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Impaired mastication reduced newly generated neurons at the accessory olfactory bulb and pheromonal responses in mice

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Abstract

Objectives: A large number of neurons are generated at the subventricular zone (SVZ) even during adulthood. In a previous study, we have shown that a reduced mastication impairs both neurogenesis in the SVZ and olfactory functions. Pheromonal signals, which are received by the vomeronasal organ, provide information about reproductive and social states. Vomeronasal sensory neurons project to the accessory olfactory bulb (AOB) located on the dorso-caudal surface of the main olfactory bulb. Newly generated neurons at the SVZ migrate to the AOB and differentiate into granule cells and periglomerular cells. This study aimed to explore the effects of changes in mastication on newly generated neurons and pheromonal responses.

Design: Bromodeoxyuridine-immunoreactive (BrdU-ir; a marker of DNA synthesis) and Fos-ir (a marker of neurons excited) structures in sagittal sections of the AOB after exposure to urinary odors were compared between the mice fed soft and hard diets.

Results: The density of BrdU-ir cells in the AOB in the soft-diet-fed mice after 1 month was essentially similar to that of the hard-diet-fed mice, while that was lower in the soft-diet-fed mice for 3 or 6 months than in the hard-diet-fed mice. The density of Fos-ir cells in the soft-diet-fed mice after 2 months was essentially similar to that in the hard-diet-fed mice, while that was lower in the soft-diet-fed mice for 4 months than in the hard-diet-fed mice.

Conclusions: The present results suggest that impaired mastication reduces newly generated neurons at the AOB, which in turn impairs olfactory function at the AOB.

1. Introduction

In human, a causal relationship between mastication and brain function has been observed.[1] Cognitively normal elderly females have more teeth and stronger bite force than cognitively impaired elderly females. Chewing ability in elder persons correlates with cognitive impairment.[2] The Nun study, a longitudinal study of aging and Alzheimer's disease, indicated that participants with the fewest teeth had the highest prevalence and risk of incidence of dementia.[3] These results suggest the significance of mastication on brain function in humans.[4] In rats, extraction of all molars or shortening of the upper molars impairs spatial memory,[5,6] suggesting that mastication also correlates with brain function in experimental animals. Ingestion of a hard diet induced a remarkable excitation in neurons at the principal sensory trigeminal nucleus (Pr5), which receive oral somatosensory information via the trigeminal nerves, but ingestion of a soft diet did not, suggesting that mastication is also impaired by offering animals only a soft diet.[7] In fact, performance on tests of working memory and space memory were lower in soft-diet-fed mice than in hard-diet-fed mice.[5,8] The number of bromodeoxyuridine-immunoreactive (BrdU-ir) cells in the hippocampus of mice fed a soft diet after weaning decrease at the age of 3 months.[9,10] In rats, feeding with a soft diet after weaning for 24 weeks reduces BrdU-ir cells in the hippocampus.[11] One explanation for these results may be that reduced sensory input influences neurogenesis at the hippocampus.[1]

Vomeronasal sensory neurons, which receive pheromones providing specific information concerning the reproductive state, and social status in a variety of mammals,[12-14] project to the accessory olfactory bulb (AOB) located on the dorso-caudal surface of the main olfactory bulb (MOB).[15] Thus, the AOB is an important site, not only for the discrimination of pheromones but also for memories concerning the reproduction.[16] Neurogenesis occurs in the forebrain subventricular zone (SVZ) as well as in the hippocampus throughout life.

Neurons generated in the SVZ migrate via the rostral migratory stream (RMS) to the MOB[17] and AOB.[18] Neurogenesis has been shown to be required for anti-predator[19] and sex-specific responses related to olfaction.[20]

The soft diet feeding reduced neurogenesis at the SVZ, decreased the number of newly generated neurons at the MOB, and impaired olfactory functions in mice.[7] In the present study, BrdU-ir structures in sagittal sections of the AOB of female mice fed a soft or hard diet were studied to explore the effects of changes in mastication on newly generated neurons. We then explored the effects of the soft-diet feeding on pheromonal responses to male urine at the AOB.

