

Title	Molecular mechanisms of vestibular compensation in the central vestibular system
Author(s)	北原, 紵
Citation	大阪大学, 1997, 博士論文
Version Type	VoR
URL	<a href="https://doi.org/10.11501/3128957">https://doi.org/10.11501/3128957</a>
rights	
Note	

*Osaka University Knowledge Archive : OUKA*

<https://ir.library.osaka-u.ac.jp/>

Osaka University

## Research report

# Effects of MK801 on Fos expression in the rat brainstem after unilateral labyrinthectomy

Tadashi Kitahara <sup>a,b,\*</sup>, Noriaki Takeda <sup>a</sup>, Takanori Saika <sup>a</sup>, Takeshi Kubo <sup>a</sup>, Hiroshi Kiyama <sup>b</sup><sup>a</sup> Department of Otolaryngology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan<sup>b</sup> Department of Neuroanatomy, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan

Accepted 11 July 1995

---

**Abstract**

Unilateral labyrinthectomy (UL) causes ocular and postural asymmetries, which disappear over time in the processes of equilibrium recovery known as vestibular compensation. It has been reported that *N*-methyl-D-aspartate (NMDA) receptors are involved in vestibular compensation. In the present study, in order to elucidate the NMDA receptor-mediated neural circuit responsible for the development of vestibular compensation, we used Fos expression as a marker of neural activation and examined the effects of MK801, a specific antagonist of NMDA receptors, on UL-induced Fos expression in the rat brainstem. After UL, Fos-like immunoreactive (-LIR) neurons were observed in the ipsilateral medial vestibular nucleus (ipsi-MVe), the contralateral prepositus hypoglossal nucleus (contra-PrH) and the contralateral inferior olive beta subnucleus (contra-IOb). Fos-LIR neurons then gradually disappeared in the processes of vestibular compensation. It is suggested that the activation of the ipsi-MVe, the contra-PrH and the contra-IOb neurons after UL are the initial event of vestibular compensation. Intraperitoneal injection of MK801 in the processes of vestibular compensation caused reappearance of UL-induced behavioral deficits. During the decompensation induced by MK801, Fos-LIR neurons appeared in the contra-MVe, the ipsi-PrH and the bilateral-IOb. It is suggested that the contra-MVe, the ipsi-PrH and the bilateral-IOb neurons are inhibited by glutamatergic synapses driving inhibitory neurons via NMDA receptors in the processes of vestibular compensation and that disinhibition of these nuclei induced by MK801 causes decompensation. However, MK801 caused neither Fos expression nor behavioral decompensation after vestibular compensation is accomplished. All these findings suggest that the NMDA receptor-mediated inhibitory modulation in the central vestibular system plays an important role for the initial processes of the development of vestibular compensation.

**Keywords:** Vestibular compensation; Neural plasticity; Decompensation; Nystagmus; *N*-Methyl-D-aspartate receptor; Immunohistochemistry

---

**1. Introduction**

Unilateral labyrinthectomy (UL) induces severe postural (barrel rotation, head tilt) and oculomotor (nystagmus) asymmetry. However, the functional deficits recover gradually after the lesion. This progressive restoration of balance is referred to as vestibular compensation [28,35]. Since UL results in a permanent loss of vestibular inputs from the ipsilateral vestibular periphery [16], vestibular compensation has been attributed to functional reorganization of the central vestibular system [35] and used as a model of lesion-induced neural plasticity in the central nervous system (CNS) [20].

The *N*-methyl-D-aspartate (NMDA) receptor is a type of glutamate receptor that plays a crucial role in the CNS

plasticity [2,39]. There are several lines of evidence that the NMDA receptor plays a key role in vestibular information processing. Electrophysiological studies have shown that the NMDA receptor is involved in neural activation of vestibular nuclei via vestibular commissures [6,11,19]. Recent morphological studies have revealed substantial expression of the NMDA receptor at both the mRNA and protein level in the rat vestibular nuclei [23,26,41]. Moreover, in guinea pigs, a specific antagonist of NMDA receptor, MK801, has been demonstrated to cause reappearance of UL-induced behavioral deficits, i.e. decompensation [31,36,40]. These findings suggested that the NMDA receptor takes part in the neural plasticity of the central vestibular system after UL.

In the present study, to clarify the NMDA receptor-mediated neural circuit responsible for the development of vestibular compensation, we used Fos expression as a marker. Fos is the protein product of *c-fos*, a nuclear

---

\* Corresponding author. Fax: (81) (6) 879-3959.

proto-oncogene, which is rapidly induced in neurons in response to various kinds of stimulation [4,21]. Previous studies by both ourselves [29] and Kaufman et al. [17] showed that asymmetrical Fos expression was induced by UL in the vestibular and vestibular-related nuclei in rats. We first examined changes in Fos expression with time after UL in the rat brainstem immunohistochemically, and then the effects of MK801 on the UL-induced Fos expression were examined.

## 2. Materials and methods

### 2.1. Labyrinthectomy

Adult Wistar rats weighing about 150 g were used. Animals were anesthetized with ether and the right tympanic membrane, malleus and incus were removed by the retroauricular approach under an operating microscope. The stapes crura were fractured and the stapes foot plate was removed to open the oval window. Then, a small opening was made on the bony horizontal semicircular canal with a small dental burr. Through these two openings, the membranous labyrinth was surgically removed with a small right-angled hook and chemically destroyed by injection of 100% ethanol. At the end of surgery, antibiotic cream (Furacin) was topically applied to the opened labyrinth to prevent infection and the temporal bone was sealed with dental cement. The operative wound was sutured and the animal was allowed to recover in the light.

At each post-operative interval of 1, 2, 6, 12 h and 1, 3, 7, 14, 28 days, four animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with 100 ml of ice-cold saline, followed by 250 ml of Zamboni's fixative.

### 2.2. Drug

At each post-UL interval of 6 h and 1, 3, 7, 14, 28 days, four animals received intraperitoneal (i.p.) injection of MK801 (Research Biochemical Inc., MA, USA). Each animal of these different post-operative groups was injected only once. The dose of 1.0 mg/kg was chosen, because previous studies reported that this dose of MK801 induced full decompensation in unilateral labyrinthectomized guinea pigs [36]. MK801 was dissolved in 0.85% saline to a volume of 1.0 ml/kg. Two and six hours after administration of MK801, animals were then treated for perfusion and fixation as described above.

### 2.3. Immunohistochemistry

The rat brain was immediately removed after perfusion, post-fixed in the same fixative at 4°C for 1–2 days, and then placed in 30% sucrose-phosphate buffer at 4°C for

2–3 days. Frozen serial sections (18  $\mu$ m thick) were cut on a cryostat. The avidin-biotin complex (ABC) method was used to visualize the immunohistochemical reaction. Briefly, sections were incubated sequentially in the following solutions at 4°C: 1% bovine serum albumin (BSA) and normal goat serum (NGS) in 0.3% Triton X-100 in PBS for 3 h; antisera against Fos (diluted 1:500) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 48 h; 0.1 M PBS for 15 min; biotinylated goat anti-rabbit IgG (Vector Labs. Inc., USA) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 24 h; 0.1 M PBS for 15 min; Vectastain reagent (Vector Labs. Inc., USA) for 24 h; diaminobenzidine tetrahydrochloride (DAB)/H<sub>2</sub>O<sub>2</sub> for 15 min, and then examined under a light microscope. The antibody raised against Fos was obtained from Oncogene Science (NY, USA; catalog number PC05). This Fos antibody is a rabbit, affinity-purified polyclonal antibody raised against the peptide S-G-F-N-A-D-Y-E-A-S-S-S-R-C corresponding to residues 4–17 of human Fos [9].

### 2.4. Cell counting

Transverse 18  $\mu$ m brainstem sections were examined under bright-field microscopy at 40 $\times$  and 100 $\times$  magnification to detect Fos-LIR cells. Only cells that had significant levels of DAB reaction product in their nucleus above tissue background levels were counted with a digital image analysis system (Universal Imaging Software).

### 2.5. Behavior

Vestibular-ocular and vestibular-spinal reflex are usually used as a marker of the development of vestibular compensation. In the present study, we chose the frequency of horizontal spontaneous nystagmus (SN) as a marker, because it is reliably measured by a video camera.

Eye movements were videotaped using a Panasonic NV-M7 video camera with a zoom lens and replayed using a Mitsubishi E7 Black Diamond video recorder and a Sony Trinitron color monitor. The frequency of SN was measured as the number of quick phase beats occurring over periods of 15 s. The eye movements were replayed and counted 3 times for each animal ( $n = 4$ ) and the means obtained. These measurements were made at 0.5, 1, 2, 6, 12, 18, 24, 42, 48 and 72 h post-UL. SN induced by MK801 at various post-operative intervals was also measured as described above.

## 3. Results

### 3.1. Vestibular compensation after UL and MK801-induced decompensation

After right UL, the quick phase of SN toward the left (intact) side appeared. The frequency of SN reached a

maximum of 27–28 beats/15 s (27–28/15 s) half-an-hour after UL. Then, the frequency of SN was gradually decreased and disappeared by 48 h post-operation (Fig. 1A). On day 3 after UL, i.p. injection of MK801 at a dose of 1.0 mg/kg caused reappearance of SN toward the intact side (the same direction as in the case after UL). The frequency of SN reached a maximum of 20–25/15 s half-an-hour after injection. The frequency of SN was quickly decreased and disappeared by 6 h post-injection (Fig. 1B).

Fig. 2 shows the maximum frequencies of SN after UL and SN induced by MK801 (1.0 mg/kg) on days 3, 7 and 14 after UL. The maximum frequency of MK801-induced SN on day 7 was less than that on day 3. MK801 did not induce SN on day 14 after UL. Thus, the decompensation (reappearance of SN) induced by MK801 was observed only when administered up to day 7 after UL.

Saline injections did not cause reappearance of SN in unilateral labyrinthectomized rats ( $n = 2$ , data not shown). In normal rats, MK801 injections did not induce any kind of nystagmus ( $n = 4$ , data not shown).

After right UL, the head of animals was tilted toward the right (lesioned) side. The degree of the head tilt (HT) was gradually, but incompletely compensated. On day 3 after UL, MK801 (1.0 mg/kg) caused exacerbation of HT toward the lesioned side (the same direction in the case after UL), which quickly disappeared by 6 h post-injection. But, MK801 did not cause the exacerbation of HT on day 14 after UL.

### 3.2. Fos expression after UL

In normal rats, Fos-like immunoreactive (-LIR) neurons were not observed in the vestibular or vestibular-related

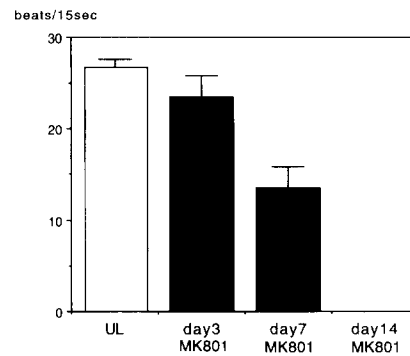


Fig. 2. The maximum number of spontaneous nystagmus (beats/15 s) after unilateral labyrinthectomy (UL) (open column) and those induced by MK801 (1.0 mg/kg, i.p.) at post-UL intervals of 3, 7 and 14 days (filled columns) in rats. Columns represent means  $\pm$  S.E.M. for four animals.

nuclei ( $n = 2$ , data not shown). One hour after UL, Fos-LIR neurons appeared in the medial vestibular nucleus ipsilateral to the lesioned side (ipsi-MVe), the prepositus hypoglossal nucleus (contra-PrH) and the inferior olive beta subnucleus (contra-IOb) contralateral to the lesioned side. The level of Fos expression in these nuclei reached a maximum by 6 h post-operation (Fig. 3), and then was gradually reduced. Fos-LIR neurons in the contra-PrH and the contra-IOb disappeared on day 3 after surgery. Residual expression of Fos was still observed in the ipsi-MVe on day 3, but not on day 7 after UL. The distributions of Fos-LIR neurons in the brainstem and the changes in their number with time after UL are shown schematically in Fig. 6A–E.

