

Temporal Changes of the Calcium-binding Proteins in the Medial Vestibular Nucleus following Unilateral Labyrinthectomy in Rats

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Calcium (Ca^{2+}) is an intracellular second messenger associated with neuronal plasticity of the central nervous system. The calcium-binding proteins regulate the Ca^{2+} -mediated signals in the cytoplasm and buffer the calcium concentration. This study examined temporal changes of three calcium-binding proteins (calretinin, calbindin and parvalbumin) in the medial vestibular nucleus (MVN) during vestibular compensation after unilateral labyrinthectomy (UL) in rats. Rats underwent UL, and the changes in the expression of these proteins at 2, 6, 12, 24, 48, and 72 h were examined by immunofluorescence staining. The expression levels of all three proteins increased immediately after UL and returned to the control level by 48 h. However, the level of calretinin showed changes different from the other two proteins, being expressed at significantly higher level in the contralateral MVN than in the ipsilateral MVN 2 h after UL, whereas the other two proteins showed similar expression levels in both the ipsilateral and contralateral MVN. These results suggest that the calcium binding proteins have some protective activity against the increased Ca^{2+} levels in the MVN. In particular, calretinin might be more responsive to neuronal activity than calbindin or parvalbumin.

Key Words: Calcium-binding proteins, Medial vestibular nuclei, Vestibular compensation

INTRODUCTION

Calcium (Ca^{2+}) plays an important role as an intracellular mediator of different physiological actions in nerve cells, including development, growth, transmitter release, transmembrane signaling, and synaptic plasticity (Ghosh & Greenberg, 1995). Since a high concentration of intracellular calcium is noxious to cells, control of the cytosolic Ca^{2+} level is of fundamental significance (Orrenius & Nicotera, 1994). An increase in the intracellular Ca^{2+} level triggers the activity of the calcium binding proteins, which have been found to regulate the intracellular calcium concentrations. They regulate the Ca^{2+} -mediated signals in the cytoplasm and buffer the calcium concentration (Chard et al, 1993).

Sensory deprivation of the sensory organs or central nervous system produces variable changes in the levels of calcium binding proteins as well as diverse molecular changes according to time. There have been many studies on the calcium-binding protein levels in the auditory brainstem and central visual nervous system (Lane et al, 1996; Caicedo et al, 1997). However, there are only a few reports on the central vestibular system, and they mainly been focused on the role of calcium-binding proteins as a marker of afferent input, by using calretinin only or calretinin and calbindin after the recovery of static vestibular symptoms

(Sans et al, 1995; Kevetter & Neouard, 1997). Sans et al (1995) reported that unilateral sensory deprivation induces permanent asymmetry in the expression of calretinin between the ipsilateral and contralateral MVN, and suggested that the calretinin expression in the MVN depends upon the integrity and activity of the sensorineural peripheral vestibular influences. Kevetter and Neouard (1997) reported that the level of calretinin and calbindin expression decreases after a vestibular neurectomy, and suggested that calretinin and calbindin are effective markers for identifying the vestibular afferents.

Some of the vestibular symptoms caused by a loss of unilateral vestibular function subside during behavioral recovery, known as vestibular compensation. Once removed peripheral vestibular receptor cells do not regenerate, and neurons in the Scarpa's ganglion do not undergo any significant functional recovery (Lacour et al, 1981), therefore, vestibular compensation has been suggested to be the result of central nervous system plasticity. Moreover, the MVN appears to be of primary importance in the neuronal plasticity of the central vestibular network that allows vestibular compensation (Kim et al, 1997). Therefore, this study was undertaken to investigate temporal changes of calcium-binding proteins, including calretinin, calbindin and parvalbumin, in the MVN during the early phase of vestibular compensation after ablation of unilateral peripheral vestibular receptors in rats.

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ABBREVIATIONS: MVN, medial vestibular nucleus; UL, unilateral labyrinthectomy; NMDA, N-methyl-D-aspartate.

METHODS

Animal preparation

Spargue-Dawley adult male rats, weighing 250~300 g, were used in this study. All the procedures were approved by the Institutional Ethical Committee on the Experimental Use of Animals. A surgical labyrinthectomy was performed, as previously described in detail (Kim et al, 1997). Briefly, a small opening was made around the oval window in the left ear under chloral hydrate anesthesia (300 mg/kg, i.p.). The membranous labyrinth was destroyed surgically through this opening using a small, right-angled hook, and aspiration with a suction pump. The UL was confirmed by the appearance of spontaneous nystagmus as well as the postural asymmetry after the recovery from anesthesia. The control animals underwent the same surgical procedure to expose the inner ear, but the membranous labyrinth was left intact. The control animals were sacrificed after surgery for immunofluorescence staining (n=6). The animals were sacrificed for immunofluorescence staining at 2 h (n=6), 6 h (n=6), 24 h (n=6), 48 h (n=6) and 72 h (n=6) after UL.