2. Materials and methods

All experiments were carried out in accordance with the Guidelines for the Use of Laboratory Animals of the Asahikawa Medical University and approved by the Committee of Asahikawa Medical University for Laboratory Animal Care and Use (approval ID: 11014).

2.1. Animals

A total of 38 C57BL/6 female mice (from 24 to 28 weeks old) were used. Five mice were fed 25 g of hard or soft diet per week in the same cage. The mice and the hard and soft diets were obtained from Sankyo Laboratory Co. (Sapporo, Japan); both diets had the same nutritional composition. The relative body weights of the mice fed the soft diet were essentially the same as those of the mice fed the hard diet (data not shown).

2.2. Stimulation with urine

Pheromones in the urine excreted from males induce various changes in gonadal function and endocrine state in females.[21] In the present study, we applied urine excreted from male mice

to female mice to explore effects of decreases in mastication on neural responses at the AOB. Urine was collected from 10 males using a metabolic cage. Five milliliters of a urine mixture taken from 10 males was sprayed on the soiled bed made of paper (SLC, Hamamatsu, Japan) in the cage. The animals were deeply anesthetized with pentobarbital sodium (35 mg/kg) 90 min after exposure to the stimulus.

2.3. Tissue processing and BrdU and Fos immunohistochemistry

The mice were injected intraperitoneally with BrdU (50 mg/g, a marker of DNA synthesis; Sigma, St. Louis, MO) during each of the 3 days from 1 week before killing. After deep anesthetization with pentobarbital sodium (35 mg/kg), the BrdU-injected mice were exsanguinated by perfusion through the heart with phosphate-buffered saline (PBS, pH 7.3), then fixed with 4% paraformaldehyde. The brain was removed and cut sagitally on a vibratome at a thickness of 100 μm. The sagittal sections were identified with a stereotaxic atlas of the mouse brain.[22] Four sections from Figure 108 of the mouse atlas (lateral 0.84 mm) to lateral were used for BrdU or Fos immunostaining. For the detection of BrdU-labeled nuclei, sections were first incubated in 2N HCl for 30 min at 37°C and rinsed in 0.1 M boric acid (pH 8.0) for 5 min, followed by washing in PBS with 0.4% Triton X-100 (PBSx). The sections were then incubated in PBSx with 0.6% H_2O_2 for 15 min, followed by washing in PBSx. After 1 h of incubation in 3% normal goat serum, the sections were incubated with mouse anti-BrdU monoclonal antibody (1:400; Roche Diagnostics, Mannheim, Germany) for 24 h at room temperature. The sections then were rinsed in PBSx and incubated with biotinylated goat anti-mouse IgG (1:100; Vector Laboratories, Burlingame, CA) for 1 h. The sections were rinsed again in PBSx, incubated with ABC (ABC Elite kit; Vector Laboratories) for 1 h, and developed with DAB/H_2O_2 (0.05% DAB and 0.003% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6) for 5 min.

For the detection of c-Fos immunoreactivity, the sections were first treated with 0.6% H_2O_2 for 15 min in PBSx, followed by two washes with PBSx. After 1 h incubation in 3% normal goat serum, the sections were incubated overnight at room temperature with rabbit anti-c-Fos polyclonal antibody (1:8000, Ab-5; Calbiochem, La Jolla, CA) in PBSx. All sections treated in this manner were rinsed with PBSx and incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) for 1 h. The sections were rinsed again in PBSx, incubated with ABC for 1 h, and developed with DAB/H_2O_2 for 12 min.

The sections were rinsed with water, mounted, and dehydrated before being covered with cover slips. The thickness of dehydrated slices was about 20 μm.[7] All BrdU-ir cells or Fos-immunoreactive (Fos-ir) cells in dehydrated sections were photographed in the same focal plane. The sections were photographed by a CCD camera (DP72; Olympus, Tokyo, Japan) attached to an inverted microscope (BX51; Olympus) to count the BrdU-ir or Fos-ir cells by the naked eye.