### 3.3. Fos expression during MK801-induced decompensation

A schematic representation of the effects of MK801 on Fos expression in the rat brainstem after UL is shown in Fig. 6F–J. In normal rats, 2 h after i.p. injection of MK801, Fos-LIR neurons were not seen in the MVe or the PrH, but they were present in the bilateral IOb symmetrically (Figs. 4 and 6F). Six hours later, however, no Fos expression was observed in the IOb. During the decompensation induced by MK801 (1.0 mg/kg), which was administered up to day 7 after UL, Fos-LIR neurons appeared in the contra-MVe and the ipsi-PrH. Two h after injection at a post-UL interval of 6 h, in addition to Fos-LIR neurons in the ipsi-MVe and the contra-PrH induced by UL, MK801-induced Fos-LIR neurons were also observed in the contra-MVe and the ipsi-PrH (Fig. 6G). On days 3 and 7 after UL, Fos-LIR neurons were observed only in the contra-MVe and the ipsi-PrH 2 h after injection of MK801 (Fig. 6H,I). At a post-UL interval of 14 d, MK801 induced neither decompensation nor Fos-LIR neurons in these regions (Fig. 6J). The Fos expression level in the contra-MVe and the ipsi-PrH induced by MK801 reached a maximum 2 h after injection and disappeared 6 h later.

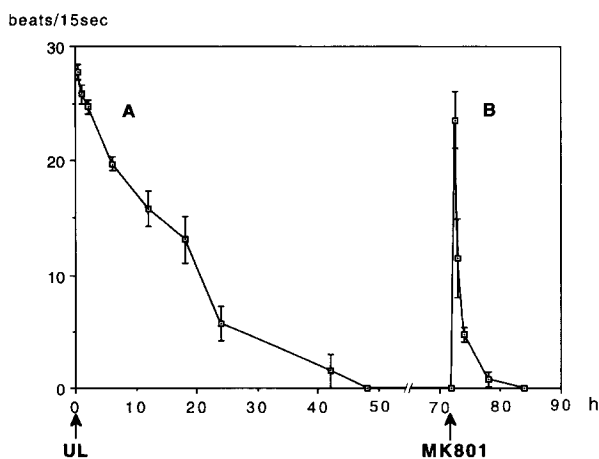


Fig. 1. Changes in frequency of spontaneous nystagmus with time after unilateral labyrinthectomy (A) and the effect of MK801 (1.0 mg/kg, i.p.) on the frequency of spontaneous nystagmus on post-operative day 3 (B) in rats. Data are expressed as mean frequency  $\pm$  S.E.M. of quick phase beats per 15 s for four animals. UL, unilateral labyrinthectomy; MK801, injection of MK801.

In the IOb, MK801 induced asymmetrical Fos expression. At a post-UL interval of 6 h, the Fos expression in the contra-IOb was stronger than that in the ipsi-IOb (Fig. 6G). Then, on day 3 after UL, the expression in the ipsi-IOb was stronger than that in the contra-IOb (Fig. 6H). The asymmetrical Fos expression became evident 2 h after injection of MK801 and disappeared after a further 6 h. On days 7 and 14 after UL, MK801-induced Fos expression was symmetrical as the case with injection into normal rats (Fig. 6I,J).

Injection of the same volume of saline into lesioned animals had no effect on Fos expression ( $n = 2$ , data not shown).

Histological examination after UL showed that the surgical and chemical destruction of the membranous labyrinth

had been achieved and that no vestibular hair cells had regenerated (data not shown).

#### 4. Discussion

In the present study, half-an-hour after UL, the frequency of SN toward the intact side reached a maximum in rats. The initial increase of SN was probably due to recovery from ether anesthesia, because the quick phase of nystagmus was suppressed by anesthesia [32,36]. Then, the frequency of SN was gradually decreased and disappeared by 48 h post-operation (Fig. 1A). Fos-LIR neurons appeared in the MVe, PrH and IOb after UL in the rat brainstem (Fig. 6A–E). The level of Fos expression in the

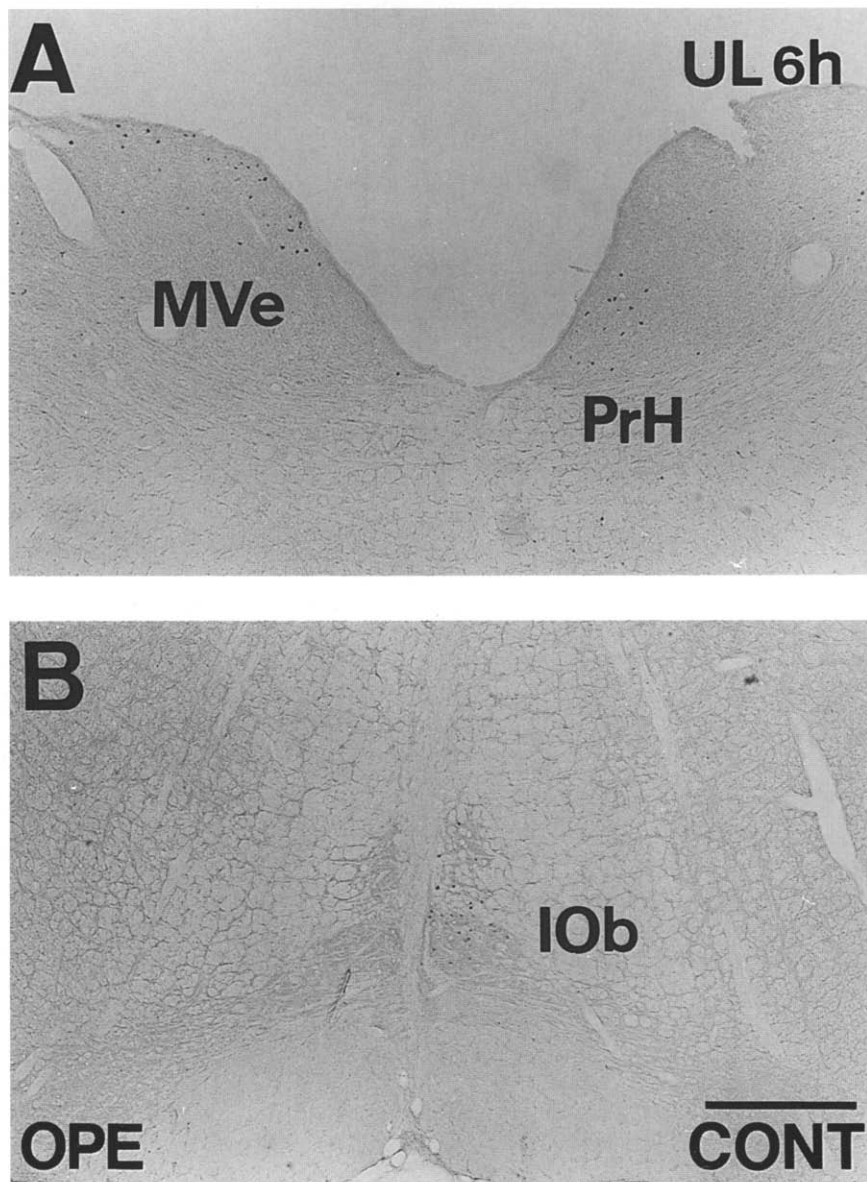


Fig. 3. Bright-field photomicrographs showing Fos expression in the ipsi-MVe, the contra-PrH (A) and the contra-IOb (B) 6 h after unilateral labyrinthectomy (UL6h). Bar = 200  $\mu$ m. OPE, operated side; CONT, control side.

ipsi-MVe, the contra-PrH and the contra-IOb reached a maximum 6 h after surgery (Fig. 3). Then, Fos expression in these nuclei was gradually reduced and hardly observed by the 3rd post-operative day. Thus, Fos expression seems to be reduced in accordance with recovery from UL-induced behavioral deficits. Since Fos is expressed in neurons following synaptic excitation by various kinds of stimulation [4,21], these findings suggest that neural activation of the ipsi-MVe, the contra-PrH and the contra-IOb is the initial event of the development of vestibular compensation. Our previous study [29] and Kaufman et al. [17] showed that asymmetrical Fos expression was induced by UL in the vestibular and vestibular-related nuclei in rats. However, we did not observe UL-induced Fos expression in the inferior vestibular nucleus or the dorsolateral central gray, which Kaufman et al. previously reported [17].

In the previous electrophysiological study [34], the resting activity in the ipsi-MVe type I neurons, which directly receive primary afferent inputs, was decreased after UL. But, the resting activity in the ipsi-MVe type II interneurons, which receive commissure inputs, was increased after UL. Thus, Fos-LIR activated neurons in the ipsi-MVe may be the type II interneurons. However, we did a preliminary experiment that showed a part of Fos-LIR neurons in the ipsi-MVe projected their axons to the vestibulo-cerebellum by means of retrograde tracing and immunohistochemical techniques (unpublished observation).

Previously, we showed that the expression of preproenkephalin (PPE) mRNA, a precursor of Met- and Leu-enkephalin, was increased in the MVe on the operated side after UL [30]. The level of PPE mRNA expression in the ipsi-MVe reached a maximum 1 day after the opera-

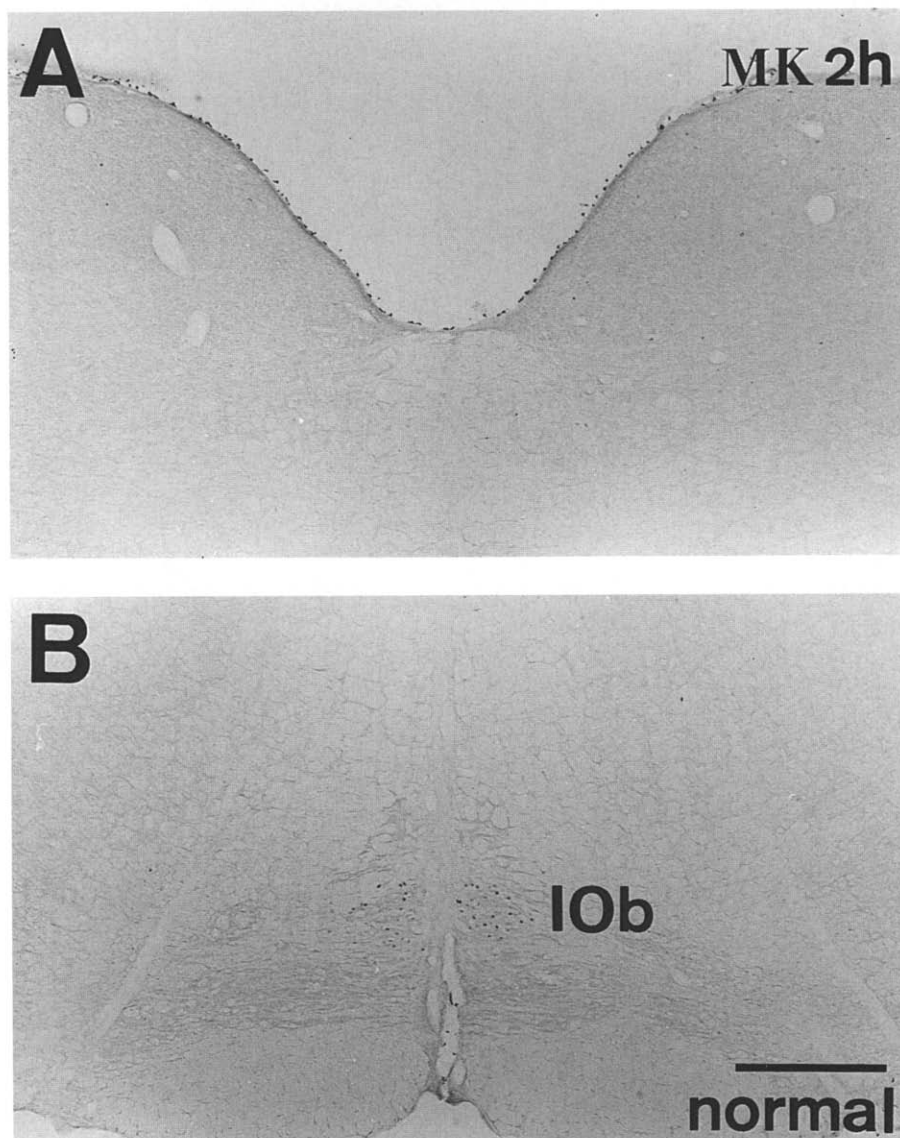


Fig. 4. Bright-field photomicrographs showing Fos expression in normal rats 2 h after injection of MK801 (1.0 mg/kg, i.p.) (MK2h). No Fos expression was observed in the MVe or the PrH (A), but in the bilateral-IOb (ipsi = contra) (B). Bar = 200  $\mu$ m.

tion, and then gradually returned to normal. Thus, changes in PPE mRNA expression in the ipsi-MVe after UL seem to follow those observed here in Fos expression. A PPE gene has a DNA binding site called AP-1 site in its promoter region and its expression is regulated by Fos–Jun complexes [38]. These findings suggest that Fos expression after UL is involved in vestibular compensation by inducing PPE mRNA expression in the ipsi-MVe.

