Prolonged Expression of c-Fos Protein in the Lateral Habenular Nucleus of the Japanese Monkey (*Macaca fuscata*) after Eye Enucleation

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Key Words

Eye enucleation · Lateral habenular nucleus · Japanese monkey · Immunocytochemistry · c-Fos

Abstract

To elucidate the effect of traumatic stress on the lateral habenular nucleus, we investigated the time course of the expression of c-Fos protein in this nucleus of the Japanese monkey (*Macaca fuscata*) after enucleation of one eye using c-Fos protein immunocytochemistry. c-Fos protein-like immunoreactive neurons were significantly increased; the increase started 1 h after the enucleation and remained high for 3–9 h in the lateral habenular nucleus on both sides. These results suggest that the prolonged expression of c-Fos protein occurred in the lateral habenular nucleus after traumatic stress through multiple transsynaptic activations.

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Introduction

Presently, it is strongly believed that the lateral habenular nucleus is involved in a variety of important biological functions and behaviors, e.g., stress processing [1], sleep disorder [2], pain [3] and reproductive behaviors [4]. These diverse biological functions of the lateral habenular nucleus were suggested to be due to its connections with the dopaminergic, serotonergic and cholinergic

systems [5–7]. Furthermore, Murray et al. [8] reported that this nucleus acts to significantly modulate the levels of the circulating hormone in relation to stress response.

Fos protein is a nuclear phosphoprotein encoded by the *c-fos* gene and visualization of c-Fos protein immunoreactivity has been used as an indicator of neuronal activation following exposure to a given stimulus [9]. This technique has been widely used to identify the central pathways that may play a role in the regulation of the stress system [10]. Furthermore, c-Fos protein-like immunoreactivity was successfully used to study the function of the lateral habenular nucleus in response to various stressors [11]. In addition, direct retinal input to this nucleus detected in rats [12, 13] was not reported in primates, including the Japanese monkey [our unpubl. results]. Moreover, compared with other species, e.g., the rat and the hamster, until now very little has been done to elucidate the function of this nucleus in response to stress in primates. Therefore, in the present study, we focused on the lateral habenular nucleus and investigated the effects of enucleation of one eye on c-Fos protein expression in this nucleus in the Japanese monkey in an attempt to clarify the role of the lateral habenular nucleus in response to traumatic stress in primates.

Materials and Methods

We used 14 adult (5 male, 9 female) monkeys (*M. fuscata*), weighing 4.4–10.4 kg. The monkeys were anesthetized with sodium pentobarbital (30 mg/kg i.p.). We enucleated one eye of 10 monkeys under

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Fig. 1. Photomicrographs showing the expression of c-Fos protein in the lateral habenular nucleus of the Japanese monkey (*M. fuscata*). Compared with an intact monkey (**A**), a significantly increased number of c-Fos-positive neurons is observed 1 h after enucleation of the right eye (**B**). Nuclei immunoreactive for c-Fos protein appear as black staining. MH = Medial habenular nucleus; LH = lateral habenular nucleus; HI = habenulo-interpeduncular tract; MD = medial dorsal nucleus. Scale bar, 200 μ m.

