuronal networks controlling gaze and posture in the guinea pig (Babalian et al. 1997) and their plasticity following unilateral labyrinthectomy (Vibert et al. 1999). The IWB of the guinea pig could be an adequate model to study the cellular mechanisms of VOR adaptation because it has been recently demonstrated that in this species the VOR gain could be plastically altered by appropriate paradigms of VOR adaptation (Serafin et al. 1999). The IWB preparation has also an advantage of much lower levels of background neuronal activity as compared with intact animals. This provides more favorable conditions for efficient, selective stimulation of various pathways and detection of plasticity than experiments in vivo.

Three types of experiments were performed in the present study. The first group of experiments was designed to study floccular modulation of vestibulocellular pathways. Recordings were performed from oculomotor nerves, abducens nerves, and nuclei, while vestibular nerves and flocculus were electrically stimulated (see Figs. 1B, 3B, and 4, A2 and B2 for the corresponding experimental designs). In the same experiments, to assess possible flocculus-dependent plasticity of vestibular inputs to FTNs, several paradigms of pairings of floccular and vestibular nerve stimulations were performed (see Results).

In the second type of experiments, using the same stimulation configuration as described in the preceding text, we searched for and intracellularly recorded FTNs to study their physiological properties. To map the location of recorded FTNs in the brain stem, the following procedure was used. Initially, the abducens nucleus was localized using the antidromic field potential evoked by stimulation of the abducens nerve. Once the nucleus was found, the glass recording electrode was positioned in its center (as judged by the largest amplitude of the antidromic field potential), and the electrode shank was broken. The extremity of the broken pipette emerging from the ventral surface of the brain stem served then as a landmark to determine the FTN locations. All recorded FTNs were found in the rostral part of the vestibular complex in the vicinity of the abducens nucleus.

Finally, a third experimental design was used to explore whether the cerebellar LTD could be induced in the IWB. The contralateral inferior olive and ipsilateral vestibular nerve were stimulated while intracellular recordings were made from Purkinje cells of the parafloccular and floccular regions (see Fig. 7A). To ensure that the inferior olive stimulation was not simultaneously activating neighboring vestibular neurons, control recordings of the activity of abducens...
and oculomotor nerves were performed prior to LTD studies. The protocol for inducing LTD in Purkinje cells consisted of the inferior olive and the following vestibular nerve single-pulse stimulations presented at an interval of 150 ms during 500–600 trials at a rate of 2 Hz. While the issue of the optimal stimulation order and timing required for inducing the LTD remain largely controversial, we applied a protocol traditionally used for that purpose by other authors (see Linden and Connor 1995).

Both vestibular nerves, the flocculus and the inferior olive, were stimulated with bipolar metallic electrodes. They were made using stainless steel microelectrodes (FHC, Brunswick, ME) and had interpolar distances of 0.5–1 mm. To stimulate vestibular nerves, the electrodes were inserted in the anterior (vestibular) stumps of the vestibulocochlear nerve. For stimulation of the flocculus, which was exposed and easily accessible in the ventral side up position of the brain, the electrode was lowered in the corresponding area of the cerebellar cortex on a depth of 0.5–1 mm. Placement of the stimulating electrode in the inferior olive was first inferred fromatlases of the guinea pig brain (Gstoettner and Burian 1987; Voitenko and Marлин-ski 1993). The electrode was inserted just below (about 0.5 mm) the ventral surface of the brain stem, 1 mm lateral to the midline, and 1–1.5 mm caudally to clearly visible cochlear nerves and nuclei. Its position was further adjusted by monitoring a characteristic field response size induced by activation of the flocculus were expressed as averaging four to eight individual traces and measuring both the peak amplitudes of averaged responses during the control period was set at 100% for each cell, and the amplitudes of averaged responses during the whole recording time were expressed as a percentage of this value. Finally, the response sizes (in percentages) were averaged over the time intervals of 5 min for the whole population of recorded Purkinje cells.

The data samples were compared using an unpaired r-test. For data including more than two samples of measurements, the ANOVA test was initially run to evaluate possible differences between the samples. If the existence of significant differences (5% confidence interval) was found, a further comparison of pairs of measurements was performed (SigmaPlot and SigmaStat, Jandel Scientific). All averaged data in the paper are expressed as means ± SD (standard deviation).

RESULTS
Effects of stimulation of the flocculus on evoked activity in the ipsilateral abducens nerve and nucleus

Floccular modulation of the VOR pathways was assessed by monitoring changes of vestibular-evoked responses in abducens and oculomotor nerves and nuclei following stimulation of the flocculus. The effects of floccular stimulation were maximal when it coincided with or slightly preceded (by up to 2 ms) the stimulation of vestibular nerves (control experiments: not shown). Therefore the flocculus was always stimulated within this optimal time window. In all experiments described in the following text, the laterality of stimulated flocculus and vestibular nerves is defined with respect to recorded nerves or nuclei.