In the present study, MK801 caused reappearance of SN toward the intact side. Half-an-hour after injection of MK801 on day 3 after UL, the frequency of SN reached a maximum. The frequency of SN was then quickly decreased and disappeared by 6 h post-injection (Fig. 1B). These results in rats are consistent with the findings of

MK801-induced decompensation in labyrinthectomized guinea pigs [36], suggesting that MK801 transiently disrupts the plastic processes in vestibular compensation in rats. As the post-UL interval became longer, the maximum frequency of SN induced by MK801 was reduced (Fig. 2). MK801 never induced SN on day 14 after UL. These results suggest that MK801 induces decompensation only before vestibular compensation is accomplished. The MK801-sensitive period during the development of vestibular compensation was also suggested by Darlington et al. [8]. Therefore, it is likely that an NMDA receptor-mediated neural circuit is involved in the initial processes of vestibular compensation.

In the present study, Fos-LIR neurons were never seen

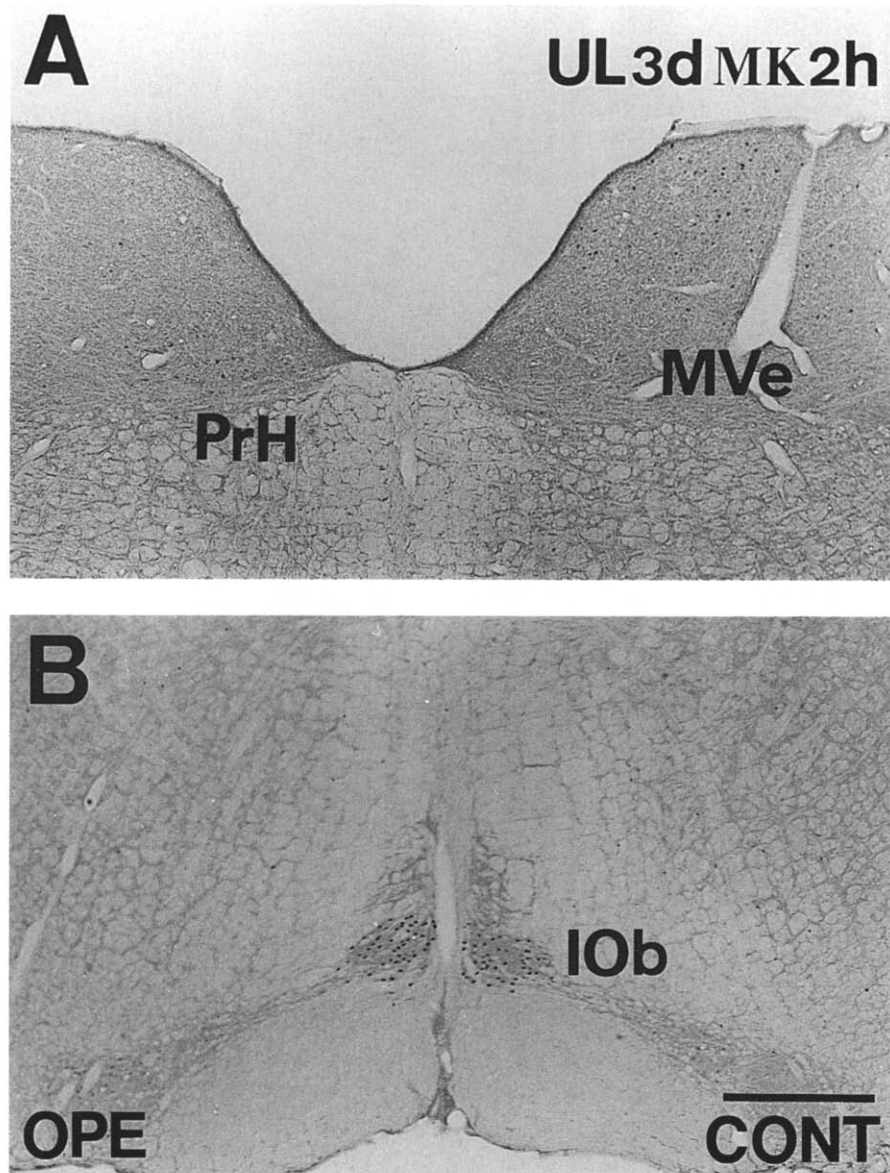


Fig. 5. Bright-field photomicrographs showing Fos expression in the contra-MVe, the ipsi-PrH (A) and the bilateral-IOb (ipsi > contra) (B) 2 h after injection of MK801 (1.0 mg/kg, i.p.) in labyrinthectomized rats on day 3 after the operation (UL6hMK2h). Bar = 200  $\mu$ m. OPE, operated side; CONT, control side.

in the MVe or the PrH after injection of MK801 in normal rats (Figs. 4 and 6F). However, during decompensation induced by MK801 in labyrinthectomized rats, Fos-LIR neurons appeared in the contra-MVe and the ipsi-PrH (Fig. 6G–I). Since Fos is induced in activated neurons, it is suggested that the contra-MVe and the ipsi-PrH were inhibited via NMDA receptors in the initial period of vestibular compensation, and that MK801 probably activated these nuclei by disinhibition, resulting in decompensation. Since the NMDA receptor is not directly mediated in inhibitory transmission, it is likely that after UL the contra-MVe and the ipsi-PrH were inhibited by certain inhibitory neurons driven by glutamatergic synapses via NMDA receptors. As vestibular compensation developed, the number of MK801-induced Fos-LIR neurons was reduced (Fig. 6G–J). MK801 never induced Fos-LIR in these neurons on day 14 after UL. This decay profile of MK801-induced Fos-LIR seems to be correlated with that of MK801-induced reappearance of SN (decompensation) (Figs. 2 and 6H–J). These results suggest that the inhibitory regulation of the contra-MVe and the ipsi-PrH after UL by certain inhibitory neurons driven by glutamatergic synapses via NMDA receptors plays an important role for the initial processes of the development of vestibular compensation.

There are three possible origins of the NMDA receptor-mediated inhibitory projection to the contra-MVe and the ipsi-PrH in the development of vestibular compensation. (1) *Commissures*: the MVe and the PrH neurons receive commissural fibers from neurons in the opposite nuclei, respectively [13]. These commissures make synaptic contacts on GABAergic interneurons via NMDA receptors [10,27,37]. Taken together with the present findings, it is suggested that the ipsi-MVe and the contra-PrH neurons activated after UL inhibit the contra-MVe and the ipsi-PrH through glutamatergic commissures driving GABAergic interneurons via NMDA receptors in the development of vestibular compensation. (2) *Mossy fibers*: the MVe and the PrH neurons give rise to their axons to the vestibulo-cerebellum [1]. The mossy fibers from the nuclei make synaptic contacts on the granule cells, which project their glutamatergic axons to Purkinje cells in the vestibulo-cerebellum via NMDA receptors [14,25,26,33]. Axons of the Purkinje cells then make GABAergic synaptic contacts on the MVe and the PrH [24]. The reciprocal projection between the vestibular nuclei and the vestibulo-cerebellum may be responsible for NMDA receptor-mediated inhibition of the contra-MVe and the ipsi-PrH in the development of vestibular compensation. By means of retrograde tracing and immunohistochemical techniques, it was revealed that enkephalinergic neurons in the MVe project their axons to the vestibulo-cerebellum [18]. We reported that the expression of PPE mRNA was increased in the ipsi-MVe after UL [30]. Therefore, it is possible that the enkephalinergic mossy fiber is a component of the NMDA receptor-mediated inhibitory projection in the development

of vestibular compensation. (3) *Climbing fibers*: the IOb neurons also project their glutamatergic axons, known as climbing fibers, directly to Purkinje cells in the vestibulo-cerebellum via NMDA receptors [3,5,14,26]. Ito et al. previously reported that climbing fibers receive visual inputs and make neuronal control of the response of cerebellar Purkinje cells to parallel fibers [14,15]. Since UL-induced nystagmus causes severe visual disturbances, the IOb neurons may be activated after UL and convey visual information to the vestibulo-cerebellum. Therefore, it is very likely that the contra-IOb neurons activated after UL also inhibit the contra-MVe and the ipsi-PrH by glutamatergic synapses of climbing fibers driving GABAergic

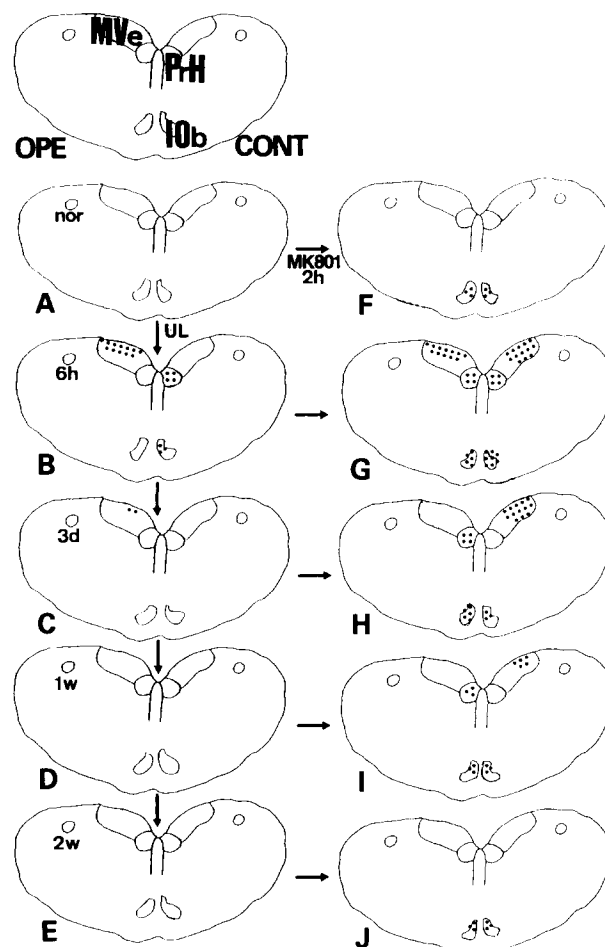


Fig. 6. Schematic representation of Fos expression in rat brainstem sections at the level of MVe, PrH and IOb. A–E: unilateral labyrinthectomized rats 6 h (B), 3 days (C), 1 week (D) and 2 weeks (E) after the operation. F: normal rats 2 h after MK801 injection (1.0 mg/kg, i.p.). G–J: unilateral labyrinthectomized rats 2 h after MK801 injection (1.0 mg/kg, i.p.) at post-operative intervals of 6 h (G), 3 days (H), 1 week (I) and 2 weeks (J). The average number of Fos-labeled neurons per nucleus is indicated by the dots ( $n = 4$ ). One dot represents twenty Fos-immunopositive neurons. Note that the rats in G, H and I showed reappearance of UL-induced behavioral deficits (decompensation) after MK801 injection. OPE, operated side; CONT, control side.



projections of Purkinje cells via NMDA receptors in the development of vestibular compensation.

During the development of vestibular compensation, there are two events for the correction of the imbalance of the resting activity between bilateral MVe neurons. One is the recovery of the resting activity in the ipsi-MVe neurons and the other is the inhibition of the contra-MVe neurons. Here, we propose a hypothesis that the NMDA receptor-mediated inhibitory projection to the contra-MVe and the ipsi-PrH plays a crucial role for the latter event.