deep anesthesia using standard surgical procedures. These monkeys were killed at various times (1 h: $n = 2$, 3 h: $n = 2$, 6 h: $n = 2$, 9 h: $n = 2$, 27 h: $n = 2$) after the enucleation and were kept under deep anesthesia until sacrifice to minimize pain perception. Monkeys of the intact group ($n = 4$) were also kept under deep anesthesia until 1, 3, 6, and 9 h prior to sacrifice. The deeply anesthetized monkeys were perfused transcardially with heparinized physiological saline, followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4, containing 0.2% picric acid for 20 min. The brains were removed and immersed in 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4, for 7 days at 4°C. The brains were cryoprotected in 25% sucrose phosphate-buffered saline (PBS). Serial 40- μ m-thick sections were cut with a freezing microtome and immersed in PBS. The immunocytochemical detection of c-Fos protein was performed using the peroxidase-antiperoxidase (PAP) detection protocol [14]. To insure penetration of antibodies, the sections were preincubated in a solution containing 0.3% Triton X-100 in PBS after blocking endogenous peroxidase. Free-floating sections were then incubated for 3 days at 4°C with rabbit antibody to c-Fos protein (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) diluted 1:4,000 in PBS containing 0.3% Triton X-100. The sections were incubated in a 1:2,000 dilution of goat anti-rabbit IgG (E-Y Laboratories Inc., San Mateo, Calif.) for 1 day at 4°C, followed by the tertiary antibody, PAP in a 1:2,000 dilution and incubated for 1 day at 4°C. The primary, secondary and tertiary antibodies were diluted to appropriate concentrations in 0.3% bovine serum albumin and 1% normal goat serum and 0.05% sodium azide. Between the incubation steps, sections were thoroughly washed with PBS. The diaminobenzidine (DAB) method for visualization of peroxidase was used. The sections were treated for 20 min at room temperature with 0.02% DAB in 0.003% hydrogen peroxide. After the final rinses in 0.05 *M* Tris-HCl buffer, pH 7.6, the sections were mounted onto gelatin-chrome-alum-coated glass slides, air-dried, dehydrated with ethanol, cleared in xylene, and coverslipped.

The specificity of the immune reaction was assessed by omitting the primary antibody in which no specific immunostaining was detected.

All experimental procedures were carried out in accordance with the Guide for Animal Experimentation of the Faculty of Medicine of Kagoshima University.

The sections were analyzed under a microscope and the labeled c-Fos-positive neurons per section were counted. Counts were obtained from a 1 in 3 series of sections and 6 sections per animal were used. Then, the mean number of c-Fos-positive neurons ($\pm$ SD) for

each time point was calculated. The statistical analysis was performed using a paired *t* test and differences at $p < 0.001$ were considered significant.

Results

After the enucleation of one eye, in addition to the supraoptic nucleus, as observed in our previous report [14], a large number of c-Fos protein-like immunoreactive neurons were found in the lateral habenular nucleus, spinal trigeminal nucleus and midbrain periaqueductal gray matter, but the time course of c-Fos protein expression in the lateral habenular nucleus was different from those of other areas.

As shown in figure 1A, almost no c-Fos-like immunoreactive nerve cell bodies were observed in the lateral habenular nucleus of intact Japanese monkeys. The semi-quantitative analysis of the number of c-Fos-positive nerve somata per section was 1.3 ± 1.5 on the right side and 1.3 ± 1.5 on the left side of the lateral habenular nuclei in intact Japanese monkeys (fig. 2). Higher numbers of densely labeled c-Fos-positive neurons were, however, observed bilaterally in this nucleus and started to increase at 1 h after enucleation of the right eye, compared with intact monkeys ($p < 0.001$); the number of c-Fos-immunoreactive cell bodies was 225 ± 11 per section on the ipsilateral side and 266 ± 6.6 per section on the contralateral side (fig. 1B, 2). This result is in good agreement with the observation that noxious stimulation induces increases in the number of c-Fos-positive neurons in the bilateral habenular nucleus of the cat [15].

As shown in figure 2, this increase in the number of the c-Fos-like immunoreactive cells remained almost as high as at 3 h (253 ± 13 per section on the ipsilateral side and 261 ± 18 per section on the contralateral side), and 6 h (250 ± 19 per section on the ipsilateral side and 256 ± 19

Fig. 2. Time course of semi-quantitative analysis of c-Fos-positive neurons in the lateral habenular nuclei of Japanese monkeys after right eye enucleation. On both sides of the lateral habenular nuclei, the number of c-Fos protein-like immunopositive neurons starts to increase significantly after 1 h compared with intact monkeys and remains high over a longer period from 3 to 9 h after enucleation ($p < 0.001$).

per section on the contralateral side) after enucleation. However, the number of c-Fos-positive immunoreactive neurons started to decline at 9 h after enucleation, whereas this expression level was still significantly increased compared with intact monkeys (206 ± 17 per section on the ipsilateral side and 212 ± 12 per section on the contralateral side). The count of c-Fos protein-like immunoreactive cells reached the level in intact monkeys at 27 h after enucleation of the right eye (0.5 ± 0.6 per section on the ipsilateral side and 0.75 ± 1 per section on the contralateral side).