In a previous study (Babalian et al. 1997), we have demonstrated that the stimulation of the ipsi- and contralateral vestibular nerves evoked characteristic responses in the abducens nerve and nucleus. Stimulation of the contralateral vestibular nerve elicited a synchronized discharge in the abducens nerve and a disynaptic negative excitatory field potential in the abducens nucleus (Fig. 1A; Vc). Activation of the ipsilateral vestibular nerve did not produce any response in the abducens nerve, while it evoked a disynaptic positive inhibitory field potential in the abducens nucleus (Fig. 1B; Vi). Stimulation of the ipsilateral flocculus alone had no visible effect on the abducens nerve and nucleus (Fig. 1A; Vc).
translation was combined with stimulation of the two vestibular nerves. As shown before (Babalian et al. 1997), stimulation of the ipsilateral vestibular nerve resulted in a strong decrease of the excitatory responses evoked from the contralateral vestibular nerve (compare Fig. 1A, Vc + Vi with Vc) in the abducens nerve (23.8 ± 16%; n = 10; P < 0.001) and nucleus (31.4 ± 8.2%; n = 6; P < 0.005). Adding the floccular stimulation to the stimulation of both vestibular nerves resulted in substantial recovery of responses in the abducens nerve and nucleus (Fig. 1A; Fi + Vc + Vi). The response magnitudes amounted to 72.2 ± 14.8% (n = 10) and 76.8 ± 11.2% (n = 6) of control values for the abducens nerve and nucleus, respectively. These responses were significantly bigger (P < 0.001) than those when two vestibular nerves were stimulated without the flocculus, but still substantially smaller (P < 0.01) than control responses.

Thus it can be concluded that the flocculus exerts inhibitory actions on inhibitory second-order vestibular neurons activated from the ipsilateral vestibular nerve and projecting to the ipsilateral abducens nucleus. The experimental design and postulated circuitry for the afore-described interactions are schematically presented in Fig. 1B, while the amplitude measures for responses in different stimulation conditions are summarized in Fig. 1C.

Intracellular recordings from abducens motoneurons (Fig. 2)
further confirmed the suggested mechanism of floccular action on responses in the ipsilateral abducens nucleus. Motoneurons were antidromically identified by stimulation of the abducens nerve (Fig. 2; anti). Stimulation of the contra- and ipsilateral vestibular nerves evoked disynaptic excitation (Fig. 2; Vc) and inhibition (Fig. 2; Vi) of motoneurons, respectively. Although stimulation of the flocculus alone had no direct effect on the motoneurons (Fig. 2; Fi), it largely or sometimes completely eliminated the disynaptic inhibitory postsynaptic potential (IPSP) evoked from the ipsilateral vestibular nerve (Fig. 2; \(F_i + V_i\)). The disynaptic EPSP from the contralateral vestibular nerve (Fig. 2; Vc), attenuated by the inhibitory action of the ipsilateral vestibular nerve (Fig. 2; Vc + Vi), was also disinhibited by the floccular stimulation (Fig. 2; Fi + Vc + Vi).

**Effects of stimulation of the flocculus on evoked activity in the contralateral abducens nerve and nucleus**

The excitatory and inhibitory responses in the abducens complex induced by stimulation of the two vestibular nerves (Fig. 3; Vc and Vi) remained unchanged when the contralateral flocculus was activated (Fig. 3; Fc + Vc and Fc + Vi). The amplitudes of abducens nerve discharge and excitatory field potential in the abducens nucleus, in response to combined stimulation of the contralateral vestibular nerve and flocculus (Fig. 3; Fc + Vc), amounted to 99 ± 1.5% (\(n = 3\)) and 101 ± 1.4% (\(n = 3\)) of control responses, respectively. Similarly, the amplitude of the positive field potential evoked in the abducens nucleus by stimulation of the ipsilateral vestibular nerve amounted to 100% (\(n = 3\)) of the control response when the vestibular nerve was stimulated together with the contralateral flocculus (Fig. 3; Fc + Vi). Consequently, floccular stimulation did not affect the responses in the abducens nerve and nucleus induced by joint stimulation of both vestibular nerves (compare Fig. 3; Vc + Vi with Fc + Vc + Vi). The inhibited discharges in the abducens nerve due to combined stimulation of both vestibular nerves (43 ± 18.4% of control Vc response; \(P < 0.05; n = 3\)) had a similar size when stimulation of the flocculus was added (42.5 ± 10.7%; \(n = 3\)). The corresponding response magnitudes in the abducens nucleus compared with control (Vc alone) values were 20 ± 7.1% (\(P < 0.01; n = 3\)) and 21 ± 8.5% (\(P < 0.01; n = 3\)). These data suggest that projections of the flocculus do not converge with vestibular afferent inputs on excitatory second-order vestibular neurons projecting to the contralateral abducens nucleus (Fig. 3B).

**Floccular modulation of vestibular-evoked activity in oculomotor nerves**

Our preliminary recordings have demonstrated that stimulation of vestibular nerves induced responses with complex waveforms in the oculomotor nuclei, suggesting a dual nature (excitatory and inhibitory) of underlying synaptic events (not shown). Moreover, response shapes varied significantly depending on the position of the recording electrode within the nucleus as predicted from the fact that the oculomotor nucleus encompasses motoneuronal populations of four different ex-
nerves of the IWB preparation, only the excitatory vestibular-evoked responses could systematically be investigated.

Stimulation of the contra- and ipsilateral vestibular nerves evoked synchronized disynaptic discharges in oculomotor nerves (Fig. 4, A1 and B1; Vc, Vi). Stimulation of the flocculus alone had no effect on oculomotor nerves on either the ipsilateral (Fig. 4A1; F) or contralateral (Fig. 4B1; Fc) side.