Immunofluorescence staining

The animals were anesthetized with chloral hydrate (100 mg/kg), perfused transcardially, fixed with 4% paraformaldehyde solution in a phosphate-buffered saline (PBS; pH 7.4) containing 0.05 M Na_2HPO_4 and 0.137 M NaCl, and decapitated. The brain was removed, post-fixed, rinsed with phosphate buffer solution, and immersed in 30% sucrose solution for 2 days. The sucrose-embedded brain was sectioned at 40 μm thickness using a cryostat. The non-specific binding sites were blocked with normal rabbit serum (1:50) for 30 min at room temperature. The primary antibodies to calretinin, calbindin and parvalbumin (Chemicon, Temecula, CA, USA) were applied overnight at 4°C. On the following day, the tissue sections were incubated with Alexa Fluor 594 goat anti-rabbit IgG (1:500; Molecular Probes, USA) for 1 h at room temperature for immunofluorescence. The sections were then washed and examined by fluorescence microscopy.

Data analysis

The areas occupied by calcium-binding proteins positive neurons, fibers and denser patches were measured using a digital image analysis system (Image-Pro plus, USA) for each section of the MVN. All data were presented as the ratio of the areas on the MVN in UL animals to those in control animals. Mean value of the areas on left MVN was used in control animals (Sans et al, 1995). All data were presented as mean \pm SE. The statistical significance of the differences was assessed using a Mann-Whitney U test (SPSS 11.5). p values < 0.05 were considered significant.

RESULTS

Calretinin

In the control animals without UL, calretinin expression was detectable near the 4th ventricle of the MVN, the parvocellular portion (MVNpc) at the rostral part of the MVN

and the magnocellular portion (MVNmc) at the caudal part of the MVN as a calretinin-immunoreactive cell. The expression was symmetrical in the bilateral sides. UL induced a significant increase in the level of calretinin expression in the bilateral MVN compared with control until 24 h after UL ($p < 0.01$ at 2, 6 h; $p < 0.05$ at 24 h). Moreover, there were significantly higher numbers of MVNpc, MVNmc, and calretinin-expressing neurons in the contralateral MVN to the lesion side than in the ipsilateral MVN 2 h after UL ($p < 0.05$). The expression of calretinin in the bilateral MVN peaked at 2 h after UL, and then decreased gradually with time. The pattern of asymmetric expression between the bilateral MVN disappeared 24 h after UL, and the level of calretinin expression returned to the control level by 48 h after UL (Fig. 1, 2).