2.4. Statistical analysis

The numbers of BrdU-ir cells and numbers of Fos-ir cells were compared by .analysis of variance (ANOVA) with Fisher's PLSD post-hoc testing. Statistical analyses were performed with StatView version 5.0 (SAS Institute, Cary, NC). Data are expressed as the mean ± SEM.

3. Results

3.1. Soft-diet feeding reduced newly generated neurons at the AOB

Neurogenesis occurs robustly in the hippocampus and SVZ even in adulthood.[23,24] In the previous study, we showed that the soft-diet feeding led to a decrease in neurogenesis activity at the SVZ.[7] Newly generated cells at the SVZ migrate to the MOB and AOB via the

RMS.[17] The previous study also showed that the numbers of BrdU-ir cells at the MOB of mice fed a soft diet were lower than those of mice fed a hard diet.[7] The olfactory information emitted from general odorants is transmitted to the MOB, while the pheromonal information is transmitted to the AOB. In the present study, BrdU-ir structures of mice fed a soft or hard diet were studied to explore the effects of changes in mastication on newly generated neurons in the AOB. Fig. 1 shows BrdU-ir cells at the AOB of mice fed a hard or soft diet for 1or 3 months. The numbers of BrdU-ir cells at the AOB of mice fed a soft diet were similar to those of mice fed a hard diet for 1 month (Fig. 1A and B). However, feeding of a soft diet for 3 months decreased BrdU-ir cells at the AOB (Fig. 1D; compare to Fig. 1C). In rodents, the rostral and caudal halves of the AOB receive different information from vomeronasal sensory neurons.[25,26] The data from each group were cast into a three-factor ANOVA as follows: hard or soft diet, periods of feeding and regions (rostral and caudal). This analysis revealed a main effect of hard diet versus soft diet ($F(1, 43) = 10.017$, $p < 0.005$). Fisher's PLSD post-hoc testing indicated that the number of BrdU-ir cells in mice fed the soft diets was less than that of mice fed the hard diets ($p < 0.01$).

BrdU-ir cells at the SVZ and MOB of mice fed the soft diet for 1 month were lower than those of mice fed the hard diet.[7] However, at the AOB, the soft diet feeding for 1 month did not reduce BrdU-ir cell (Fig. 2A and B). The number of BrdU-ir cell at the rostral half of the AOB of mice fed soft diet for 3 or 6 months was lower than that of mice fed hard diet, but not significant. At the caudal half of AOB, the number of BrdU-ir cells in mice fed soft diet for 3 or 6 months was lower than that of mice fed hard diet ($p < 0.05$).

3.2. Soft-diet feeding impaired pheromonal responses to male urine at the female AOB

Pheromonal information transmitted via mitral/tufted cells (MT) is modified by GABAergic interneurons, which soma exit in the periglomerular cell (PG) layer or in granule cells (GC)

layer.[12-14] Next, we examined Fos-ir structures at the AOB of hard or soft diet fed mice after exposure to male urinary pheromones. Exposure to urinary odor induced Fos-immunoreactivity, which is correlated with cellular activity, in various cells of the AOB of mice fed the hard diet for 2 or 4 months or soft diet for 2 months (Fig. 3A, B and C). In contrast, Fos-immunoreactivity at the AOB of mice fed the soft diet for 4 months was low (Fig. 3D). Fig. 4 shows the number of Fos-ir cells at the PG, MT and GC layers of the rostral and caudal regions of the AOB after exposure to urinary odor. The data from each group were cast into a four-factor ANOVA as follows: hard or soft diets, periods of feeding, regions (rostral and caudal) and layers (PG, MT and GC). Four-factor ANOVA revealed the main effect of diet ($F(1, 96) = 63.752$, $p < 0.0001$). Fisher's PLSD post-hoc testing indicated that mice fed the hard diet had the larger number of Fos-ir cells than the mice fed the soft diet ($p < 0.0001$). Four-factor ANOVA also revealed the interaction between diets and periods ($F(1, 96) = 15.545$, $p < 0.0005$).