Stimulation of the ipsilateral flocculus did not significantly modify the size (101.3 ± 9.4%; n = 6) of the responses evoked from the contralateral vestibular nerve (Fig. 4A1; Fi + Vc). In contrast, activation of the flocculus inhibited (to 45.7 ± 20.5%; P < 0.005; n = 6) the responses from the ipsilateral vestibular nerve (Fig. 4A1; FFi + Vi). The suggested schema of neuronal wiring for these interactions and relative amplitudes of the responses in different stimulation conditions are presented in Fig. 4A2 and A3, respectively.

Stimulation of the contralateral flocculus substantially reduced (58.3 ± 10.7%; P < 0.005; n = 3) the responses from the vestibular nerve of the same side (Fig. 4B1; Fc + Vc), but it had no effect (99.3 ± 4%; n = 3) on responses from the ipsilateral vestibular nerve (Fig. 4B1; Fc + Vi). Proposed neuronal connections underlying these floccular actions are depicted in Fig. 4B2, while B3 shows the distribution of response sizes due to different combination of stimuli.

Together our data suggest the convergence of homolateral inputs from the flocculus and vestibular afferents on excitatory second-order vestibular neurons projecting to both ipsi- and contralateral oculomotor nerves. The flocculus exerted a stronger inhibitory action on vestibular-evoked responses in the ipsilateral oculomotor nerve than in the contralateral one (compare the sizes of flocculus-induced depressed responses in the preceding text). Finally, as in the case of recordings from abducens nerves and nuclei, no signs of interaction between the flocculus and contralateral vestibular afferents were observed.

Physiological properties of FTNs

Altogether, successful intracellular recordings were made from 11 FTNs. All these cells were found in a small region located slightly caudal (0.3–0.8 mm) and lateral (0.5–0.8 mm) to the abducens nucleus, 0.5–1 mm below the nucleus level (see in Methods the way to localize FTNs). According to the atlases of the guinea pig brain (Gstoettner and Burian 1987; Voitenko and Marlinskii 1993), this area should correspond to the rostral part of the medial vestibular nucleus and to the medial parts of inferior (descending) and lateral vestibular nuclei. Penetrations of FTNs were rather rare. Only one of about five to seven impaled vestibular neurons
in this region was a FTN. The neurons were identified as FTNs when receiving monosynaptic inhibitory input from the ipsilateral flocculus. All recorded FTNs were monosynaptically excited by stimulation of the ipsilateral vestibular nerve and were thus characterized as second-order vestibular neurons. The recorded neurons had resting membrane potentials not less than −55 mV and spike amplitudes of 50 mV or more. Recordings from a typical FTN are shown in Fig. 5A. Stimulation of the ipsilateral vestibular nerve evoked a monosynaptic EPSP and superimposed spikes in this neuron (Fig. 5A1), whereas activation of the homolateral flocculus elicited a short-latency monosynaptic IPSP (Fig. 5A2) in the same cell. The inhibitory nature of the hyperpolarizing response from the flocculus could be demonstrated by combined stimulation of the ipsilateral vestibular nerve and the flocculus. The monosynaptic EPSP evoked by stimulation of the ipsilateral vestibular nerve was substantially inhibited by simultaneous floccular stimulation (Fig. 5A3). Moreover, polarizing tests applied to FTNs demonstrated that hyperpolarizing flocculus-induced responses could be reduced and even reversed by hyperpolarization of the neuronal membrane using currents passed through the intracellular microelectrode (Fig. 5A5). Finally, in two pharmacologically tested FTNs, the hyperpolarizing responses were suppressed by bath application of bicuculline (Fig. 5A4), suggesting that they were mediated by GABAergic transmission. These results indicate that hyperpolarizing responses induced from the flocculus are true IPSPs and not disfacilitations.

The IPSPs could follow high stimulation frequencies (100 Hz; not shown); they had an average latency of 2.28 ± 0.22 ms (range 2–2.6 ms; n = 11; see about their monosynaptic nature

![Figure 5](http://jn.physiology.org/)

**FIG. 5.** Physiological properties of flocculus target neurons (FTNs). Intracellular recordings from 2 (A and B) FTNs at resting membrane potentials of −59 and −57 mV, respectively. A1: monosynaptic excitatory postsynaptic potentials (EPSPs) with superimposed spikes evoked by stimulation of the ipsilateral vestibular nerve (top traces) and the corresponding extracellular records (bottom traces). Note that the stimulation artifact is bigger in the extracellular records due to slightly higher stimulation intensity. A2: inhibitory postsynaptic potentials (IPSPs) evoked in the same neuron by floccular stimulation (top traces) and the corresponding extracellular records (bottom traces). A3: response to the combined stimulation of the vestibular nerve and flocculus. A4: effect of perfusion of the brain (9 min) with bicuculline (25 μM) on the flocculus-evoked IPSP (compare with A2). A5: decrease and reversal of the flocculus-evoked IPSP by hyperpolarization of the neuronal membrane. The numbers on the left side of the panel indicate the membrane potential values. The reversal potential for IPSP was around −90 mV. Records in A1–A4 are superpositions of several individual traces, whereas records in A5 are averages of 16 individual responses for each membrane potential value. B1: antidromic activation of a neuron at threshold intensity stimulation of the spinal cord (spinal). B2: antidromic action potentials following triple stimulation of the spinal cord (spinal x3) at frequency of 200 Hz. B3: monosynaptic activation of the neuron by stimulation of the ipsilateral vestibular nerve (top traces) and the corresponding extracellular records (bottom traces). B4: monosynaptic IPSPs evoked by floccular activation (top traces) and the corresponding records outside of the cell (bottom traces). B5: effect of combined stimulation of the flocculus and vestibular nerve. Records in B1–B5 are superpositions of several individual responses. Amplitude calibration is 20 mV for records in B1 and B2 while it is 5 mV for the rest of records in A and B.
in DISCUSSION) and an amplitude of 3.8 ± 1.4 mV (range 1.5–5.8 mV; n = 11). The latency of monosynaptic EPSPs evoked in FTNs by the vestibular nerve stimulation was 1.68 ± 0.18 ms (range 1.3–1.9 ms; n = 11). The input resistance and the time constant of FTNs measured in five cells were 70 ± 10.6 MΩ and 15.9 ± 5.6 ms, respectively.