Calbindin

The expression of calbindin was significantly weak com-

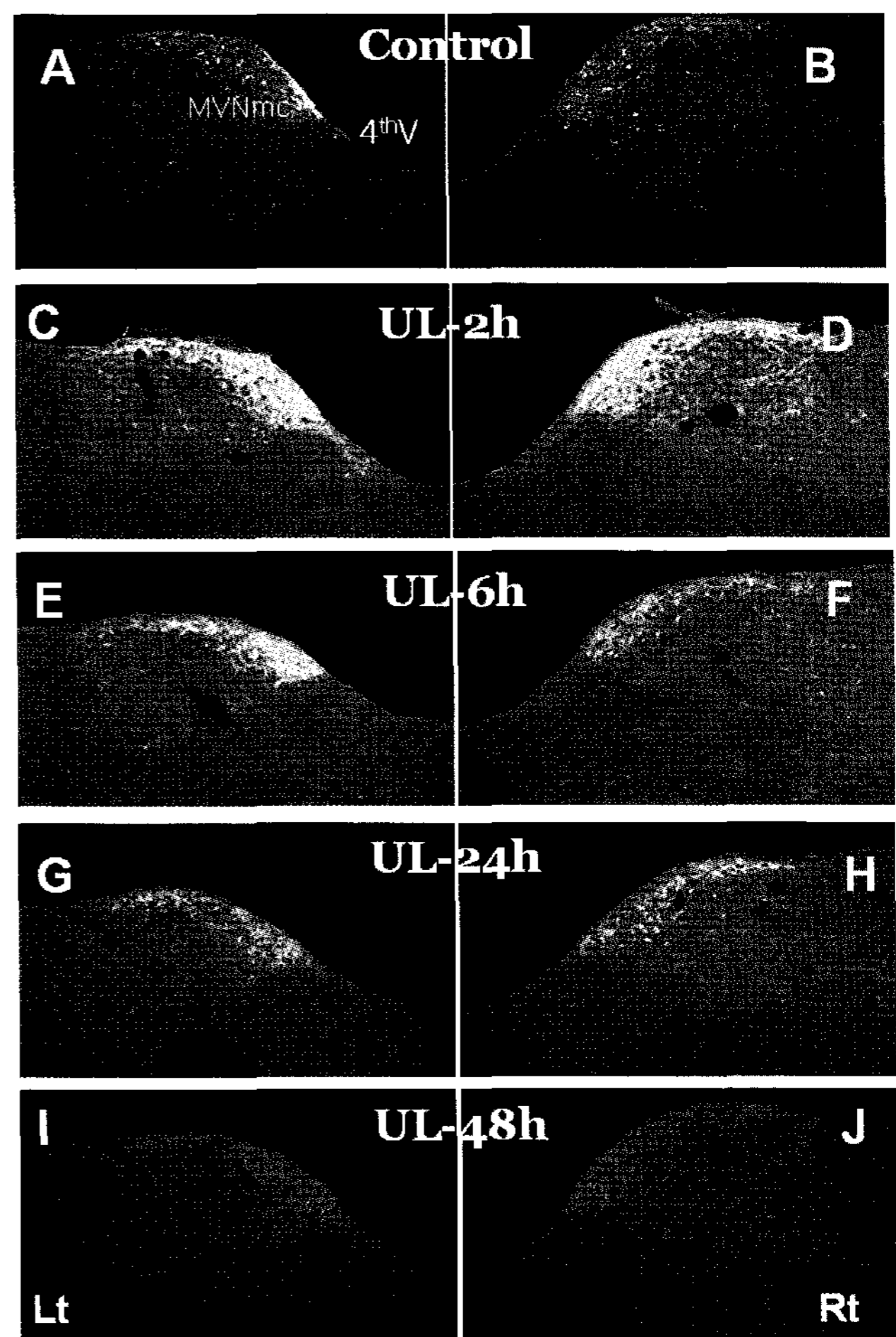


Fig. 1. Immunofluorescence findings of calretinin in the medial vestibular nucleus after unilateral labyrinthectomy (UL). A & B, left (Lt) & right (Rt) MVN, respectively, in control rat; C & D, ipsilateral (left) & contralateral (right) MVN to the lesion, respectively, 2 h after UL; E & F, ipsilateral & contralateral MVN, respectively, 6 h after UL; G & H, ipsilateral & contralateral MVN, respectively, 24 h after UL; I & J, ipsilateral & contralateral MVN, respectively, 48 h after UL; MVNmc, magnocellular portion MVN; 4thV, 4th ventricle.