After injection of MK801 into normal rats, Fos-LIR neurons were moderately detected in the bilateral IOB symmetrically (Figs. 4 and 6F). Previous morphological studies also showed that injection of MK801 into normal rats leads to induction of Fos in some nuclei in the CNS [12,22]. A possible mechanism of activation of these neurons is that NMDA antagonism results in disinhibition of glutamatergic synapses which lead inhibitory cells via NMDA receptors [7,42]. During the decompensation after MK801 injection into labyrinthectomized rats, Fos expression in the IOB was asymmetrical. Fos expression in the contra-IOB was stronger than that in the ipsi-IOB when MK801 was administered 6 h after UL (Fig. 6G), and then vice versa when MK801 was administered 3 d after UL (Figs. 5 and 6H). On day 7 after UL, MK801 never induced asymmetrical Fos expression in the IOB (Fig. 6I). Thus, the IOB neurons may also be involved in the decompensation induced by MK801.

In conclusion, after UL, the ipsi-MVe, the contra-PrH and the contra-IOB neurons are first activated and then inhibit the contra-MVe, the ipsi-PrH and the bilateral-IOB by glutamatergic synapses driving inhibitory neurons via NMDA receptors. The NMDA receptor-mediated inhibitory modulation in the central vestibular system plays an important role for the initial processes of the development of vestibular compensation.

## Acknowledgements

We thank Prof. M. Tohyama (Department of Anatomy II, Osaka University Medical School) for his helpful advice and support. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan, Senri Life Science Foundation (Osaka, Japan), and Brain Science Foundation (Tokyo, Japan).

## References

- [1] Allen, G.I. and Tsukahara, N., Cerebro cerebellar communication systems, *Physiol. Rev.*, 54 (1974) 957.
- [2] Artola, A. and Singer, W., Long-term potentiation and NMDA receptors in rat visual cortex, *Nature*, 330 (1987) 649.
- [3] Beitz, A.J., Larson, A.A., Monaghan, P., Alschuler, A., Mullett, M.M. and Madl, J.E., Immunohistochemical localization of glutamate, glutaminase and aspartate aminotransferase in neurons of the pontine nuclei of the rat, *Neuroscience*, 17 (1986) 741–753.
- [4] Bullitt, E., Induction of *c-fos* like protein within the lumbar spinal cord and thalamus of the rat following peripheral stimulation, *Brain Res.*, 493 (1989) 391–397.
- [5] Clements, J.R., Magnusson, K.R. and Beitz, A.J., Ultrastructural description of glutamate-, aspartate-, taurine- and glycine-like immunoreactive terminals from five rat brain regions, *J. Electron Microsc. Techn.*, 15 (1990) 49–66.
- [6] Cochran, S.L., Kasik, P. and Precht, W., Pharmacological aspects of excitatory synaptic transmission to second order vestibular neurons in the frog, *Synapse*, 1 (1987) 102–123.
- [7] Criswell, H.E., Johnson, K.B., Mueller, R.A. and Breese, G.R., Evidence for involvement of brain dopamine and other mechanisms in the behavioral action of the *N*-methyl-D-aspartic acid antagonist MK801 in control and 6-hydroxydopamine-lesioned rats, *J. Pharmacol. Exp. Ther.*, 265 (1993) 1001–1010.
- [8] Darlington, C.L., Flohr, H. and Smith, P.F., Molecular mechanism of brainstem plasticity, *Mol. Neurobiol.*, 5 (1991) 355–368.
- [9] Delogni, P., Niman, H., Raymond, V., Sawchenko, P. and Verma, I.M., Detection of Fos protein during osteogenesis by monoclonal antibodies, *Mol. Cell. Biol.*, 8 (1988) 2251–2256.
- [10] Dieringer, N. and Precht, W., Mechanism of compensation for vestibular deficits in the frog. II. Modifications of the inhibitory pathways, *Exp. Brain Res.*, 36 (1979) 329–341.
- [11] Doi, K., Tsumoto, T. and Matsunaga, T., Actions of excitatory amino acid antagonists on synaptic inputs to the rat medial vestibular nucleus: an electrophysiological study in vitro, *Exp. Brain Res.*, 82 (1990) 254–262.
- [12] Dragunow, M. and Faull, R.L.M., MK801 induces *c-fos* protein in thalamic and neocortical neurons of rat brain, *Neurosci. Lett.*, 113 (1990) 144–150.
- [13] Galiana, H.L., Flohr, H. and Melvill Jones, G., A reevaluation of intervestibular nuclear coupling: Its role in vestibular compensation, *J. Neurophysiol.*, 51 (1984) 242–259.
- [14] Ito, M. (Ed.), *The Cerebellum and Neural Control*, Ravan Press, New York, 1984.
- [15] Ito, M., Sakurai, M. and Tongroach, P., Climbing fibre induced depression both mossy fibre responsiveness and glutamate sensitivity of cerebellar Purkinje cells, *J. Physiol.*, 324 (1982) 113.
- [16] Jensen, D.W., Survival of function in the deafferented vestibular nerve, *Brain Res.*, 273 (1983) 175–178.
- [17] Kaufman, G.D., Anderson, J.H. and Beitz, A.J., Brainstem Fos expression following acute unilateral labyrinthectomy in the rat, *NeuroReport*, 3 (1983) 829–832.
- [18] King, J.S., Ho, R. and Bishop, G., The origin and distribution of enkephalin-like immunoreactivity in the opossum's cerebellum. In J.S. King (Ed.), *New Concepts in Cerebellar Neurobiology*, Alan R. Liss, New York, 1987, pp. 1–28.
- [19] Knopfel, T., Evidence for *N*-methyl-D-aspartate acid receptor-mediated modulation of the commissural input to central vestibular neurons of the frog, *Brain Res.*, 426 (1987) 212–224.
- [20] Lacour, M. and Xerri, C., Vestibular compensation: new perspectives. In H. Flohr and W. Precht (Eds.), *Lesion Induced Neuronal Plasticity in Sensorimotor Systems*, Springer, Berlin, 1981, pp. 240–253.
- [21] Lee, J.H. and Beitz, A.J., The distribution of brainstem and spinal cord nuclei associated with different frequencies of electroacupuncture analgesia, *Pain*, 52 (1993) 11–28.
- [22] Lee, S., Rivier, C. and Torres, G., Induction of *c-fos* and CRF mRNA by MK-801 in the parvocellular paraventricular nucleus of the rat hypothalamus, *Mol. Brain Res.*, 24 (1994) 192–198.
- [23] Nakanishi, S., Molecular diversity of glutamate receptors and implications for brain function, *Science*, 258 (1992) 597–603.
- [24] Obata, K. and Takeda, K., Release of GABA into the IVth ventricle induced by stimulation of the cat's cerebellum, *J. Neurochem.*, 16 (1969) 1043–1047.

- [25] Ottersen, O.P., Laake, J.H. and Storm-Mathisen, J., Demonstration of a releasable pool of glutamate in cerebellar mossy and parallel fibre terminals by means of light and electron microscopic immunocytochemistry, *Arch. Ital. Biol.*, 128 (1990) 111–125.
- [26] Petralia, R.S., Yokotani, N. and Wenthold, R.J., Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody, *J. Neurosci.*, 14 (1994) 667–696.
- [27] Precht, W., Schwindt, P.C. and Baker, R., Removal of vestibular commissural inhibition by antagonists of GABA and glycine, *Brain Res.*, 62 (1973) 222–226.
- [28] Precht, W. and Dieringer, N., Neuronal events paralleling functional recovery (compensation) following peripheral vestibular lesions. In A. Berthoz and G. Melvill Jones (Eds.), *Adaptive Mechanism in Gaze Control, Facts and Theories*, Elsevier, Amsterdam, 1985, pp. 251–268.
- [29] Saika, T., Kiyama, H., Senba, E. and Tohyama, M., Expression of c-fos-like protein following unilateral labyrinthectomy, *Neurosci. Res.*, Suppl., 16 (1991) S36.
- [30] Saika, T., Takeda, N., Kiyama, H., Kubo, T., Tohyama, M. and Matsunaga, T., Changes in preproenkephalin mRNA after unilateral and bilateral labyrinthectomy in the rat medial vestibular nucleus, *Mol. Brain Res.*, 19 (1993) 237–240.
- [31] Sansom, A.J., Darlington, C.L. and Smith, P.F., Intraventricular injection of an NMDA antagonist disrupts vestibular compensation, *Neuropharmacology*, 29 (1990) 83–84.
- [32] Sansom, A.J., Darlington, C.L. and Smith, P.F., Pretreatment with MK-801 reduces spontaneous nystagmus following unilateral labyrinthectomy, *Eur. J. Pharmacol.*, 220 (1992) 123–129.
- [33] Sekiguchi, M., Okamoto, K. and Sakai, Y., NMDA receptors on Purkinje cell dendrites in guinea pig cerebellar slices, *Brain Res.*, 437 (1987) 402–406.
- [34] Smith, P.F. and Curthoys, I.S., Neuronal activity in the ipsilateral medial vestibular nucleus of the guinea pig following unilateral labyrinthectomy, *Brain Res.*, 444 (1988) 308–319.
- [35] Smith, P.F. and Curthoys, I.S., Mechanism of recovery following unilateral labyrinthectomy: a review, *Brain Res. Rev.*, 14 (1989) 155–180.
- [36] Smith, P.F. and Darlington, C.L., The NMDA antagonist MK801 and CPP disrupt compensation for unilateral labyrinthectomy in the guinea pig, *Neurosci. Lett.*, 94 (1988) 309–313.
- [37] Smith, P.F. and Darlington, C.L., Neurochemical mechanism of recovery from peripheral vestibular lesions (vestibular compensation), *Brain Res. Rev.*, 16 (1991) 117–133.
- [38] Sonnenberg, J.L., Rauscher III, F.J., Morgan, J.I. and Curran, T., Regulation of proenkephalin by Fos and Jun, *Science*, 246 (1989) 1622–1625.
- [39] Vezzani, A., Wu, H.W., Moneta, E. and Samanin, R., Role of the N-methyl-D-aspartate-type receptors in the development and maintenance of hippocampal kindling in rats, *Neurosci. Lett.*, 87 (1988) 63–68.
- [40] de Waele, C., Vibert, N., Baudrimont, M. and Vidal, P.P., NMDA receptors contribute to the resting discharge of vestibular neurons in the normal and hemilabyrinthectomized guinea pig, *Exp. Brain Res.*, 81 (1990) 125–133.
- [41] de Waele, C., Abitbol, M., Chat, M., Menini, C., Mallet, J. and Vidal, P.P., Distribution of glutamatergic receptors and GAD mRNA-containing neurons in the vestibular nuclei of normal and hemilabyrinthectomized rats, *Eur. J. Neurosci.*, 6 (1994) 565–576.
- [42] Zhang, J., Chiodo, L.A. and Freeman, A.S., Electrophysiological effects of MK-801 on rat nigrostriatal and mesoaccumbal dopaminergic neurons, *Brain Res.*, 590 (1992) 153–163.



## ROLE OF THE FLOCCULUS IN THE DEVELOPMENT OF VESTIBULAR COMPENSATION: IMMUNOHISTOCHEMICAL STUDIES WITH RETROGRADE TRACING AND FLOCCULECTOMY USING FOS EXPRESSION AS A MARKER IN THE RAT BRAINSTEM

T. KITAHARA,\*†‡ N. TAKEDA,† T. SAIKA,† T. KUBO,† and H. KIYAMA\*

\*Department of Neuroanatomy, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka, 565, Japan

†Department of Otolaryngology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka, 565, Japan

**Abstract**—After unilateral labyrinthectomy in rats, Fos-like immunoreactive neurons appeared in the ipsilateral medial vestibular nucleus, contralateral prepositus hypoglossal nucleus and contralateral inferior olive beta subnucleus, and thereafter gradually disappeared in accordance with the development of vestibular compensation. This finding indicated that the activation of these nuclei is the initial event of vestibular compensation. In the present study, retrograde tracing experiments revealed that these Fos-like immunoreactive neurons project a proportion of their axons to the vestibulocerebellum (uvula-nodulus, flocculus). Before vestibular compensation was accomplished, right, left or bilateral flocculectomy was performed in right-labyrinthectomized rats. All these treatments caused reappearance of unilateral labyrinthectomy-induced behavioral deficits and Fos expression in the left medial vestibular nucleus and right prepositus hypoglossal nucleus. Since floccular efferents are GABAergic, these results indicate that the neurons in which Fos expression was detected by flocculectomy had been inhibited after unilateral labyrinthectomy by floccular Purkinje neurons and that disinhibition of these neurons induced by flocculectomy caused decompensation.