Figure 3 shows the semi-quantitative analysis of the number of c-Fos protein-like immunoreactive nerve cell bodies in the spinal trigeminal nucleus after enucleation of the right eye of Japanese monkeys. Compared with intact monkeys, the number of the c-Fos-positive neurons significantly increased and peaked at 1 h on the ipsilateral side after enucleation of one eye (14.2 ± 1.6 per section in the intact monkey and 23.4 ± 1.1 per section in the enucleated monkey; $p < 0.001$). This peaked c-Fos expression, however, decreased to the level of the intact monkey at 3 h (12.2 ± 0.8 per section in the ipsilateral side) after enucleation of one eye.

In the midbrain periaqueductal gray matter, c-Fos protein expression also peaked at 1 h after right eye enucleation compared with intact monkeys (29 ± 2 per section in the intact and 112 ± 2 per section in the enucleated monkey; $p < 0.001$). The count of these c-Fos immunoreactive

Fig. 3. The time course of semi-quantitative analysis of c-Fos-positive neurons in the spinal trigeminal nuclei of Japanese monkeys after right eye enucleation. The number of c-Fos protein-like immunopositive neurons peaks after 1 h compared with intact monkeys ($p < 0.001$) and returns to the level of the intact monkeys 3 h after enucleation.

cells reached the level of intact monkeys at 3 h (3 ± 0.7 per section) after enucleation of one eye. These results suggested the effect of the eye enucleation on these areas is mediated by the ophthalmic nerve. These findings are consistent with the previously published reports that c-Fos protein expression was induced in the spinal trigeminal nucleus and midbrain periaqueductal gray after noxious or traumatic stress [16–19].

Discussion

In addition to the lateral habenular nucleus, we demonstrated that c-Fos like immunoreactive neurons were significantly increased as well in the spinal trigeminal nucleus and midbrain periaqueductal gray matter in enucleated monkey 1 h after enucleation compared with intact monkeys; these structures receive a direct sensory input from the eye which is believed to be involved in the process of antinociception. Furthermore, in our previous study we observed significantly increased numbers of c-Fos-positive vasopressinergic neurons in the supraoptic nucleus 1 h after eye enucleation, and as it is known, the supraoptic nucleus plays a key role in stress response [14]. However, the time course of c-Fos protein expression in the lateral habenular nucleus was different from those of other areas. Interestingly, in the lateral habenular nucleus, c-Fos protein expression remained high over a longer

period until 9 h postinjury on both sides, although c-Fos protein expression is generally believed to reach a peak 1–2 h after the onset of stimulation and then to quickly decrease to the basal level [20]. In fact, prolonged induction of c-Fos has been reported in unusual conditions [21, 22]. Previous studies [1] observed that chronic stimulants of different types, e.g., exposure of rats to longer periods of immobilization and continuous administration of dopamine agonists or cocaine resulted in the labeling of greater numbers of c-Fos protein-like immunoreactive neurons in the lateral habenular nucleus. Furthermore, several lines of evidence suggest that the lateral habenular nucleus is the site of functional interactions between the different parts of the central nervous system [5–7, 23]. Therefore, the long-term effect in c-Fos expression detected in the present study suggests that traumatic stress causes prolonged activation of this nucleus through multiple synapses [24] and also that the lateral habenular

nucleus may act as a site of key neuronal circuitry in the process of long-term stress.

It is worth mentioning that c-Fos couples with another nuclear protein, Jun, to form a heterodimeric complex which binds to the AP-1 DNA site, and then regulates the downstream expression of other target genes [25].

Overall, it is suggested that further studies are needed to elucidate the nature of this prolonged c-Fos protein expression observed in the present study in an attempt to reveal the underlying mechanisms of traumatic stress-induced transsynaptic hyperactivation in addition to clarifying the function of the lateral habenular nucleus.

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