In two cells, stimulation of the spinal cord evoked antidromic spikes, suggesting the existence of spinal projections from some of FTNs. Responses of one of these cells are illustrated in Fig. 5B. In the neuron, stimulation of the spinal cord evoked action potentials appearing in an all-or-none manner at stable latencies without any subthreshold potentials (Fig. 5B1). Moreover, these spikes could follow high frequencies of spinal stimulation (200 Hz, Fig. 5B2), and were concluded to be antidromic. Stimulation of the ipsilateral vestibular nerve evoked a monosynaptic EPSP with superimposed spikes in the neuron (Fig. 5B3), whereas floccular activation induced monosynaptic inhibition of the cell (Fig. 5B4). The IPSP from the flocculus completely shunted the vestibular-induced EPSP when both structures were stimulated together (Fig. 5B5).

Of nine FTNs in which the commissural input from the contralateral vestibular nerve was tested, seven cells were inhibited (mainly disynaptically) at latencies of 3.7 ± 0.55 ms and the remaining two neurons responded with EPSPs at latencies of 2.9 and 4 ms.

All recorded FTNs discharged action potentials with double-component afterhyperpolarization (Fig. 5A1 and B3), did not exhibit low-threshold spikes when the membrane potential was manipulated with current pulses, and were thus tentatively characterized as type B neurons according to the “in vitro” classification of vestibular neurons (see Babalian et al. 1997; Serafin et al. 1991). Most of the FTNs (7 of 11) were spontaneously active at resting membrane potentials, and their discharge rate was 9.8 ± 2.6 Hz.

**Pairings of floccular and vestibular nerve stimulations: search for plasticity at the FTN level**

To test the hypothesis of whether coactivation of converging floccular and vestibular nerve projections could lead to long-term plastic changes of the efficiency of the synaptic input from vestibular afferents to FTNs, pairings of floccular and vestibular nerve stimulations were performed. We monitored the effect of pairing on either the positive inhibitory field potentials in the abducens nucleus or the discharges in the oculomotor nerve evoked by the ipsilateral vestibular nerve stimulation. Both these responses were strongly inhibited by stimulation of the ipsilateral flocculus applied approximately 1.5 ms before the vestibular nerve stimulation (Fig. 6A2; Fi + Vi). However, after 1 h of such joint stimulation of the flocculus with vestibular nerve at a rate of 0.5 Hz (altogether about 1,800 trials), the response in the oculomotor nerve remained unchanged (Fig. 6A2, 2nd trace from the bottom). Another paradigm of pairing was performed in the same experiment: five-pulse stimulation of the flocculus with the interstimulus interval of 10 ms (100 Hz) followed the vestibular nerve stimulation after a 150-ms time interval. The trials were repeated each 2 s (0.5 Hz) during 50 min (about 1,500 trials). Again, after the pairing procedure, the response in the oculomotor nerve evoked by the ipsilateral vestibular nerve stimulation was not modified (Fig. 6A2, bottom).

Similarly, different paradigms of pairings were tested while recording from the ipsilateral abducens nucleus. The corresponding experimental arrangement and neuronal interactions are presented in Fig. 6B1. Stimulation of the ipsilateral vestibular nerve evoked a positive field potential in the abducens nucleus (Fig. 6B2; Vi, control) corresponding to the disynaptic inhibition of abducens motoneurons. As shown in the preceding text, floccular stimulation strongly suppressed this response (Fig. 6B2; Fi + Vi), acting on FTNs that are in turn the inhibitory second-order, relay vestibular neurons projecting to the ipsilateral abducens nucleus. In the illustrated experiment, simultaneous stimulation of the flocculus and vestibular nerve were performed at three different frequencies (1, 10, and 100 Hz), in 450–600 trials for each frequency. However, the pairing did not result in any latency, amplitude or shape changes of the field potential as compared with the control response (Fig. 6B2; 3rd–5th traces from the top). In the same experiment, another pairing protocol, in which the vestibular nerve stimulation preceded the floccular stimulation by 10 ms in 700 trials at 1 Hz, also did not affect the response in the abducens nucleus (Fig. 6B2; bottom).

Altogether, in the present study, 18 pairing sessions with different timing patterns of the vestibular nerve and floccular stimulation were performed. However, none of these paradigms summarized in the Table 1 led to significant long-term changes of the efficiency of transmission from vestibular afferents to the abducens nucleus and oculomotor nerve as one could conclude from the response amplitudes, slopes, and latencies.