Based on our present findings, we propose a hypothesis that the bilateral flocculus serves the restoration of balance between intervestibular nuclear activities to induce vestibular compensation after unilateral labyrinthectomy. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

**Key words:** labyrinthectomy, uvula-nodulus, Fluoro-Gold dye, decompensation, nystagmus, neural plasticity.

Unilateral labyrinthectomy induces severe postural (barrel rotation, head tilt) and oculomotor (nystagmus) asymmetry. The functional deficits recover gradually after the lesion. This progressive restoration of balance is referred to as vestibular compensation.<sup>15,17</sup> Since unilateral labyrinthectomy results in a permanent loss of vestibular input from the ipsilateral vestibular periphery,<sup>8</sup> vestibular compensation has been attributed to functional reorganization of the central vestibular system<sup>17</sup> and used as a model of lesion-induced neural plasticity in the CNS.<sup>13</sup>

In previous studies in rats, Fos-like immunoreactive (LIR) neurons appeared in the ipsilateral medial vestibular nucleus (MVe), contralateral prepositus

hypoglossal nucleus (PrH) and contralateral inferior olive beta subnucleus (IOb) after unilateral labyrinthectomy, and then gradually disappeared in accordance with the development of vestibular compensation.<sup>3,9,12,16</sup> This finding indicated that the activation of these nuclei is the initial event of vestibular compensation. Intraperitoneal injection of dizocilpine maleate (MK-801), a specific antagonist of *N*-methyl-D-aspartate (NMDA) receptors, caused reappearance of unilateral labyrinthectomy-induced behavioral deficits. During the decompensation induced by MK-801, Fos-LIR neurons appeared in the contralateral MVe, ipsilateral PrH and bilateral IOB.<sup>11</sup> These findings suggest that the activated neurons in the ipsilateral MVe, contralateral PrH and contralateral IOB after unilateral labyrinthectomy inhibit the contralateral MVe, ipsilateral PrH and bilateral IOB by inhibitory neurons via NMDA receptors and that the NMDA receptor-mediated inhibitory modulation in the central vestibular system plays an important role in the initial processes of the development of vestibular compensation.

‡To whom correspondence should be addressed.

**Abbreviations:** BSA, bovine serum albumin; ChAT, choline acetyltransferase; FG, Fluoro-Gold; IOB, inferior olive beta subnucleus; LIR, -like immunoreactive; MK-801, dizocilpine maleate; MVe, medial vestibular nucleus; NGS, normal goat serum; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PrH, prepositus hypoglossal nucleus; SN, spontaneous nystagmus.

There are two possible origins of the inhibitory projections to the contralateral MVe, ipsilateral PrH and bilateral IOb in the development of vestibular compensation. One is the vestibular commissures and the other is the vestibulocerebellum (uvula-nodulus, flocculus). Previous works have demonstrated that the latter plays an important role in vestibular compensation.<sup>6,7</sup> In the present study, to clarify the involvement of the vestibulocerebellum in the inhibitory circuits responsible for the development of vestibular compensation, we first examined the projection of Fos-LIR neurons after unilateral labyrinthectomy to the vestibulocerebellum by means of retrograde tracing and immunohistochemical techniques. We also studied the effects of flocculectomy on unilateral labyrinthectomy-induced Fos expression.

### EXPERIMENTAL PROCEDURES

#### *Retrograde tracing*

Adult male Wistar rats (Kiwa Experimental Animal Co. Ltd, Japan) weighing about 150 g were used. Fluoro-Gold dye (FG; Fluorochrome Inc., U.S.A.) was dissolved in saline (100 µg/5 µl saline) and used as a retrograde tracer. The caudal cerebellum was exposed and FG was injected through a glass micropipette connected to a 1-µl syringe (Hamilton). For uvula-nodulus injections ( $n=4$ ), a posterior surgical approach was adopted. The posterior part of the bone situated under the occipital bone crest was removed and the dura incised. The micropipette was inserted through the dorsal uvula into the ventral aspect of the uvula and into the nodulus at several medial-lateral locations, as described previously by Barmack *et al.*<sup>1</sup> For injections into the right or left flocculus ( $n=4$ ), an ipsilateral retroauricular approach was adopted. A small opening of the temporal bone was made at the cristata petrosa near the external auditory meatus with a small dental finishing burr and then the flocculus covered with the dura was exposed. The micropipette was inserted through this opening to inject FG into the flocculus. A total of 0.25 µl was pressure injected at each site. After three days postoperative survival, animals were right-labyrinthectomized and then perfused for immunohistochemical analysis.

#### *Labyrinthectomy*

Three days after FG injection, animals were anesthetized with ether and the right tympanic membrane, malleus and incus were removed by the retroauricular approach under an operating microscope. The stapes crura were fractured and the stapes foot plate was removed to open the oval window. Then, a small opening was made on the bony horizontal semicircular canal with a small dental finishing burr. Through these two openings, the membranous labyrinth was surgically removed with a small right-angled hook and chemically destroyed by injection of 100% ethanol. At the end of surgery, antibiotic cream (Furacin) was topically applied to the opened labyrinth to prevent infection and the temporal bone was sealed with dental cement. The operative wound was sutured and the animal was allowed to recover in the light.

#### *Immunohistochemistry by the indirect immunofluorescence method*

At a post-unilateral labyrinthectomy interval of 6 h, FG-injected animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with 100 ml of ice-cold saline, followed by 250 ml of Zamboni's fixative.

The brain was immediately removed after perfusion with Zamboni's fixative, postfixed in the same fixative at 4°C for one to two days and then placed in 30% sucrose-phosphate buffer at 4°C for two to three days. Frozen serial sections (18 µm thick) were cut on a cryostat and processed using the indirect immunofluorescence method. Briefly, sections were incubated sequentially in the following solutions at 4°C: 1% bovine serum albumin (BSA) and normal goat serum (NGS) in 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 3 h; antisera against Fos (diluted 1:500) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 48 h; 0.1 M PBS for 15 min; donkey anti-rabbit immunoglobulin G conjugated with Texas Red (diluted 1:250) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 24 h; 0.1 M PBS for 15 min. They were then examined under a light microscope. FG has gold fluorescence when viewed using a V dichroic mirror filter. Neurons with Fos-LIR on the same section showed red fluorescence under a G dichroic filter. The antibody raised against Fos was obtained from Oncogene Science (New York, U.S.A.; catalog no. PC05). This Fos antibody is a rabbit, affinity-purified polyclonal antibody raised against the peptide S-G-F-N-A-D-Y-E-A-S-S-R-C corresponding to residues 4–17 of human Fos.<sup>5</sup>

#### *Flocculectomy*

At each post-unilateral labyrinthectomy interval of three, seven and 14 days, animals were anesthetized with ether and received right, left or bilateral flocculectomy using the same approach as in the cases with FG injection into the flocculus ( $n=4$  in each case). A part of the temporal bone near the crista petrosa was carefully removed and the flocculus was exposed. Through the hole on the temporal bone, a small right-angled hook and a suction pipette were inserted to destroy mechanically and absorb the posterior part of the flocculus. Then, proceeding anteriorly along the petrous bone, the lesion could be extended to the anterior floccular lobules.

Two and six hours after flocculectomy, animals were treated for perfusion and fixation as described above.

#### *Immunohistochemistry using the avidin-biotin complex method*

The avidin-biotin complex method was used to visualize Fos expression after uni- or bilateral flocculectomy. Briefly, sections were incubated sequentially in the following solutions at 4°C: 1% BSA and NGS in 0.3% Triton X-100 in PBS for 3 h; antisera against Fos (diluted 1:500) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 48 h; 0.1 M PBS for 15 min; biotinylated goat anti-rabbit immunoglobulin G (diluted 1:250; Vector Labs, Inc., U.S.A.) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 24 h; 0.1 M PBS for 15 min; Vectastain reagent (diluted 1:500; Vector Labs, Inc., U.S.A.) for 24 h; diaminobenzidine tetrahydrochloride/ $H_2O_2$  for 15 min. Sections were then examined under a light microscope.

#### *Cell counting*

To detect FG-fluorescent and Fos-LIR neurons, transverse 18-µm brainstem sections were examined under a fluorescence microscope at  $\times 40$  and  $\times 100$  magnification using the indirect immunofluorescence method. On the other hand, to detect Fos-LIR cells after flocculectomy, sections were examined under a bright-field microscope at  $\times 40$  and  $\times 100$  magnification using the avidin-biotin complex method. Only cells that had significant levels of immunofluorescent or diaminobenzidine reaction product in their nucleus above tissue background levels were counted with a digital image analysis system (Universal Imaging Software).

#### *Behavior*

Vestibulo-ocular and vestibulospinal reflexes are usually used as markers of the development of vestibular

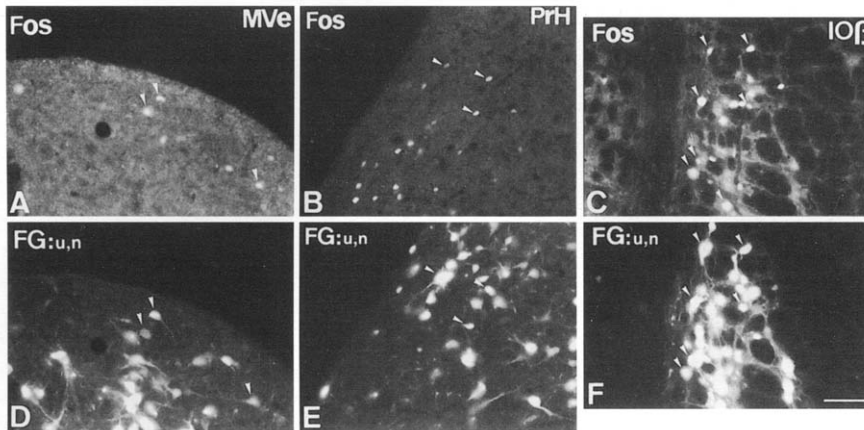


Fig. 1. (A–C) Fos-LIR cells in the ipsilateral MVe (A), contralateral PrH (B) and contralateral IOb (C) 6 h after unilateral labyrinthectomy. (D–F) FG-labeled cells in the same fields as A (D), B (E) and C (F) after injection of FG into the uvula-nodulus (u,n). Arrowheads indicate double-labeled cells. Scale bar=50 μm.

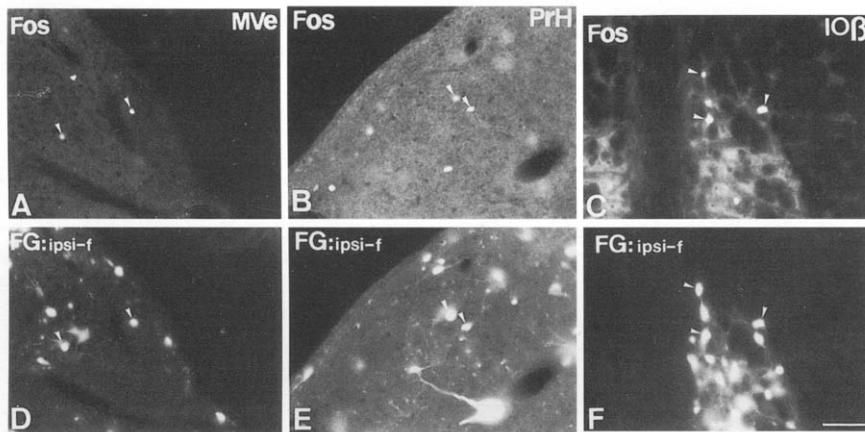


Fig. 2. (A–C) Fos-LIR cells in the ipsilateral MVe (A), contralateral PrH (B) and contralateral IOb (C) 6 h after unilateral labyrinthectomy. (D–F) FG-labeled cells in the same fields as A (D), B (E) and C (F) after injection of FG into the ipsilateral flocculus (ipsi-f). Arrowheads indicate double-labeled cells. Scale bar=50 μm.

compensation. In the present study, we chose the frequency of horizontal spontaneous nystagmus (SN) as a marker, because it can be reliably measured using a video camera.