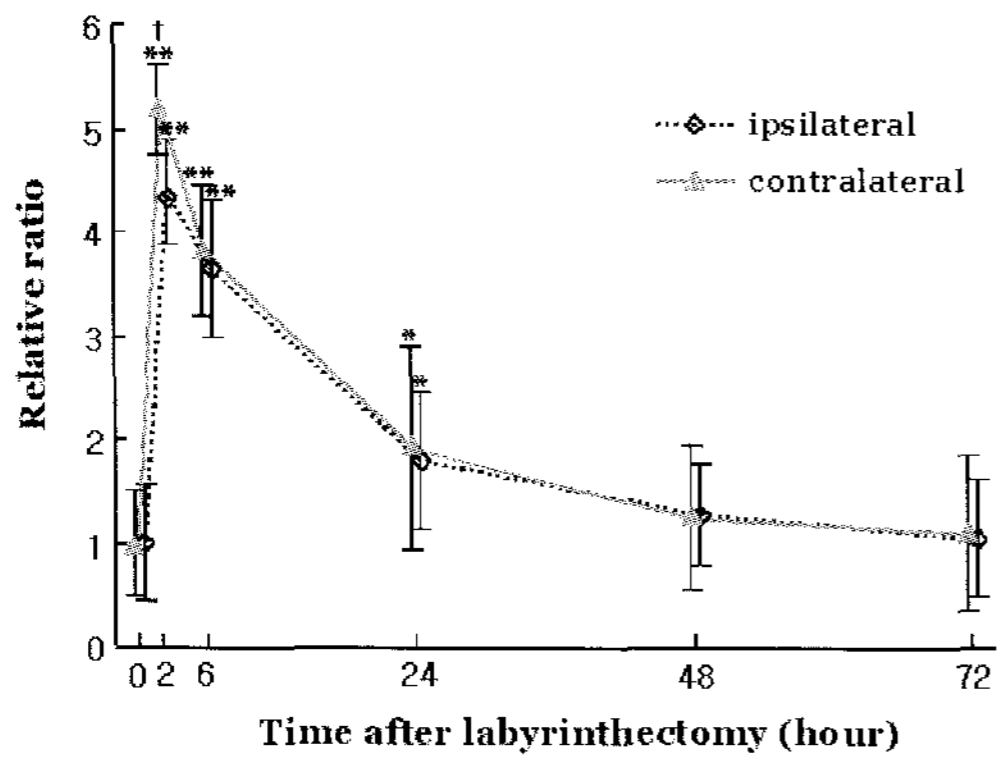


Fig. 2. Changes in the area of calretinin staining in the medial vestibular nucleus after UL. 0 hour represents control; ipsilateral, ipsilateral MVN to the lesion; contralateral, contralateral MVN to the lesion. Relative ratio was calculated from the ratio of the areas in UL animals to those in control animals. Values are mean±SE. * $p < 0.05$, ** $p < 0.01$, significantly different from control; † $p < 0.05$, significantly different from the opposite side.

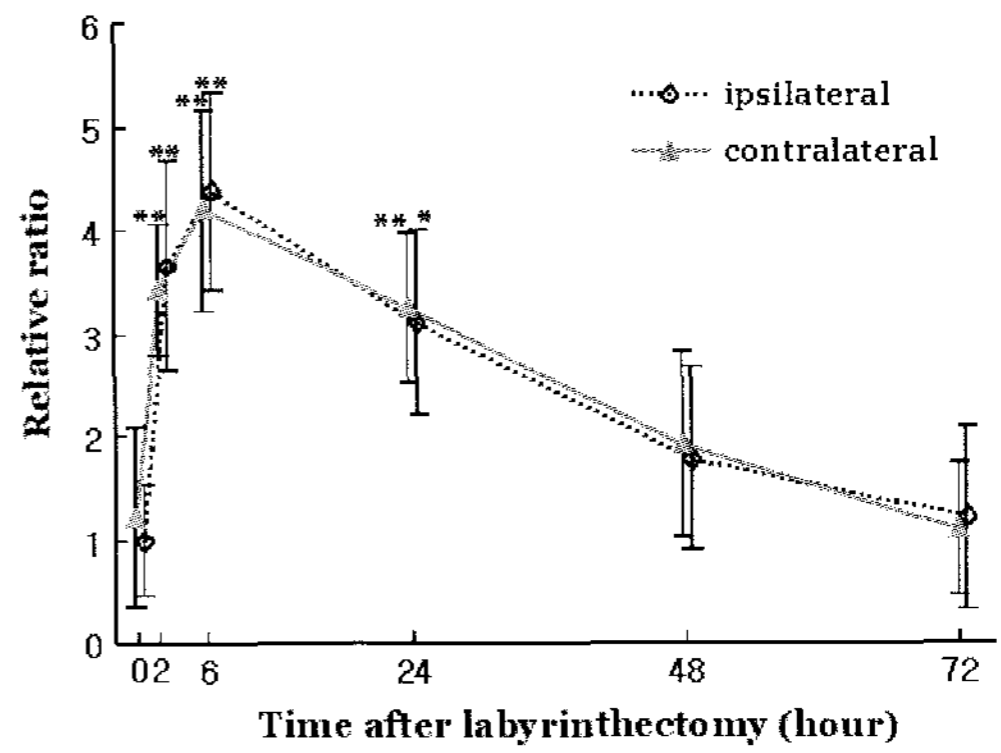


Fig. 4. Changes in the area of calbindin staining in the medial vestibular nucleus after UL. 0 hour represents control; ipsilateral, ipsilateral MVN to the lesion; contralateral, contralateral MVN to the lesion. Relative ratio was calculated from the ratio of the areas in UL animals to those in control animals. Values are mean±SE. * $p < 0.05$, ** $p < 0.01$, significantly different from control.