Soft diet feeding for 2 months slightly decreased Fos-ir cells at the AOB after exposure to urinary pheromones, but not significant. At the MT and GC layers of the rostral and caudal halves of the AOB, the numbers of Fos-ir cells of mice fed soft diet for 4 months were lower than those of mice fed hard diet, indicating that soft diet feeding impaired pheromonal responses transmitted to the rostral and caudal halves of the AOB.

4. Discussion

The numbers of BrdU-ir cells in the AOB of adult female mice fed the soft diet for 1 month were similar to those of the mice fed the hard diet. The soft diet also did not affect the expression of Fos-ir cell at the AOB of mice fed the soft diet for 2 months after exposure to male urine. However, mice fed the soft diet for 3 months showed lower expression of BrdU-ir cells in the AOB. The responses to urinary odor at the AOB of mice fed soft diet for 4

months were lower than those of mice fed hard diet.

Reduction of neurogenesis by impaired mastication has been observed at the hippocampus in rodents.[9,10] The effects of decreases in mastication by the soft-diet feeding or by shortening of the upper molars have been studied in order to explore the effects of impaired mastication on the brain functions in mice. Feeding of soft diet after weaning decrease the level of synaptophysin in the whole cortex,[8] those of brain-derived neurotrophic factor (BDNF), and the number of BrdU-ir cells in the hippocampus.[9-11] The abilities of the working memory tested with an eight-arm maze[8] and space memory tested with a water maze[27] are reduced in mice fed a soft diet after weaning. Rats that undergo extraction of the molar teeth show an impairment of space memory at 24 weeks of age.[5] The shortening of the upper molars of elderly mice reduces neurogenesis in the dentate gyrus (DG) of hippocampus, and spatial learning ability.[6,28] In the separate study, we showed that soft-diet feeding reduced neurogenesis not only at the hippocampus but also at the SVZ of mice.[7]

Adult neurogenesis controls behaviors under physiological changes[24] such as pregnancy and aging.[18,29,30,31] The neurogenesis activities at the SVZ is lower in aged mice than young ones.[30] Aging in humans and mice impairs various olfactory functions such as smell identification of general odors, olfactory discrimination learning, fine olfactory discrimination, and sensitivity to general odors.[31,32] In the separate study, we showed that mice fed a soft diet showed low neurogenesis and did not avoid the odor of 50% butyric acid, while mice which fed only a hard diet or a hard diet after a soft one, showed normal or recovered neurogenesis and avoided the odor, respectively.[7] These results suggest that the decrease in adult neurogenesis induced by a soft diet impaired the ability of odor cognition for avoidance via the main olfactory system.

BrdU-ir cells, which correspond to the neuronal precursors originating from the SVZ,[33,34] were also found in the AOB, but their density was low (about 9% of those at the GC

in the MOB).[18,35] At the rat MOB, the density of BrdU-ir cells in the region close to the rostral end of the AOB is higher than that distant from the AOB.[18] Sexually experienced male rats have been shown to prefer oestrous to dioestrous female urine, while sexually inexperienced males do not exhibit these preferences.[36,37] Sexual experience in males enhances the transmission of reproductively salient information concerning potential estrous status to the localized region (lateral and rostral sections) of the PG layer of the AOB.[20] It is possible that newly generated cells in the AOB concern with changes in transmission of pheromonal information at the PG layer. Soft diet feeding decreased newly generated cells at the AOB and impaired olfactory responses to urinary pheromones. Therefore, it is possible that the soft-diet feeding impairs pheromone-related sexual and/or social behaviors in rodents.