Eye movements were recorded using a Panasonic NV-M7 video camera with a zoom lens, and replayed using a Mitsubishi E7 Black Diamond video recorder and a Sony Trinitron color monitor. The frequency of SN was measured as the number of quick phase beats occurring over periods of 15 s. The eye movements were replayed and counted three times for each animal and the means obtained. These measurements were made at 0.5, 1, 2, 6, 12, 18, 24, 42, 48 and 72 h post-unilateral labyrinthectomy. SN induced by flocculectomy at various postoperative intervals was also measured as described above.

The statistical significance of differences was evaluated using Student's *t*-test.

## RESULTS

### *Fos expression in cerebellar afferent neurons after unilateral labyrinthectomy*

Pressure injections of FG into the vestibulo-cerebellum (uvula-nodulus, flocculus) labeled a large

number of vestibular and vestibular-related nuclei. Following FG injections into the uvula-nodulus, MVe, inferior vestibular nucleus, superior vestibular nucleus, PrH and IOb neurons were labeled bilaterally. In the case of FG injections into the unilateral flocculus, MVe, inferior vestibular nucleus, nucleus X and PrH neurons were labeled almost symmetrically and IOb neurons were labeled only contralaterally.

Unilateral labyrinthectomy-induced Fos-LIR neurons 6 h after unilateral labyrinthectomy in the ipsilateral MVe, contralateral PrH and contralateral IOb are shown in Figs 1A–C and 2A–C. FG-labeled neurons are shown in Figs 1D–F (after FG injections into the uvula-nodulus) and 2D–F (after FG injections into the flocculus ipsilateral to the unilateral labyrinthectomized side). Arrowheads indicate double-labeled neurons. About 20% of ipsilateral MVe, 15% of contralateral PrH and 70% of contralateral IOb Fos-LIR neurons were double-labeled by FG injections into the uvula-nodulus. In the ipsilateral MVe, most of these double-labeled neurons

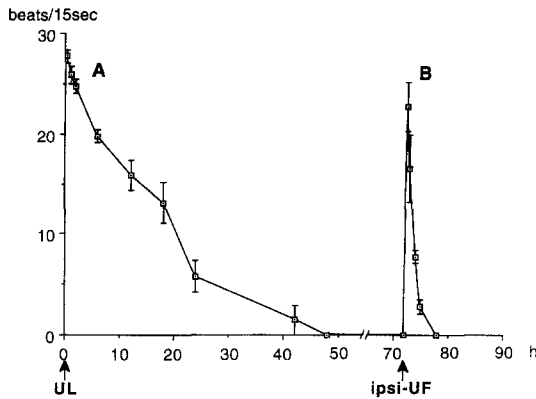


Fig. 3. Changes in frequency of SN with time after right unilateral labyrinthectomy (A) and the effects of right unilateral flocculectomy on the frequency of spontaneous nystagmus on postoperative day 3 (B) in rats. Data are expressed as mean frequencies  $\pm$  S.E. of quick phase beats per 15 s for four animals. UL, unilateral labyrinthectomy; ipsi-UF, unilateral flocculectomy ipsilateral to the unilateral labyrinthectomized side.

were dorsolateral to caudally located. Following FG injection into the ipsilateral flocculus, about 20% of ipsilateral MVe, 20% of contralateral PrH and 30% of contralateral IOb Fos-LIR neurons were FG fluorescent. In the ipsilateral MVe, most of these double-labeled neurons were dorsomedial to caudally distributed near the ventricular surface. Following FG injection into the flocculus contralateral to the lesioned side, double-labeled neurons were rarely observed.

#### *Vestibular compensation after unilateral labyrinthectomy and flocculectomy-induced decompensation*

After right unilateral labyrinthectomy, the quick phase of SN toward the left (intact) side appeared. The frequency of SN reached a maximum 30 min after unilateral labyrinthectomy. Then, the frequency of SN gradually decreased and disappeared by 48 h post-unilateral labyrinthectomy (Fig. 3A). On day 3 after right unilateral labyrinthectomy, right unilateral flocculectomy caused reappearance of SN toward the left side (the same direction as in the case after right unilateral labyrinthectomy). The frequency of SN reached a maximum 30 min after unilateral flocculectomy. The frequency of SN decreased and disappeared by 6 h post-unilateral flocculectomy (Fig. 3B). Left or bilateral flocculectomy gave similar results with regard to the direction and the number of reappearing SN (data not shown).

Figure 4A shows the direction and maximum frequencies of SN following right unilateral flocculectomy alone, right unilateral labyrinthectomy alone and right unilateral flocculectomy on days 3, 7 and 14 after right unilateral labyrinthectomy. When right unilateral flocculectomy was performed in normal rats, the quick phase of SN toward the right (lesioned) side appeared. The maximum frequency of

right unilateral flocculectomy-induced SN in normal rats was significantly less than that of right unilateral labyrinthectomy-induced SN ( $P < 0.05$ ; ignoring the direction of SN). In right-labyrinthectomized rats, right unilateral flocculectomy caused SN toward the left (intact) side, the same direction as after right unilateral labyrinthectomy. The maximum frequency of unilateral flocculectomy-induced SN on day 7 after right unilateral labyrinthectomy was significantly less than that on day 3 ( $P < 0.01$ ). Right unilateral flocculectomy induced not left- but right-directed SN on day 14 after right unilateral labyrinthectomy, as was the case with right unilateral flocculectomy performed in normal rats. Thus, the decompensation (reappearance of SN toward the left side) induced by right unilateral flocculectomy was observed only when performed up to day 7 after right unilateral labyrinthectomy.

Figure 4B shows the direction and maximum frequencies of SN following left unilateral flocculectomy alone, right unilateral labyrinthectomy alone and left unilateral flocculectomy on days 3, 7 and 14 after right unilateral labyrinthectomy. When left unilateral flocculectomy was performed in both normal and right-labyrinthectomized rats, the quick phase of SN toward the left (flocculectomized) side appeared. The maximum frequency of left unilateral flocculectomy-induced SN in normal rats was significantly less than that of right unilateral labyrinthectomy-induced SN ( $P < 0.05$ ). Unilateral flocculectomy-induced SN on day 3 after unilateral labyrinthectomy was slightly, but not significantly, stronger than that in normal rats.

When bilateral flocculectomy was performed in normal rats, the direction of SN was very unstable and the number of SN could not be counted.

After right unilateral labyrinthectomy, the head of the animals was tilted toward the right (lesioned) side. The degree of head tilt showed gradual but incomplete recovery. On day 3 after right unilateral labyrinthectomy, right, left or bilateral flocculectomy caused exacerbation of head tilt toward the right side (the same direction as after right unilateral labyrinthectomy), which almost disappeared by 6 h post-flocculectomy. Flocculectomy caused little exacerbation of head tilt on day 14 after unilateral labyrinthectomy, as observed with flocculectomy performed in normal rats (data not shown).

#### *Fos expression during flocculectomy-induced decompensation*

In normal rats, 2 h after right unilateral flocculectomy, Fos-LIR neurons were seen in the bilateral MVe, PrH and left IOb (Figs 5, 9C). The right MVe, left PrH and left IOb contained significantly more Fos-LIR neurons than the opposite sides ( $P < 0.01$ ; Fig. 6, Table 1). Six hours later, no Fos expression was observed.

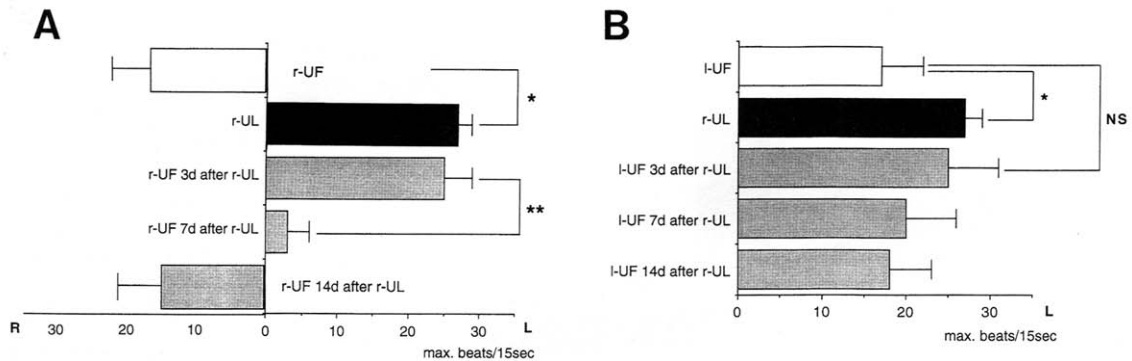


Fig. 4. (A) The direction and the maximum number of SN (beats/15 s) after right unilateral flocculectomy (r-UF; open column), after right unilateral labyrinthectomy (r-UL; filled column), and those induced by right unilateral flocculectomy at post-unilateral labyrinthectomy intervals of three, seven and 14 days (stippled columns) in rats. (B) The direction and the maximum number of SN after left unilateral flocculectomy (l-UF; open column), after right unilateral labyrinthectomy (filled column), and those induced by left unilateral flocculectomy at post-unilateral labyrinthectomy intervals of three, seven and 14 days (stippled columns) in rats. Columns represent means  $\pm$  S.E. for four animals. R, right direction; L, left direction. \* $P < 0.05$ , \*\* $P < 0.01$ . Note that the direction of SN is neglected in A.

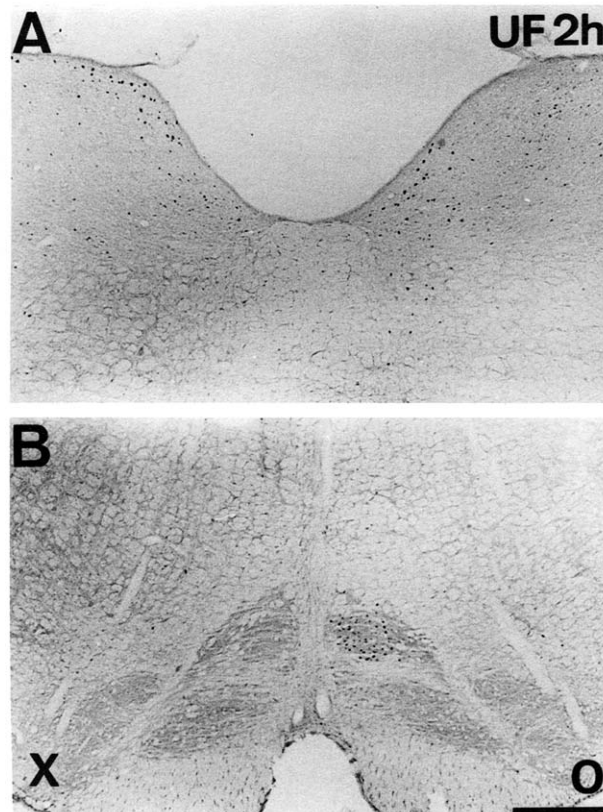


Fig. 5. Bright-field photomicrographs showing Fos expression in the bilateral MVe, PrH (A) and contralateral IOb (B) 2 h after unilateral flocculectomy (UF2h). Scale bar=200  $\mu$ m. X, unilateral flocculectomized side; O, intact side.