**Intracellular recordings from Purkinje cells: search for LTD in the IWB preparation**

Stable intracellular recordings were obtained from 12 Purkinje cells in which the responses from both the contralateral inferior olive and the ipsilateral vestibular nerve could be tested. The membrane potential of recorded cells was at least −60 mV. The design of the experiments and the involved relevant pathways are depicted in Fig. 7A. Stimulation of the contralateral inferior olive evoked a characteristic complex spike (a broad, 15- to 25-ms lasting discharge with several superimposed fast deflections) in Purkinje cells (Fig. 7B1). A strict selection of recorded Purkinje cells was made based on two requirements for the inferior olive stimulation. First, its intensity had to be low enough to induce the complex spikes in Purkinje cells without any other subthreshold potentials in the
recorded cell (Fig. 7B1). Second, the stimulation should not have induced any short-latency discharges in abducens and oculomotor nerves. Thus we ensured the placement of stimulating electrodes in the inferior olive and avoided stimulation of neighboring vestibular structures by a current spread. The Purkinje cell recordings that did not meet these criteria were discarded from the present study. As a result of this selection, we could evoke complex spikes in Purkinje cells at thresholds as low as 20–30 μA. The mean latency of the complex spikes was 8.2 ± 1.5 ms (range 5.7–11 ms; n = 12), and their average amplitude was 43.5 ± 7.6 mV (range 33–56 mV; n = 12).

Stimulation of the ipsilateral vestibular nerve evoked mainly EPSPs in the recorded Purkinje cells at thresholds as low as 20–30 μA. The mean latency of the complex spikes was 8.2 ± 1.5 ms (range 5.7–11 ms; n = 12), and their average amplitude was 43.5 ± 7.6 mV (range 33–56 mV; n = 12).

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The pairing protocol, which consisted of the inferior olive and the following vestibular nerve single-pulse stimulations presented at an interval of 150 ms during 500–600 trials at a
rate of 2 Hz, could be applied to eight Purkinje cells. Usually, only one cell per experiment was subjected to the pairing procedure. In two experiments, an exception was made and two cells in both experiments underwent the pairing. This was due to the fact that in both cases the threshold intensity of the inferior olive stimulation required for complex spike generation was significantly higher for the second cells than for the first ones. This implied that the second cells were simply not recruited during the first pairing session. The stability of recordings was assessed by small fluctuations of the resting membrane potential (not more than 6.2 mV) and by stable amplitude of complex spikes tested in between vestibular nerve stimulations both before and after the pairing. The pairing resulted in a LTD of EPSPs induced by the vestibular nerve stimulation in seven of eight tested cells. In the remaining one cell, we also observed an overall reduction of the vestibular-evoked EPSP following pairing. However, responses in this cell largely fluctuated (even after averaging) during both the control period and after pairing, so it was discarded from the analysis. Figure 7B2 shows an averaged control EPSP evoked by the vestibular nerve stimulation in a Purkinje cell in which the inferior olive stimulation generated a complex spike (Fig. 7B1). The pairing strongly reduced the vestibular-evoked EPSP, and this reduction could still be observed 43 min later (Fig. 7B3). The reduction of responses was usually obvious after 150–250 trials and persisted throughout the entire recording time following pairing, which lasted from 11 to 47 min in different neurons. The degree of reduction was not uniform among the cells varying from 55 to 20%. The mean amplitude of depressed EPSPs calculated for all cells was 71.3 ± 10.5% of the control value. The data of pairing experiments pooled for the whole population of tested Purkinje cells are presented in Fig. 7C. The reduction of EPSPs was statistically significant (P < 0.01) at all times measured following the pairing as compared with the responses during the control period. There was no difference between reduction of EPSPs recorded in normally perfused brains and those obtained after addition of bicuculline to the perfusate. The observed LTD was pairing-specific, i.e., requiring paired presentation of stimuli. Indeed, activation of cells by alternating sets of the vestibular nerve and inferior olive test stimulations (16–32 pulses at 1–1.5 min intervals) during the control period and after pairing did not lead to changes in vestibular-evoked EPSP amplitudes. It must also be noted that the amplitude of vestibular-induced discharge responses in the abducens and oculomotor nerves remained unchanged after the pairing, indicating the absence of synaptic modifications in the disynaptic pathways from vestibular afferents to oculomotor nuclei. This observation suggests that depression of EPSPs in Purkinje cells and plasticity resulting from pairing take place in the cerebellar cortex, at the site of interaction of mossy and climbing fibers in Purkinje cells, and do not reflect possible changes at the first synapse (between vestibular afferents and second-order vestib-

<table>
<thead>
<tr>
<th>No.</th>
<th>Stimulation pattern</th>
<th>Frequency of trials</th>
<th>Number of trials</th>
<th>Oculomotor nerve</th>
<th>Abducens nucleus</th>
<th>Number of Brains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0–2 ms F V</td>
<td>0.5/1 Hz</td>
<td>450–4000</td>
<td>5</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>0–2 ms F V</td>
<td>10/100 Hz</td>
<td>500–2500</td>
<td>—</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>10–150 ms V F</td>
<td>0.5/1/10 Hz</td>
<td>700–1600</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>10–150 ms F x 5 (100 Hz) V</td>
<td>0.5/1 Hz</td>
<td>600–3600</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* First and second rows include paradigms in which stimulation of the flocculus (F) and vestibular nerve (V) occurred at the same time (time interval in a range of 0–2 ms). Third row includes paradigms in which single or 5 pulse train (100 Hz) stimulation of the flocculus followed the vestibular nerve stimulation by 10–150 ms. Last row comprises paradigms in which single or train stimulation of the flocculus preceded the vestibular nerve stimulation by 10–150 ms.
Floccular modulation of the vestibuloocular pathways in the guinea pig

Our results demonstrate that the neural circuitry linking the flocculus to the brain stem vestibuloocular network in the guinea pig is similar to what has been previously described in other mammalian species.