At this time, it is not certain that a change in masticatory ability affects neurogenesis at the SVZ or the pheromone related olfactory functions. Mechanical stimulation of the tooth induced excitation of periodontal mechanosensitive neurons in the trigeminal sensory complex at the brain stem in cats and rats.[38,39] In the separate study, we showed that ingestion of a hard diet induced excitation of neurons at the principal sensory trigeminal nucleus (Pr5) in the brain stem.[7] Mechanosensory information from the oral receptors, then, is transmitted to the pedunculopontine tegmental nucleus (PTg) via the thalamus,[39] somatosensory cortex,[40] and motor cortex.[41] Neurons at the substantia nigra pars compacta (SNc), where cholinergic and gulutamatergic neurons of the PTg innervate,[42,43] were activated by ingestion of a hard diet.[7] Proliferative precursors in the SVZ express dopamine receptors and receive dopaminergic afferents from the SNc.[44,45] Dopamine increases the proliferation of precursor cells at the SVZ by releasing epidermal growth factor in vitro.[45] Ingestion of a hard diet induced remarkable excitation of neurons at the PTg and SNc.[7] Therefore, it is possible that the feeding with a hard diet maintained neurogenesis at the SVZ via the Pr5, PTg

and SNc and olfactory functions at the AOB.

5. Conclusions

The density of BrdU-ir cells and Fos-ir cells in the AOB after exposure to odors were lower in the soft-diet-fed mice than in the hard-diet-fed mice. The present results suggest that impaired mastication reduces newly generated neurons at the AOB, which in turn impairs olfactory function at the AOB.

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Competing interests

The authors declare no conflict of interests.

Ethical approval

Not required.

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Figure Legends

Fig. 1 - BrdU-ir cells in the AOB of mice fed a hard or soft diet.

Sagittal sections of the AOB, which are surrounded by line in the photographs, of mice fed a hard diet (A and B) or soft diet (C and D) for 1 and 3 months, respectively. Scale bar: 500 μm.

Fig. 2 - The number of BrdU-ir cells in the AOB of mice fed a hard or soft diet.

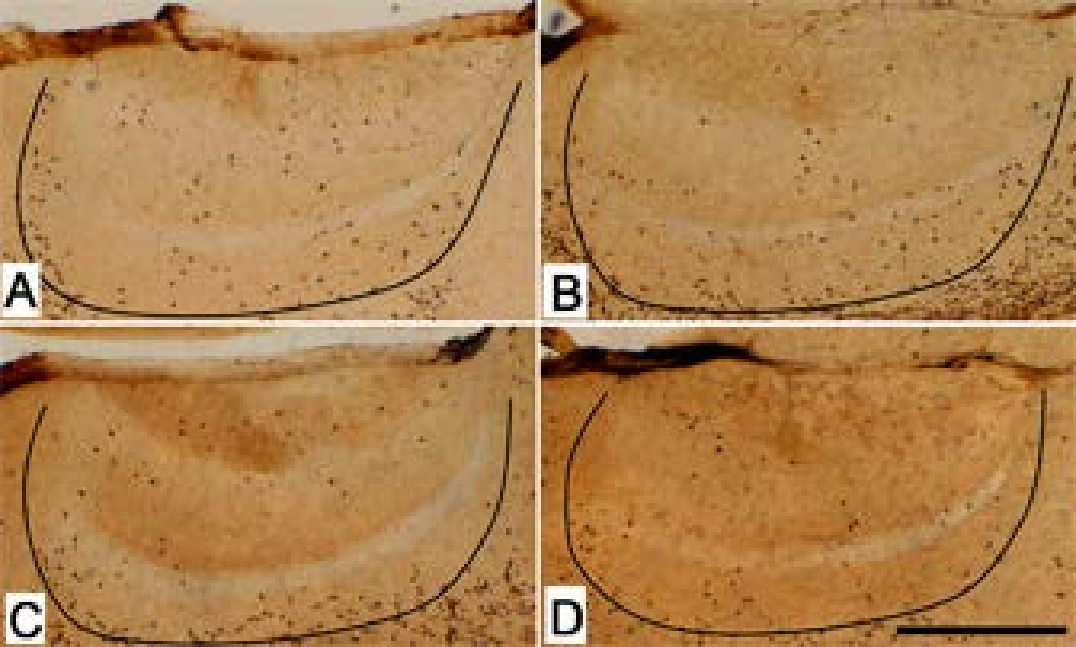
The number of BrdU-ir cells at 400 μm thickness from Figure 108 of the mouse atlas (lateral 0.84 mm) of the rostral (A) and caudal (B) AOB to the lateral side of mice fed the hard diet (black column) or soft diet (white column) for 1, 3, and 6 months. *: $p < 0.05$.