In right-labyrinthectomized rats, during reappearance of SN induced by right or left unilateral flocculectomy, which was performed up to day 7 after right unilateral labyrinthectomy, Fos-LIR neurons appeared only in the left MVe, right PrH and bilateral IOb (Figs 7, 9E, F). Fos-LIR neurons were rarely observed in the right MVe and left PrH. The numbers

of Fos-LIR neurons induced by right unilateral flocculectomy in the left MVe and the right PrH in right-labyrinthectomized rats (Fig. 9E) increased significantly in comparison with those in normal rats (Fig. 9C, Table 1A,  $P < 0.01$ ). In contrast, the numbers of Fos-LIR neurons induced by left unilateral flocculectomy in the right MVe and the left PrH in

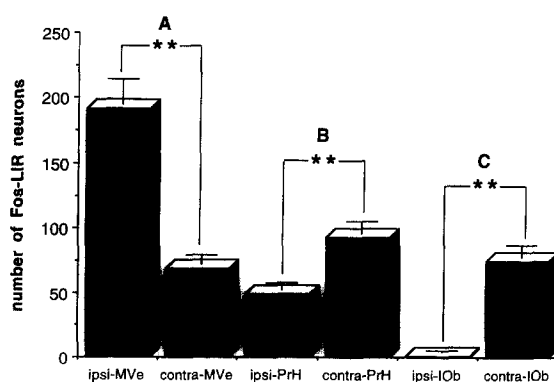


Fig. 6. Numbers of Fos-LIR neurons in MVe, PrH and IOb neurons after unilateral flocculectomy on normal rats. Columns represent means  $\pm$  S.E. for four animals. The ipsilateral MVe, contralateral PrH and contralateral IOb contain significantly more Fos-LIR neurons than the opposite sides (\*\* $P < 0.01$ ).

Table 1. The numbers of Fos-like immunoreactive neurons after unilateral flocculectomy in normal and unilateral labyrinthectomized rats

		r-MVe	l-MVe	r-PrH	l-PrH
(A)	Normal-rUF	191.05 $\pm$ 19.05	68.98 $\pm$ 7.10	48.88 $\pm$ 4.98	93.20 $\pm$ 8.99
	rUL-rUF	9.88 $\pm$ 3.23	197.25 $\pm$ 27.89	79.52 $\pm$ 9.91	9.65 $\pm$ 3.05
(B)	Normal-lUF	68.98 $\pm$ 7.10	191.05 $\pm$ 19.05	93.20 $\pm$ 8.99	48.88 $\pm$ 4.98
	rUL-lUF	8.27 $\pm$ 2.58	200.38 $\pm$ 25.18	88.78 $\pm$ 9.98	7.44 $\pm$ 3.53

(A) The numbers of Fos-LIR neurons induced by right unilateral flocculectomy in the left MVe and right PrH in right unilateral labyrinthectomized animals were increased significantly in comparison with those in normal animals ( $P < 0.01$ ). (B) The numbers of Fos-LIR neurons induced by left unilateral flocculectomy in the right MVe and left PrH in right unilateral labyrinthectomized animals were decreased significantly in comparison with those in normal animals ( $P < 0.01$ ). Numbers represent means  $\pm$  S.E. for four animals. Normal-rUF, right unilateral flocculectomized animals only (cf. Fig. 9C); rUL-rUF, right unilateral flocculectomy on right unilateral labyrinthectomized animals (cf. Fig. 9E); normal-lUF, left unilateral flocculectomized animals only (cf. Fig. 9D); rUL-lUF, left unilateral flocculectomy on right unilateral labyrinthectomized animals (cf. Fig. 9F); r-MVe, right medial vestibular nucleus; l-MVe, left MVe; r-PrH, right prepositus hypoglossal nucleus; l-PrH, left PrH.

right-labyrinthectomized rats (Fig. 9F) decreased significantly in comparison with those in normal rats (Fig. 9D, Table 1B,  $P < 0.01$ ). At a post-unilateral labyrinthectomy interval of 14 days, unilateral flocculectomy showed similar SN induction as with unilateral flocculectomy performed in normal rats (Fig. 4). Furthermore, the pattern of unilateral flocculectomy-induced Fos expression in labyrinthectomized rats (Fig. 8) was also very similar to that seen following unilateral flocculectomy in normal rats (Fig. 5). The Fos expression level in the MVe and PrH induced by unilateral flocculectomy reached a maximum 2 h after unilateral flocculectomy and disappeared 6 h later. To make the results much more accessible, the effects of right or left flocculectomy on Fos expression in the rat brainstem after right unilateral labyrinthectomy are shown in Fig. 9 and Table 1.

#### DISCUSSION

Thirty minutes after unilateral labyrinthectomy, the frequency of SN toward the intact side reached a maximum in rats. The frequency of SN then

gradually decreased and disappeared by 48 h post-operation (Fig. 3A). The recovery from unilateral labyrinthectomy-induced behavioral deficits is referred to as vestibular compensation. Fos-LIR neurons appeared in the ipsilateral MVe, contralateral PrH and contralateral IOb after unilateral labyrinthectomy in the rat brainstem (Figs 1A–C, 2A–C, 9B) and then gradually disappeared by the third postoperative day. Thus, Fos expression seems to be reduced in accordance with the development of vestibular compensation.<sup>3,9,12,16</sup> As Fos is expressed in neurons following synaptic excitation by various stimuli,<sup>2,10,14</sup> these findings suggest that neural activation of the ipsilateral MVe, contralateral PrH and contralateral IOb is the initial event in the development of vestibular compensation after unilateral labyrinthectomy.

The present combined retrograde tracing and immunohistochemical study revealed that substantial numbers of Fos-LIR neurons after unilateral labyrinthectomy project their axons directly into the uvula-nodulus and ipsilateral flocculus (Figs 1D–F, 2D–F). These results suggest that the vestibulo-cerebellum contributes to the initial event of



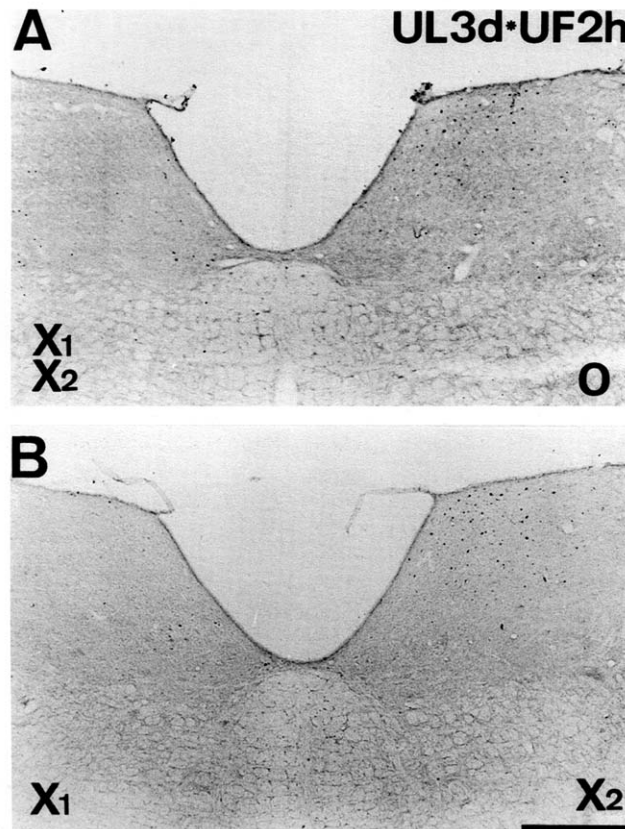


Fig. 7. Bright-field photomicrographs showing Fos expression in the left MVe and right PrH 2 h after right labyrinthectomy (A) and left labyrinthectomy (B) on day 3 after right labyrinthectomy (UL3d\*UF2h). Scale bar=200  $\mu$ m. X<sub>1</sub>, unilateral labyrinthectomized side (right); X<sub>2</sub>, unilateral flocculectomized side (A, right; B, left); O, intact side (left).

the development of vestibular compensation after unilateral labyrinthectomy. It has been reported that choline acetyltransferase (ChAT)-containing secondary vestibular neurons project their axons into the vestibulocerebellum.<sup>1</sup> The pattern of the distribution of ChAT-containing cerebellar afferent neurons is very similar to that of unilateral labyrinthectomy-induced Fos-LIR cerebellar afferent neurons. These findings suggest that secondary vestibular cholinergic neurons which have synaptic effects on the vestibulocerebellum are activated in the development of vestibular compensation after unilateral labyrinthectomy. We also performed a preliminary immunohistochemical experiment which showed that some of the unilateral labyrinthectomy-induced Fos-LIR neurons contained ChAT-LIR (unpublished observation).

In normal rats, 30 min after unilateral flocculectomy, the frequency of SN toward the unilateral flocculectomy side reached a maximum (Fig. 4). The frequency of SN then decreased and disappeared by 6 h post-unilateral flocculectomy. At that time, Fos-LIR neurons appeared in the bilateral MVe (ipsilateral>contralateral), PrH (contralateral>ipsilateral) and contralateral IOb in the rat brainstem (Figs 5, 6, 9C, D, Table 1), then disappeared by 6 h

post-unilateral flocculectomy. Since Fos is a marker of synaptic excitation by various stimuli,<sup>2,10,14</sup> it is suggested that unilateral flocculectomy-induced asymmetrical cerebellar disinhibition of the MVe, PrH and IOb neurons causes unilateral flocculectomy-induced oculomotor asymmetry. However, the number of unilateral flocculectomy-induced SN in normal rats was significantly less than that in unilateral labyrinthectomy-induced animals ( $P<0.05$ ; Fig. 4). Moreover, the barrel rotation and the obvious head tilt were hardly observed after unilateral flocculectomy, unlike after unilateral labyrinthectomy. These differences between behavioral deficits after unilateral flocculectomy and after unilateral labyrinthectomy may be due to the extent of imbalance of intervestibular nuclear activities caused by unilateral flocculectomy and unilateral labyrinthectomy. The disappearance of unilateral flocculectomy-induced SN and Fos expression by 6 h after unilateral flocculectomy may be due to the recovery from intervestibular imbalance by some other components, such as the uvula-nodulus and commissures.

In right-labyrinthectomized rats, right flocculectomy was performed before vestibular compensation was accomplished. This treatment caused reappearance of unilateral labyrinthectomy-induced behav-

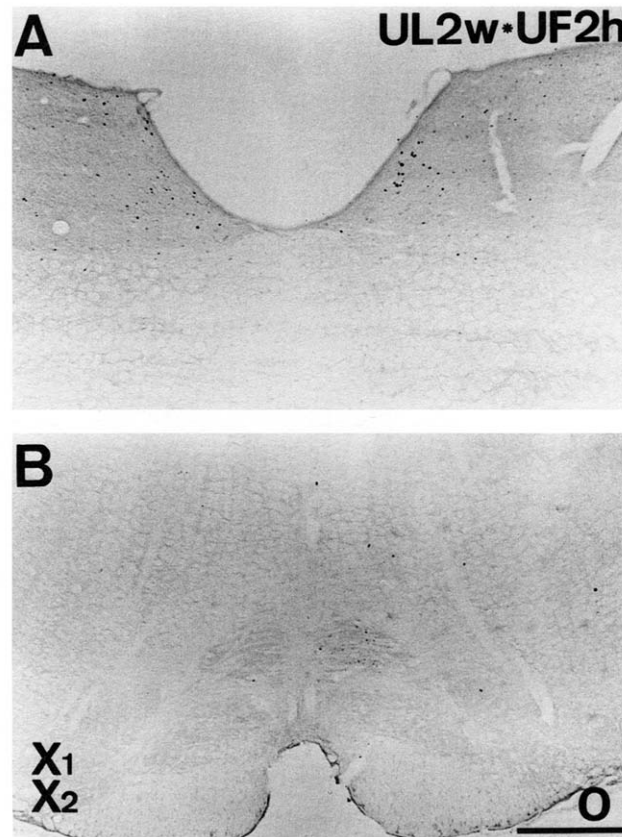


Fig. 8. Bright-field photomicrographs showing Fos expression in the bilateral MVe, PrH (A) and left IOb (B) 2 h after right flocculectomy on day 14 after right labyrinthectomy (UL2w\*UF2h). Scale bar=200  $\mu$ m. X<sub>1</sub>, unilateral labyrinthectomized side (right); X<sub>2</sub>, unilateral flocculectomized side (right); O, intact side (left).

ioral deficits and Fos expression in the left MVe, right PrH and bilateral IOb (Figs 7A, 9E). Since Fos is induced in activated neurons, it is suggested that the left MVe, right PrH and bilateral IOb had been inhibited via floccular projections during vestibular compensation after right unilateral labyrinthectomy, and that flocculectomy probably activated these nuclei by disinhibition, resulting in decompensation. Therefore, the flocculus ipsilateral to the unilateral labyrinthectomized side is a component of inhibitory circuits for the development of vestibular compensation after unilateral labyrinthectomy.