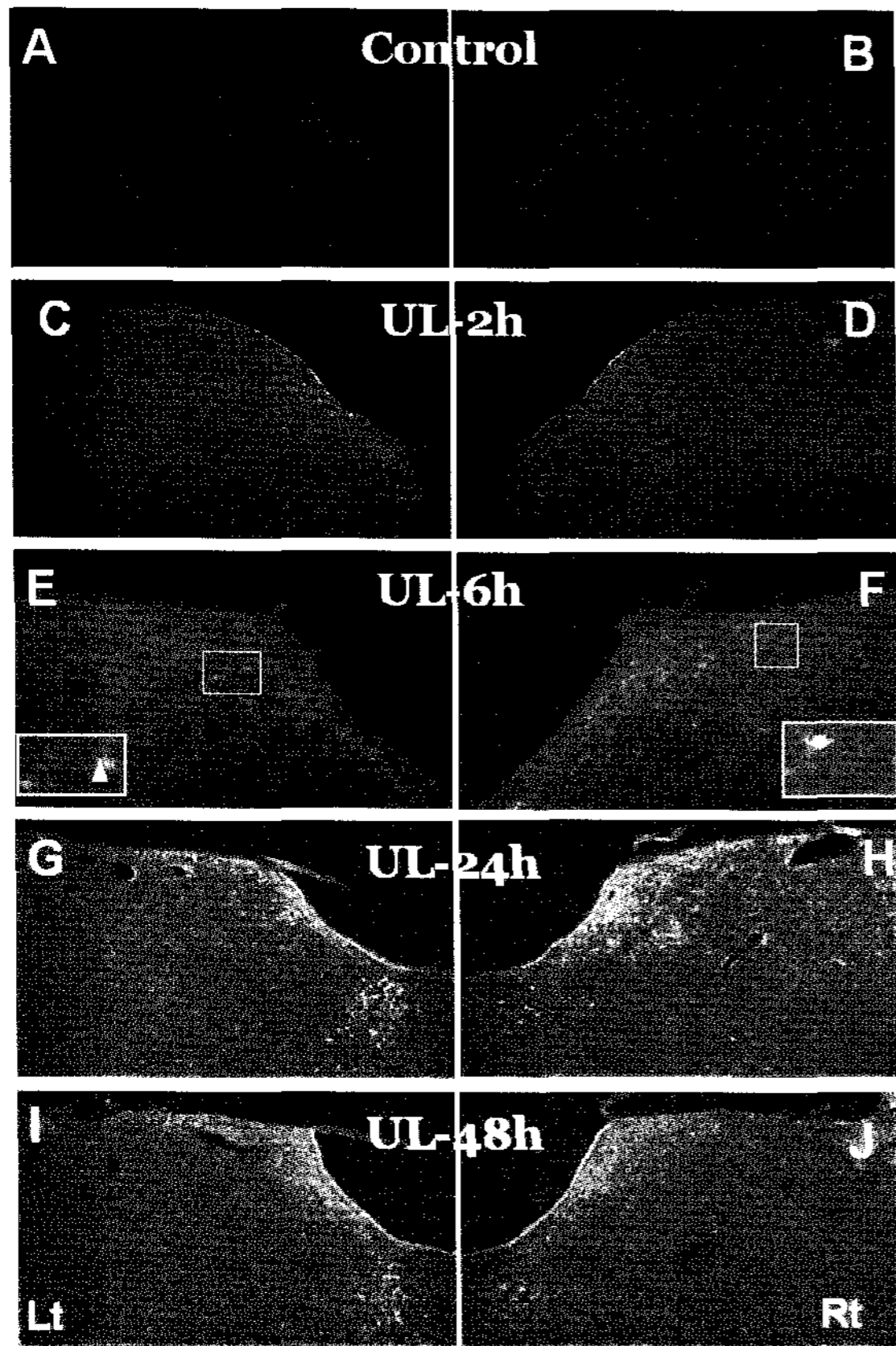


Fig. 3. Immunofluorescence findings of calbindin in the medial vestibular nucleus after UL. A & B, left (Lt) & right (Rt) MVN, respectively, in control rat; C & D, ipsilateral (left) & contralateral (right) MVN to the lesion, respectively, 2 h after UL; E & F, ipsilateral & contralateral MVN, respectively, 6 h after UL; G & H, ipsilateral & contralateral MVN, respectively, 24 h after UL; I & J, ipsilateral & contralateral MVN, respectively, 48 h after UL. In E & F, the large square in the lower panel is a magnification of the small square in the upper panel. Triangle indicates the nerve terminals and arrow indicates the nerve fibers.