Fig. 3 - Fos-ir cells in the AOB of mice fed a hard or soft diet.

Sagittal sections of the AOB of mice fed a hard diet (A and B) or soft diet (C and D) for 2 and 4 months. Scale bar: 500 μm.

Fig. 4 - The number of Fos-ir cells in the AOB of mice fed a hard or soft diet.

The number of Fos-ir cells at the PG (A and B), MT (C and D) and GC (E and F) layers of the rostral (A, C, E) and caudal (B, D, F) AOB. Fos-ir cells were counted in 400 μm thickness from the section corresponding Figure 108 of the mouse atlas (lateral 0.84 mm) of to the lateral side of mice fed the hard diet (black column; $n = 5$) or soft diet (white column; $n = 5$) for 2 and 4 months. *: $p < 0.05$; **: $p < 0.01$.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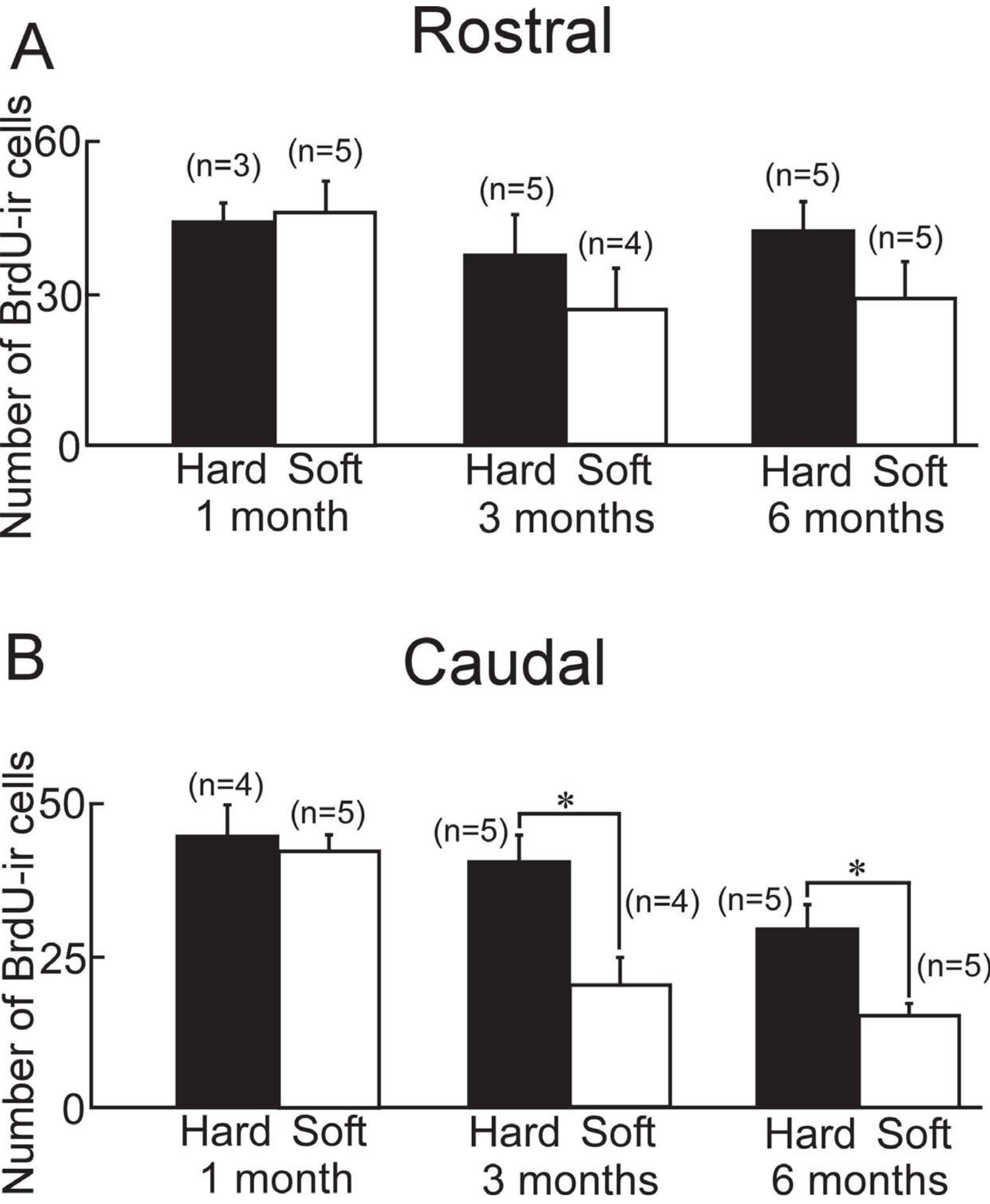
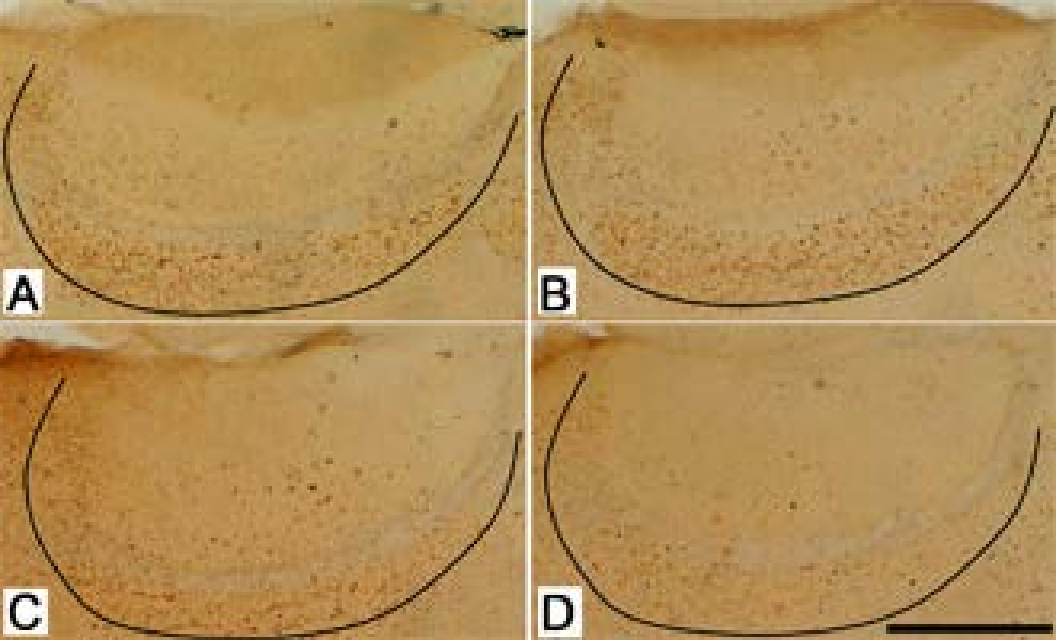