Labyrinthectomy changed the floccular inhibitory control on the brainstem vestibular system. The numbers of Fos-LIR neurons induced by unilateral flocculectomy ipsilateral to the unilateral labyrinthectomized side in the MVe contralateral to the unilateral flocculectomized side and the PrH ipsilateral to the unilateral flocculectomized side in labyrinthectomized rats (Fig. 9E) were increased significantly in comparison with those in normal rats (Fig. 9C, Table 1A,  $P<0.01$ ). Since Fos expression after unilateral flocculectomy is due to unilateral flocculectomy-induced disinhibition, the numbers of unilateral flocculectomy-induced Fos-LIR neurons are correlated with the degree of floccular inhibition of the

brainstem vestibular system before flocculectomy. Therefore, these findings suggest that inhibitory effects of the flocculus ipsilateral to the unilateral labyrinthectomized side on the contralateral MVe and ipsilateral PrH neurons were increased after unilateral labyrinthectomy. In contrast, the numbers of Fos-LIR neurons induced by unilateral flocculectomy contralateral to the unilateral labyrinthectomized side in the MVe contralateral to the unilateral flocculectomized side and the PrH ipsilateral to the unilateral flocculectomized side in labyrinthectomized rats (Fig. 9F) were decreased significantly in comparison with those in normal rats (Fig. 9D, Table 1B,  $P<0.01$ ). These findings suggest that the flocculus contralateral to the unilateral labyrinthectomized side removes inhibitory effects on ipsilateral MVe and contralateral PrH neurons after unilateral labyrinthectomy. It has been reported that the neural activity of the ipsilateral MVe second-order neurons was markedly suppressed by deafferentation and that of the contralateral MVe second-order neurons was slightly increased by commissural disinhibition in acute labyrinthectomized guinea-pigs.<sup>4</sup> Therefore, it is reasonable that the bilateral flocculus serves an equilibrative function by inhibition and disinhibition of second-order MVe neurons at the acute stage after

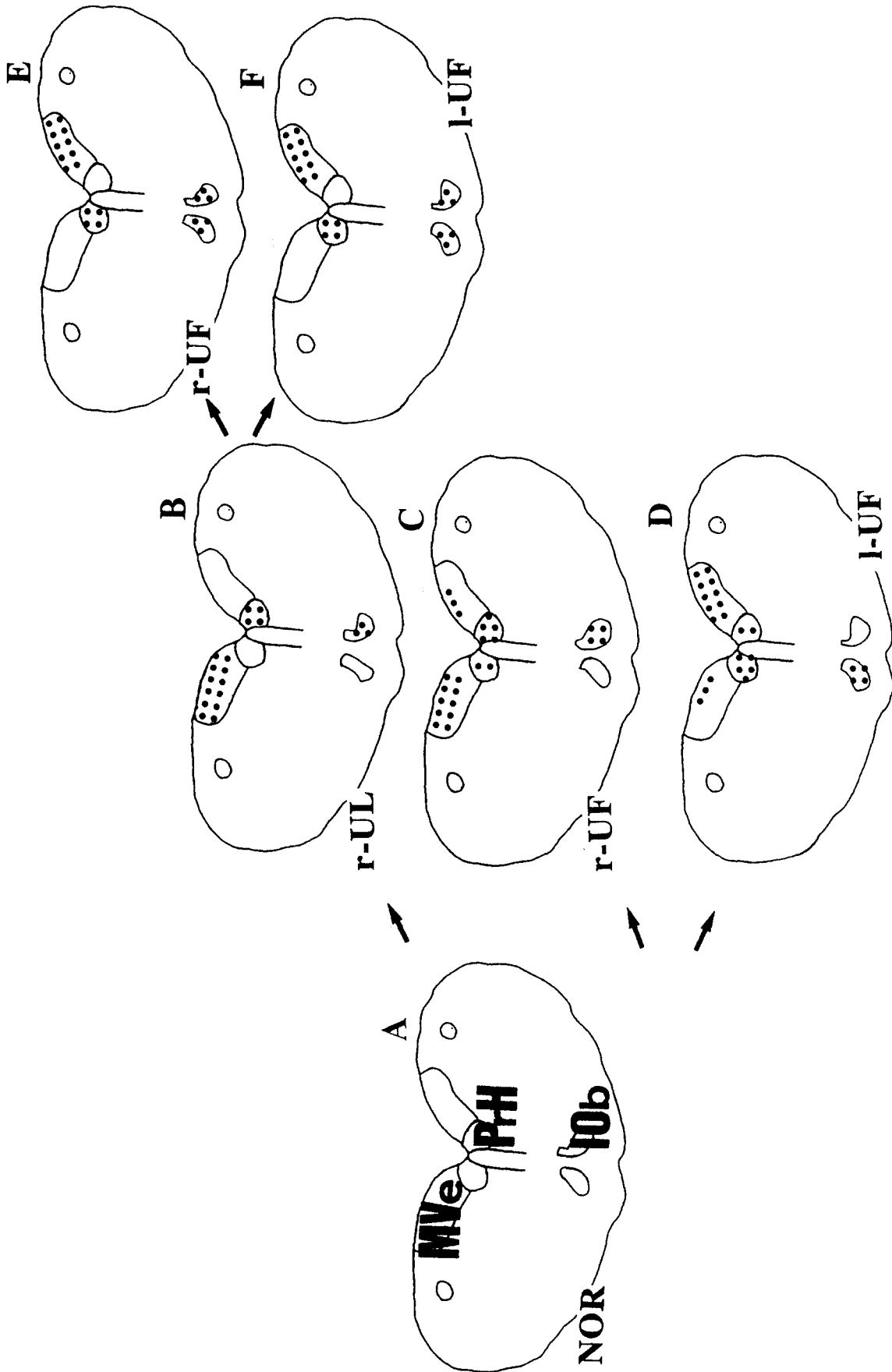


Fig. 9. Schematic representation of Fos expression in rat brainstem sections at the level of the MVe, PrH and IOb. (A) Normal rats. (B) Unilateral labyrinthectomized rats 6 h after unilateral labyrinthectomy. (C, D) Unilateral flocculectomized rats 2 h after ipsilateral (E) and contralateral (F) flocculectomy at a postoperative interval of three days. The average number of Fos-labeled neurons per nucleus is indicated by the dots ( $n=4$ ). One dot represents 20 Fos-immunopositive neurons. Note that the rats in E and F showed reappearance of unilateral labyrinthectomy-induced behavioral deficits after unilateral flocculectomy. NOR, normal rats; r-UL, right unilateral labyrinthectomy; l-UL, left unilateral labyrinthectomy; r-UF, right unilateral flocculectomy; l-UF, left unilateral flocculectomy.

unilateral labyrinthectomy. As vestibular compensation developed, the pattern of unilateral flocculectomy-induced Fos expression in labyrinthectomized rats (Fig. 8) became more like that in normal rats (Figs 5, 9C). This reversible profile of unilateral flocculectomy-induced Fos expression was well correlated with that of unilateral flocculectomy-induced reappearance of SN (decompensation; Fig. 4A). These findings suggest that labyrinthectomy-induced changes in the floccular inhibitory control on the brainstem vestibular system play an important role in the initial processes of vestibular compensation to restore a balance between intervestibular nuclear activities.

### CONCLUSION

In our recent study, the injection of MK-801 (a specific antagonist of NMDA receptors) into right-labyrinthectomized rats before vestibular compensation was accomplished caused reappearance of SN toward the intact side. During the decompensation, Fos-LIR neurons appeared in the left MVe, right PrH and bilateral IOb.<sup>11</sup> These results are well correlated with those following flocculectomy in labyrinthectomized rats in the present study. In

floccular Purkinje cells, parallel fiber afferents are mediated by NMDA receptors and Purkinje efferents are GABAergic. Taken together, it is suggested that the neurons in which Fos expression was detected by flocculectomy had been inhibited after unilateral labyrinthectomy partially by mossy and/or parallel glutamatergic fibers driving floccular GABAergic neurons via NMDA receptors, and that disinhibition of these neurons induced by flocculectomy caused decompensation. Therefore, it is likely that the floccular inhibitory control on the brainstem vestibular system was changed by floccular afferent neurons activated after unilateral labyrinthectomy via NMDA receptors, resulting in the restoration of balance between intervestibular nuclear activities.

*Acknowledgements*—We thank Prof. M. Tohyama (Department of Anatomy and Neuroscience, Osaka University Medical School) and Dr M. Yamano (Physical Therapy Osaka Pref. College of Health Science) for their helpful advice and support. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan, and the Japan Brain Foundation (Tokyo, Japan).

### REFERENCES

- Barmack N. H., Baughman R. W., Eckenstein F. P. and Shoujaku H. (1992) Secondary vestibular cholinergic projection to the cerebellum of rabbit and rat as revealed by choline acetyltransferase immunohistochemistry, retrograde and orthograde tracers. *J. comp. Neurol.* **317**, 250–270.
- Bullitt E. (1989) Induction of c-fos like protein within the lumbar spinal cord and thalamus of the rat following peripheral stimulation. *Brain Res.* **493**, 391–397.
- Cirelli C., Pompeiano M., D'ascanio P., Arrighi P. and Pompeiano O. (1996) c-Fos expression in the rat brain after unilateral labyrinthectomy and its relation to the uncompensated and compensated stages. *Neuroscience* **70**, 515–546.
- Darlington C. L., Flohr H. and Smith P. F. (1991) Molecular mechanisms of brainstem plasticity. *Molec. Neurobiol.* **5**, 355–368.
- Delogni P., Niman H., Raymond V., Sawchenko P. and Verma I. M. (1988) Detection of fos protein during osteogenesis by monoclonal antibodies. *Molec. cell. Biol.* **8**, 2251–2256.
- Haddad G. M., Friendlich A. R. and Robinson D. A. (1977) Compensation of nystagmus after VIIIth nerve lesions in vestibulo-cerebellectomized cats. *Brain Res.* **135**, 192–196.
- Jeannerod M. and Courjon J. H. (1981) Vestibular compensation: new sensorimotor perspectives. In *Lesion Induced Neuronal Plasticity in Systems* (eds Flohr H. and Precht W.), pp. 208–220. Springer, Berlin.
- Jensen D. W. (1983) Survival of function in the deafferented vestibular nerve. *Brain Res.* **273**, 175–178.
- Kaufman G. D., Anderson J. H. and Beitz A. J. (1992) Brainstem Fos expression following acute unilateral labyrinthectomy in the rat. *NeuroReport* **3**, 829–832.
- Kaufman G. D., Anderson J. H. and Beitz A. J. (1993) Otolith-brain stem connectivity: evidence for differential neural activation by vestibular hair cells based on quantification of Fos expression in unilateral labyrinthectomized rats. *J. Neurophysiol.* **70**, 117–127.
- Kitahara T., Takeda N., Saika T., Kubo T. and Kiyama H. (1995) Effects of MK801 on Fos expression in the rat brainstem after unilateral labyrinthectomy. *Brain Res.* **700**, 182–190.
- Kitahara T., Saika T., Takeda N., Kubo T. and Kiyama H. (1995) Changes in Fos and Jun expression after labyrinthectomy in the rat brainstem. *Acta otolar.* **520**, Suppl., 401–404.
- Lacour M. and Xerri C. (1981) Vestibular compensation: new sensorimotor perspectives. In *Lesion Induced Neuronal Plasticity in Systems* (eds Flohr H. and Precht W.), pp. 240–253. Springer, Berlin.
- Lee J. H. and Beitz A. J. (1993) The distribution of brain stem and spinal cord nuclei associated with different frequencies of electroacupuncture analgesia. *Pain* **52**, 11–28.
- Precht W. and Dieringer N. (1985) Neuronal events paralleling functional recovery (compensation) following peripheral vestibular lesions. In *Adaptive Mechanism in Gaze Control, Facts and Theories* (eds Berthoz A. and Melvill Jones G.), pp. 251–268. Elsevier, Amsterdam.
- Saika T., Kiyama H., Senba E. and Tohyama M. (1991) Expression of c-fos-like protein following unilateral labyrinthectomy. *Neurosci. Res.* **16**, Suppl., S36.
- Smith P. F. and Curthoys I. S. (1989) Mechanism of recovery following unilateral labyrinthectomy: a review. *Brain Res. Rev.* **14**, 155–180.