Stimulation of the flocculus affected only the responses evoked from the ipsilateral vestibular nerve. This suggests strict ipsilateral projections of the flocculus to vestibular nuclei which is consistent with previous physiological (Ito et al. 1977) and morphological data (see for reviews De Zeeuw et al. 1994; Sato and Kawasaki 1991).

Floccular stimulation depressed both the inhibitory field potentials in the abducens nucleus and disynaptic IPSPs in abducens motoneurons evoked by the ipsilateral vestibular nerve stimulation. On the other hand, activation of the flocculus did not modulate excitatory responses in the contralateral abducens nerve and nucleus. Hence in the guinea pig as in the rabbit (Highstein 1973; Ito et al. 1977), the inhibitory pathway from vestibular afferents to motoneurons of the ipsilateral lateral rectus muscle is under inhibitory control of the flocculus, whereas the excitatory pathway to the contralateral lateral rectus muscle is free from such control.

Due to the inherent limitations of the IWB (low levels of spontaneous activity in the nerves, impossibility of identifying motoneurons of different extraocular muscles and stimulating separately different branches of the vestibular nerve), the floccular modulation of the vestibuloocular pathways to the oculomotor nucleus could only be assessed in the excitatory reflex branches of the network by monitoring evoked discharges of oculomotor nerves. Assuming a similar neuronal organization of vestibuloocular pathways in the guinea pig and rabbit (Ito et al. 1976a,b; see also for review Ito 1984) our results could be interpreted as follows. The discharges induced in the ipsilateral oculomotor nerve by the vestibular nerve stimulation would correspond to the excitation of motoneurons innervating the medial rectus and superior rectus muscles, whereas the discharges of the contralateral oculomotor nerve would be due to activation of the inferior oblique and inferior rectus motoneurons. It is well established, however, that the flocculus inhibits reflex pathways originating only from horizontal and anterior canal afferents (Ito et al. 1977; Sato and Kawasaki 1991).

Therefore the excitatory pathway linking the posterior canal afferents to the motoneurons of the ipsilateral oculomotor nerve and only one of the two excitatory pathways to the motoneurons of the contralateral oculomotor nerve (see Fig. 4, A2 and B2). In agreement with this assumption, stimulation of the flocculus led to a stronger inhibition of discharges in the ipsilateral oculomotor nerve than in the contralateral one.

Considering floccular influences on the activity in the oculomotor nerves one must take into account the existence of internuclear neurons in the abducens nucleus projecting to the medial rectus subdivision of the contralateral oculomotor nucleus. Our data demonstrate that the excitatory pathway to the abducens internuclear neurons on the contralateral side is free from the inhibitory influence of the flocculus. Indeed the excitatory (negative) field potential in the contralateral abducens nucleus, to which both abducens motoneurons and internuclear neurons contribute, remain unchanged after floccular stimulation (see Fig. 3, Fc + Vc). Floccular influences on the inhibitory vestibular pathway to internuclear neurons of the ipsilateral abducens nucleus cannot be directly assessed from our data. However, stimulation of the flocculus strongly reduced the inhibitory (positive) field potential in the abducens nucleus.
(see Figs. 1, Fi + Vi, and 6B2, Fi + Vi), suggesting that most of cells in the nucleus were involved. Therefore it is very likely that the inhibitory vestibular neurons projecting to ipsilateral abducens motoneurons and interneurons are both under the inhibitory influence of the flocculus. More detailed evaluation of floccular influences on internuclear neurons would require intracellular recordings from these neurons as we did for abducens motoneurons (see Fig. 2).

Physiological properties of FTNs

To our knowledge, this is a first study in which intracellular synaptic response properties of physiologically identified FTNs have been systematically characterized. In only one previous report (Fukuda et al. 1972), the intracellular investigation of FTNs was limited to recordings of the flocculus-induced IPSPs. All recorded FTNs in our study were second-order vestibular neurons. Despite the extensive recordings in a restricted area, the FTN sample is rather small (11 neurons). It corresponds to 15–20% of impaled vestibular neurons, indicating that FTNs represent only a small proportion of these cells. FTNs were found in the brain stem region, which most likely corresponds to the rostral part of the medial vestibular nucleus and can include medial parts of adjacent lateral and inferior (descending) vestibular nuclei (Gstoettner and Burian 1987; Voitenko and Marlinskii 1993). Our results are consistent with extracellular recording data in rabbits showing that FTNs constituted a small proportion of vestibular neurons and that the highest densities of FTNs were restricted to the rostral parts of the vestibular complex, mainly to the medial vestibular nucleus and the medial part of lateral vestibular nucleus (Kawaguchi 1985; Stahl and Simpson 1995).