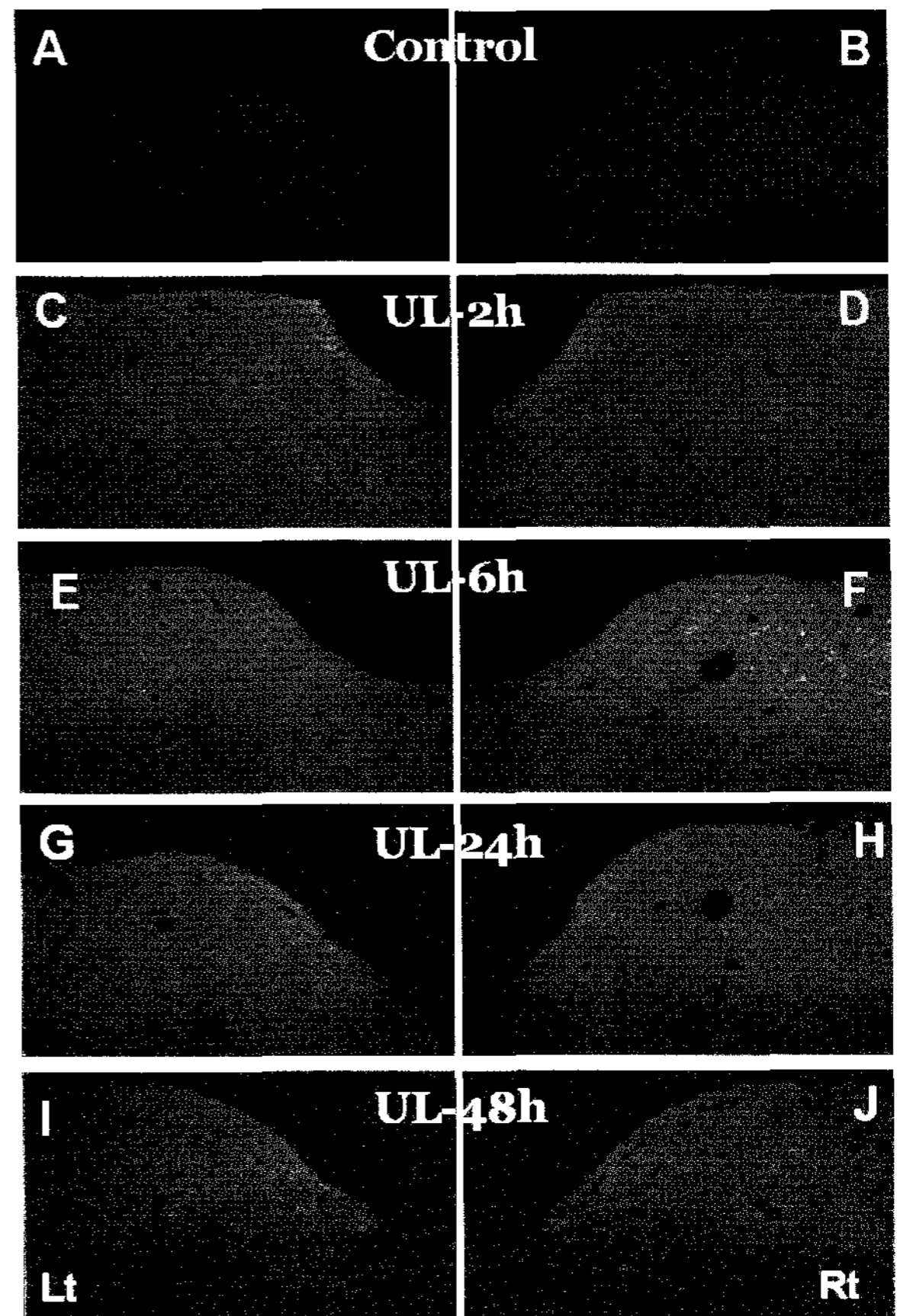


Fig. 5. Immunofluorescence findings of calbindin in the medial vestibular nucleus after UL. A & B, left (Lt) & right (Rt) MVN, respectively, in control rat; C & D, ipsilateral (left) & contralateral (right) MVN to the lesion, respectively, 2 h after UL; E & F, ipsilateral & contralateral MVN, respectively, 6 h after UL; G & H, ipsilateral & contralateral MVN, respectively, 24 h after UL; I & J, ipsilateral & contralateral MVN, respectively, 48 h after UL.