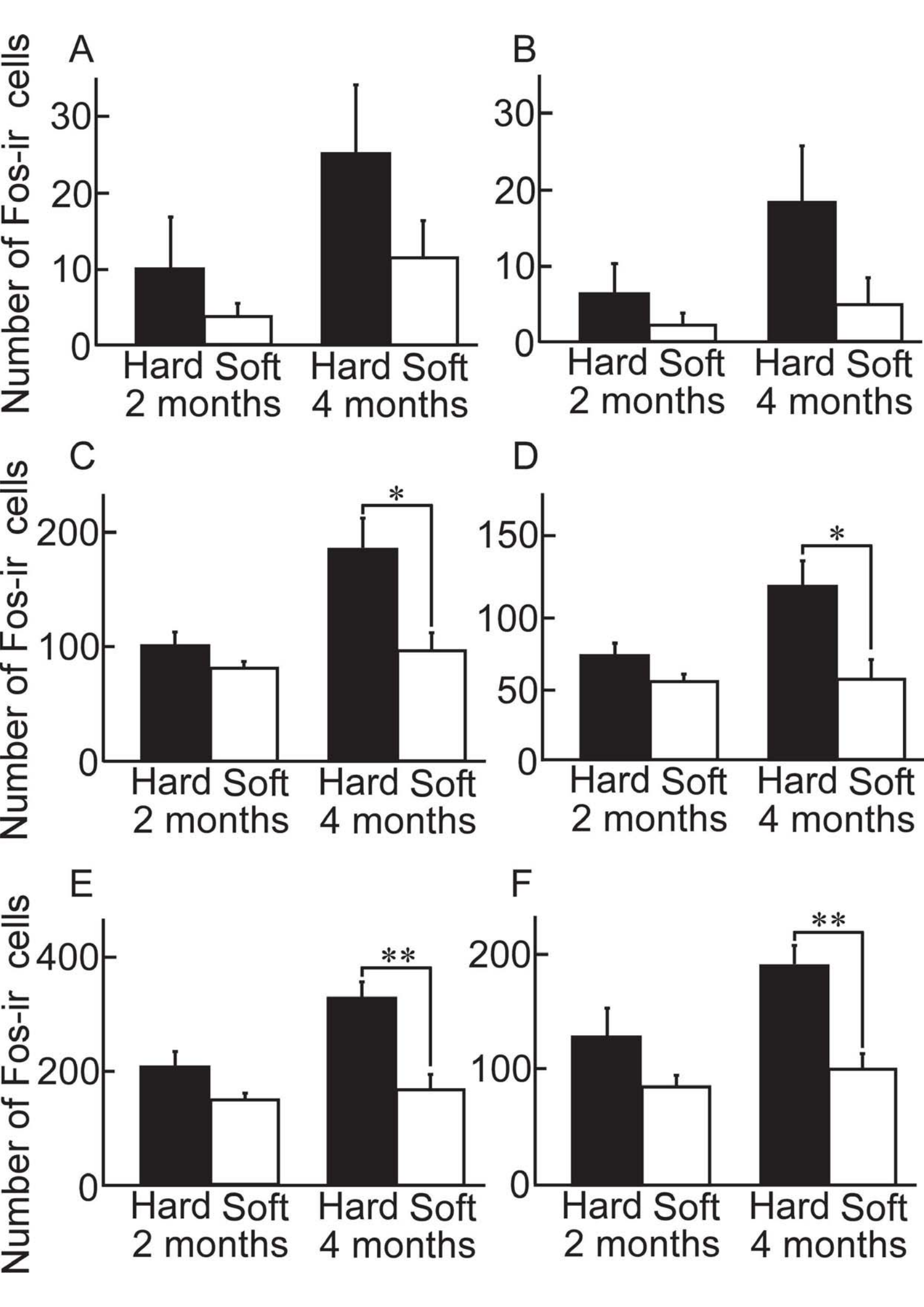
C
D


A
Rostral
Number of BrdU-ir cells
60
30
0
(n=3)
(n=5)
(n=5)
(n=4)
(n=5)
(n=5)
Hard Soft
1 month
Hard Soft
3 months
Hard Soft
6 months
B
Caudal
Number of BrdU-ir cells
50
25
0
(n=4)
(n=5)
(n=5)
*
(n=4)
(n=5)
*
(n=5)
Hard Soft
1 month
Hard Soft
3 months
Hard Soft
6 months


A
B
C
D


A
B
C
D
E
F
Number of Fos-ir cells
0
10
20
30
100
200
50
150
400
*
**
Hard Soft
2 months
Hard Soft
4 months