IPSPs evoked in FTNs by floccular stimulation had average latencies of 2.28 ms (range 2–2.6 ms). This is slightly longer than the upper latency limit of 1.9 ms set for another monosynaptic connection in the IWB: the EPSPs evoked in second-order vestibular neurons by the vestibular nerve stimulation (Babalian et al. 1997). Nevertheless flocculus-evoked IPSPs can be considered as monosynaptic for the following reasons. First, these IPSPs could follow high stimulation frequencies without latency fluctuations and without significant amplitude potentiation or depression, the properties characteristic for monosynaptic transmission. Second, it has been demonstrated in rabbits and cats that conduction velocities of Purkinje cell axons are substantially lower than those of afferent vestibular fibers (Fukuda et al. 1972; Ito and Yoshida 1966). This resulted in 30–50% longer latencies of flocculus-induced monosynaptic IPSPs in vestibular neurons as compared with the latencies of vestibular-evoked monosynaptic EPSPs in the same neurons (Fukuda et al. 1972). Similar observation has been made for monosynaptic IPSPs in Deiters’ neurons induced by stimulation of the anterior vermis (Ito and Yoshida 1966). Assuming similar differences in the guinea pig, the latencies of flocculus-induced IPSPs in the present study were well within the monosynaptic range. Another indication, although indirect, of the monosynaptic nature of flocculus-induced IPSPs in FTNs is the sensitivity of these responses to bicuculline. The IPSPs were blocked by bicuculline, indicating GABA\textsubscript{A} receptor-mediated synaptic transmission. It is well known that Purkinje cells constitute the only output of the cerebellar cortex and use GABA as a neurotransmitter. Therefore the inhibitory action of bicuculline on flocculus-induced IPSPs in FTNs is most likely due to direct drug action on synapses between Purkinje cells and FTNs. This result is in agreement with previous observations that systemic injections of picROTOxin depressed the inhibitory actions of the flocculus on VORs in the rabbit (Fukuda et al. 1972).

An unexpected finding of our study was the observation that two FTNs could be antidromically activated from the spinal cord. The cerebellar monosynaptic inhibitory control of vestibulospinal neurons in the Deiters nucleus was previously demonstrated to originate from the anterior vermis in cats (Ito and Yoshida 1964, 1966). Distant locations of the flocculus and the anterior vermis, as well as low current intensities used in our study, rule out the possibility of activation of the vermis by a current spread. Therefore there might be two alternative explanations of our finding. One possibility is that in the guinea pig the flocculus contributes to the inhibitory control of some of vestibulospinal neurons. The second explanation would be that some FTNs involved in gaze control, apart from their projections to the nuclei of extraocular muscles, give rise to axon collaterals to the spinal cord. This would imply an interesting possibility of existence of a subset of flocculus-modulated neurons simultaneously involved in the control of gaze and posture.

Our results demonstrate that FTNs in the guinea pig receive both inhibitory and excitatory commissural inputs from the contralateral vestibular afferents with predominance of inhibitory ones. In the cat, target neurons of rostral and caudal zones of floccular inhibition were found to receive exclusively excitatory polysynaptic inputs from contralateral vestibular afferents (Sato and Kawasaki 1987, 1990). On the other hand, the nature of commissural inputs to the target neurons of the middle zone inhibition, contributing mainly to the control of horizontal eye movements, was not reported (Sato et al. 1988). Therefore the presence of FTNs with inhibitory commissural inputs in the guinea pig could be attributed to species differences and/or to the involvement of these cells in the control of horizontal eye movements. The latter possibility is in accordance with assumed location of FTNs in the medial vestibular nucleus (see preceding text) and with the fact that most of medial vestibular nucleus neurons involved in the horizontal gaze control receive commissural inhibition (Babalian et al. 1997; Kasahara and Uchino 1974; Shimazu and Precht 1966).

The spontaneous discharge rate of FTNs was about 10 Hz and was similar to the spontaneous activity of a larger sample of second-order vestibular neurons recorded in the IWB preparation (Babalian et al. 1997; Vibert et al. 1999). However, this value is about four times lower than the spontaneous discharge rates of second-order vestibular neurons in the alert guinea pig (Ris and Godaux 1998; Ris et al. 1995). Furthermore, a substantial portion of second-order vestibular neurons (15–25%) (Babalian et al. 1997) including FTNs (4 of 11 cells, present study) is silent in the IWB, whereas this is not the case for intact animals. Apparently the spontaneous activity of FTNs and of other vestibular neurons in the IWB is not sufficient to maintain significant tonic activity in the motoneurons of abducens and oculomotor nerves and causes mainly subthreshold changes of the motoneuronal membrane potential. Indeed spontaneous activity of the abducens and oculomotor nerves is
very low in the IWB (Babalian et al. 1997; present study); it is not regular and usually does not exceed a few discharges per second in these nerves, which contain hundreds of fibers. These observations explain why floccular stimulation induces responses in FTNs and modulates the evoked nerve discharges, but its effect is undetectable in the background activity of almost silent nerves.

Altogether our data demonstrate that, in the IWB preparation, many characteristics of FTNs could be studied in greater detail than using other methods. It is also clear, however, that further progress in describing physiological properties of FTNs in the isolated brain would require identification of their projections to the nuclei of various extraocular muscles and the nature of their action (excitatory or inhibitory) on targets in the oculomotor nuclei.