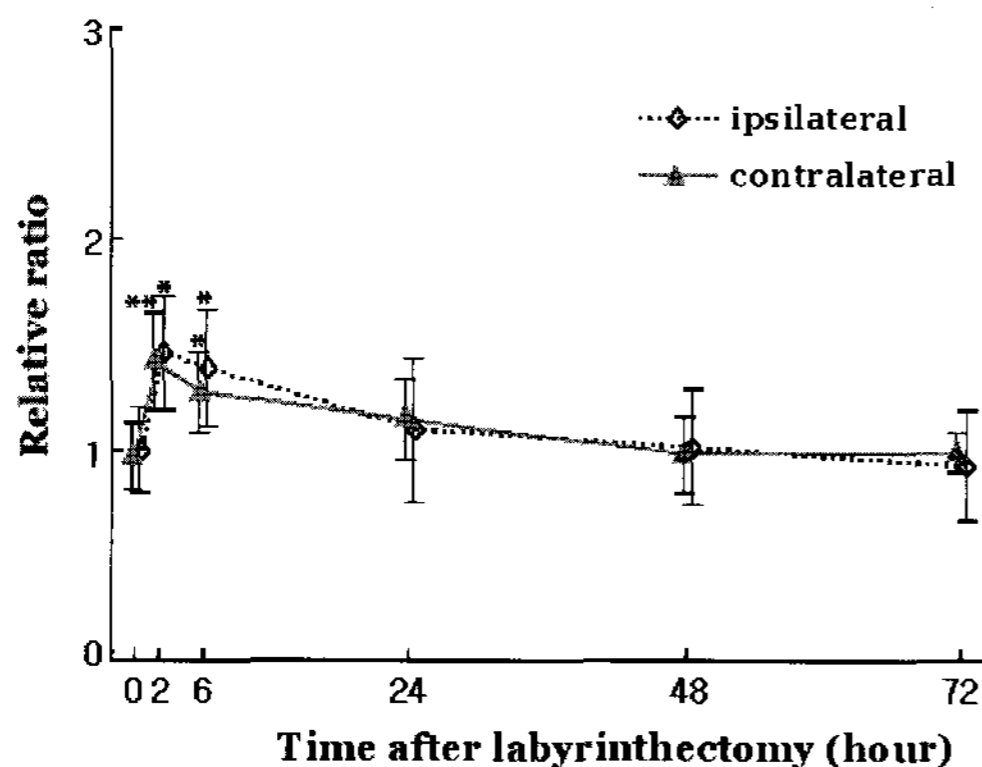


Fig. 6. Changes in the area of parvalbumin staining in the medial vestibular nucleus after UL. 0 hour represents control; ipsilateral, ipsilateral MVN to the lesion; contralateral, contralateral MVN to the lesion. Relative ratio was calculated from the ratio of the areas in UL animals to those in control animals. Values are mean \pm SE. * $p < 0.05$, ** $p < 0.01$, significantly different from control.

pared with that of calretinin and distributed mainly at the ventral part of the MVNmc in the control animals. There was more calbindin-immunoreactivity in the fibers and terminals than in the cell body throughout the MVN. Two hours after UL, the level of calbindin expression increased in the MVNpc, particularly along the border of the 4th ventricle. UL produced a significant increase in the level of calbindin expression in the bilateral MVN compared with control until 24 h after UL ($p < 0.01$ in bilateral MVN at 2, 6 h and in contralateral MVN at 24 h; $p < 0.05$ in ipsilateral MVN at 24 h). In contrast to calretinin, the expression of calbindin was slightly increased in the ipsilateral MVN to the lesion side compared to the contralateral MVN, however, there was no significant difference between the bilateral MVN. The expression of calbindin in the bilateral MVN peaked at 6 h after UL, and decreased gradually 24 h after UL. The expression decreased to the control level 48 h after UL (Fig. 3, 4).

Parvalbumin

Parvalbumin-immunoreactivity was observed mainly in the fibers and spots throughout the bilateral MVN, and a few cells were stained in the control animals. The expression of parvalbumin began to increase in the ventral side of the MVN, particularly in the fibers and spots rather than cells. The peak expression of parvalbumin was observed 2 h after UL, but the relative ratio of expression after UL was the lowest among the three calcium-binding proteins. UL produced a significant increase in the level of parvalbumin expression in the bilateral MVN, compared with control until 6 h after UL ($p < 0.01$ in contralateral MVN at 2 h; $p < 0.05$ in ipsilateral MVN at 2 h and bilateral MVN at 6 h). The expression level returned to the control level 24 h after UL (Fig. 5, 6).