Cerebellum-related plasticity

Pairings of the vestibular nerve and floccular stimulations failed to induce synaptic plasticity at the level of FTNs. This raises a question whether the interactions of vestibular afferents and cerebellar inhibitory inputs in FTNs can induce such plastic changes at all. Indeed, we used pairing paradigms in which the vestibular afferent stimulation preceded, coincided with, or followed the flocculus stimulation while the stimuli were delivered at low (0.5–1 Hz), medium (10 Hz), and high (100 Hz) frequencies. In other words, our pairing paradigms roughly covered the range of all possible orders and frequencies of stimulation. Even assuming that the timing of presented stimuli and/or the frequency of stimulations were not optimal we think that if plasticity exists, it would manifest itself, at least in a reduced form, in one or another pairing paradigm. In this context, it must be noted that the cerebellar LTD, optimally induced at a time interval of 125–250 ms between climbing and parallel fiber stimulations (Ekerot and Kano 1989), could also be triggered using different timing or even a reverse order of stimuli (Schreurs and Alkon 1993; Schreurs et al. 1996).

The absence of plasticity at the level of FTNs was probably not related to our using the guinea pig as an animal model. It has been recently demonstrated that VOR adaptation did occur in the alert guinea pig and led to modifications of the activity of second order vestibular neurons (Serafin et al. 1999). If plastic changes in FTNs underlie the VOR adaptation, it can be concluded that the cellular substrate of such plasticity must exist in the brain of the guinea pig.

Another possible reason why we failed to find plasticity in FTNs could be that physiological conditions in the IWB were not appropriate for this type of plasticity. Such an explanation seems to be very unlikely since another type of cellular plasticity, the cerebellar LTD, could reliably be induced in this preparation. Alternatively, it should be assumed that physiological conditions in different parts of the isolated brain were favorable for the cerebellar LTD and not appropriate for the brain stem plasticity. However, this argument is not supported by our present and previous data (Babalian et al. 1997; Vibert et al. 1999) showing perfect viability of all levels of the vestibuloocular pathways in the IWB preparation. We also think that the absence of plasticity in the brain stem was not related to low levels of spontaneous activity in the vestibular nuclei. First, the LTD could be induced in our experiments, although spontaneous activity of Purkinje cells is also much lower (or is absent) in the IWB preparation as compared with intact animals. Second, in some of our pairing paradigms designed for inducing brain stem plasticity (see Table 1), the stimulation was delivered at high frequencies (100 Hz) to “compensate” for low levels of spontaneous activity in the IWB. However, these protocols also failed to induce plasticity at the level of FTNs.

Although our results do not favor the hypothesis of flocculus-dependent plasticity in FTNs, we cannot exclude the possibility of plastic changes in these cells and other brain stem sites under other experimental conditions using different experimental approaches. In this context, it must be noted that learned responses resulting from VOR adaptation could be partially or completely maintained and expressed after inactivation of the flocculus, suggesting a brain stem site of plasticity in the vestibular nuclei (Luebke and Robinson 1994; Partalsis et al. 1995; Pastor et al. 1994). There are also indications that synaptic modifications related to VOR adaptation could be retained at an alternative (than FTN) site in the vestibular nuclei (Quinn et al. 1998) or could take place at different anatomical sites depending on different combinations of neural signals (Raymond and Lisberger 1998). Deep cerebellar nuclei with their mechanisms of cellular plasticity (Aizenman et al. 1998; Sastry et al. 1997) can be considered as another potential site of motor learning during VOR adaptation. It is clear however that VOR adaptation must be caused by interactions within a limited class of neurons of the VOR circuitry (Highstein 1998).

The LTD observed in the IWB preparation shared many common properties with the well-known cerebellar LTD. For instance, the pairing paradigm (timing of stimulations, the duration, and rate of pairing), similar to those used in previous studies (see for reviews Ito 1989; Linden and Connor 1995), was also efficient for LTD induction in the isolated brain. Furthermore, the observed average reduction of EPSPs (about 30% of control responses) closely matches the values obtained for depression of parallel fiber-induced EPSPs in Purkinje cells in different in vitro preparations using various stimulation paradigms (Hirano 1990; Sakurai 1987; Schreurs et al. 1996). The LTD in our experiments could be induced both in conditions of normally perfused brains and when bicuculline was added to the perfusate. A recent study in slices has also demonstrated that the block of postsynaptic inhibition of Purkinje cells by bicuculline (or other GABA_A receptor antagonists) was not a necessary condition for induction of LTD as was thought earlier (Schreurs et al. 1996).

On the other hand, the LTD in our study had an important distinctive feature: it was induced by stimulation of the vestibular afferents and inferior olive and not by coactivation of parallel and climbing fibers. In fact, this may be the first clear demonstration that the LTD in Purkinje cells could be induced in intact brains, without any pharmacological (bicuculline) manipulation, by stimulation of identified neuronal inputs known to be activated during the VOR adaptation. In the one comparable study, where stimulation of vestibular afferents was paired with activation of climbing fibers in intact animals, the extracellularly recorded activity of Purkinje cells was only occasionally modified (Ito et al. 1982). Our results strongly
suggest that the cerebellar LTD could serve as a cellular substrate of different forms of motor learning in intact animals.

Altogether, even if the present data cannot exclude the possibility of plasticity in FTNs and/or at other brain stem sites, they support the hypothesis that the cerebellar LTD is one of the main mechanisms of cellular plasticity underlying vestibular adaptation. From this point of view, our results are in agreement with the recent elegant study in knockout mice suggesting that activation of the protein kinase C is necessary for cerebellar LTD induction and that cerebellar LTD is required for adaptation of the VOR (De Zeeuw et al. 1998).

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REFERENCES