DISCUSSION

Calcium-binding proteins are useful markers of specific cells and fiber populations in a variety of locations within the nervous system. For example, calretinin-immunor-

eactive neurons have been found in the retina (Jeon & Jeon, 1998), hippocampus (Murakawa & Kosaka, 1999) and cerebellum (Fortin et al, 1998). In the auditory system, the level of calbindin expression increased after a unilateral cochleotomy (Forster & Illing, 2000), and the level of parvalbumin expression increased after temporary sensory deprivation of hearing, however, there was a decrease in the level of calretinin expression (Caicedo et al, 1997). The physiological roles of the different calcium-binding proteins are unclear (Arai et al, 1991). In the vestibular system, there are a few reports on the calcium-binding proteins after unilateral deafferentation. Sans et al (1995) examined the expression of calretinin mRNA in the MVN of guinea pigs after UL, and revealed permanent asymmetry in the expression of calretinin between the bilateral MVN, which was not abolished after vestibular compensation. Their study on the early stage of vestibular compensation is different from the present study, because they examined the mRNA level only and did not quantify calcium-binding protein level in the bilateral MVN, which was the only difference observed between the ipsilateral and contralateral MVN in the present study. Furthermore, Arai et al (1991) reported that calcium-binding proteins were markers for the vestibular afferent input, however, they did not examine the levels at the critically important times when considerable changes in symptoms, molecules or other proteins had occurred during vestibular compensation.

The changes in diverse proteins such as extracellular signal-regulated kinase (ERK) and c-Fos occur mainly in the contralateral MVN to the lesion side during the early stages of vestibular compensation after UL (Kim et al, 2002, 2004). In this study, the level of calretinin expression in the bilateral MVN increased 2 h after UL, with more increase in the contralateral MVN than in the ipsilateral MVN. The increased level of calretinin expression in the ipsilateral MVN to the lesion side may be due to increased intracellular Ca^{2+} influx through the activation of glutamate receptors (Sansom et al, 2000) and disinhibition through Purkinje cells from the cerebellum (Kitahara et al, 1997). Furthermore, the higher level of calretinin in the contralateral MVN 2 h after UL can be explained by the increased neuronal activity in the contralateral MVN, caused by the decreased inhibitory input from the commissural connections, resulting from a loss of input to the ipsilateral MVN. The level of calretinin expression decreased in the bilateral MVN 6 h after UL and returned gradually to the control level with time. The pattern of the changes in calretinin expression was similar to the recovery process of the static vestibular symptoms and the expressions of the c-Fos protein and pERK during vestibular compensation in rats (Kim et al, 2002, 2004). Calretinin might play not only a complicated role in calcium regulation (Billing-Marczak & Kuanicki, 1999), but also in the response to the level of neuronal activity (Winsky & Jacobowitz, 1995): The up-regulation of calretinin by the neuronal activity may support the above notion.

Vestibular nuclear complex showed a heterogeneous distribution of calcium-binding proteins since the nucleus has a complex internal organization (Baizer & Baker, 2005). Histologically, the 8th nerve is one source of immunoreactive fibers in the vestibular nuclei for calbindin and parvalbumin, but not calretinin. A second major source is Purkinje cells in the cerebellum (Celio, 1990). These characteristic structures can contribute to the immunoreactive findings, because unlike calretinin, calbindin and parvalbu-

min can be expressed as a dense network of labeled fibers in the MVN mixed with labeled cells, which is difficult to precisely identify. Therefore, the area, but not the number of cells, was used as a parameter of the immunoreactivity of calcium-binding protein.

In this study, the increased immunoreactivity of both calbindin and parvalbumin might have resulted from the ablation of the peripheral vestibular receptors, causing a redistribution of the NMDA receptors of glutamate, which opens the calcium channels and increases the Ca^{2+} influx. Therefore, it is possible that the enhanced calcium-binding proteins perform a protective role against an increase in the Ca^{2+} concentration. Calbindin and parvalbumin were expressed at higher levels in the fibers and spots than in the cells, and the expression of calbindin and parvalbumin was similar in the ipsilateral and contralateral MVN. Although calbindin and parvalbumin are believed to act as homeostatic calcium buffers that increase in response to increased Ca^{2+} levels, they do not reflect the neuronal activity like calretinin.

The changes of immunoreactivity are known to result from the masking or unmasking of epitopes, the intracellular redistribution of protein, or variations in its expression and turnover. Although the mechanisms involved in these changes are unclear, the coincidence with the other alterations in morphology and physiology after deaf-ferentation indicates their functional significance (Lane et al, 1996). The levels of three calcium-binding proteins increased in the MVNpc near the 4th ventricle, suggesting that MVNpc plays a role in vestibular compensation.

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