Occlusal Disharmony Transiently Impairs Learning and Memory in the Mouse by Increasing Dynorphin A Levels in the Amygdala

Kentaro Yamada,1,2 Yumie Ono,1,2,* Kin-ya Kubo,3 Toshiharu Yamamoto2,4 and Minoru Onozuka1,2

1Department of Physiology and Neuroscience, Kanagawa Dental College, Yokosuka, Kanagawa, Japan
2Research Center of Brain and Oral Science, Kanagawa Dental College, Yokosuka, Kanagawa, Japan
3Seijo University Graduate School of Health Care Studies, Tokai, Aichi, Japan
4Department of Human Biology, Kanagawa Dental College, Yokosuka, Kanagawa, Japan

Occlusal disharmony sometimes causes not only stiffness of neck but also psychiatric depression, suggesting that the condition of oral cavity may affect the central nervous system. Dynorphin A is an endogenous opioid peptide that specifically binds the κ-opioid receptor and has a protective role against stress. Dynorphinergic nervous system is intensely distributed in the amygdala and hippocampus that are coping areas with stress. As a model of malocclusion, we placed dental resin on the molars to increase the occlusal vertical dimension (bite-raise). After various survival times, we analyzed the amygdala and hippocampus by immunohistochemistry and immunosorbent assay (ELISA). Furthermore, the effects on learning and memory were assessed by Morris water maze test. In the amygdala, the levels of dynorphin A were increased on the 1st day after increasing the vertical dimension as indicated by immunohistochemical and ELISA assessments. The levels of dynorphin A returned to control levels on the 5th day. In the hippocampus, there were no noticeable changes in dynorphin A levels. The water maze test indicated that increasing the vertical dimension caused longer escape latency times on the 3rd day compared to those of sham-operated group. However, the bite-raised mice treated with a dynorphin antagonist, nor-binaltorphimine, showed similar escape latency times to the times of sham-operated group, even on the 3rd day. These results suggest that occlusal disharmony causes stress resulting in a transient increase of dynorphin A levels at least in the amygdala and that the increased dynorphin A levels transiently impair learning and memory.

Keywords: amygdala; dynorphin; hippocampus; malocclusion; stress


Introduction

Dynorphin is an opioid peptide. Its precursor, prodynorphin, produces α- and β-endorphin, dynorphin A and B (rimorphin), Leu-enkephalin, and a C-terminal-extended dynorphin B (leumorphin) (Day and Akil 1989; Day et al. 1998). Dynorphin is widely distributed in the brain, especially in the limbic system, hypothalamus, striatum, locus coeruleus and nucleus accumbens in the rat (Fallon and Leslie 1986; Ma et al. 2003) and mouse (Jamensky and Gianoulakis 1997; Ploj et al. 2000). Dynorphin specifically binds the κ-opioid receptor and has a protective role for stress through the κ-receptor. Gene expression of prodynorphin is increased in the limbic system by stress (Shirayama et al. 2004). In addition, injection of an antagonist of the κ-receptor causes stress-inducing behavior similar to those caused by immobility and forced swim stress (Mague et al. 2003; McLaughlin et al. 2003; Shirayama et al. 2004).

The amygdala, a part of the limbic system, forms the floor of the telencephalon in most mammals and locates in deep to the uncus in humans (Voogd et al. 1998). Information associated with emotion are sent to the thalamus and cerebral cortex, from which nerve fibers project to the hypothalamus, hippocampus and cingulate cortex, and affect emotional behavior, learning and memory (Davis 1992; LeDoux 1996; Adolphs and Tranel 2000). Anatomically, the amygdala is subdivided into central, medial, cortical, basomedial and basolateral nuclei. Among these nuclei, the central nucleus has abundant proenkephalin-containing neurons.

The hippocampal formation can be divided in the dentate gyrus, CA1, CA2, CA3 and hilus. Three major fiber systems, the angular bundle, fimbria-fornix pathway and hippocampal commissure, are involved in hippocampal functions such as information processing and certain forms of learning and memory (Amaral and Lavenex 2007). For example, hippocampal lesions in rats induce an impairment in various learning and memory tasks (Morris et al. 1982), and lesions of the CA3 region in the hippocampus disrupt spatial memory (Handleman and Olton 1981). The different regions in the hippocampus are innervated by both dynorphinergic and enkephalinergic neurons. Dynorphinergic neurons are present in the mossy fiber projection from the dentate gyrus to the CA3 region (McGinty et al. 1982; Chavkin et al. 1983). Enkephalinergic neurons are mainly present in the perforant path, which supplies the hippocampus and dentate gyrus with fibers from the entorhinal cortex (McGinty et al. 1982).

Occlusal disharmony occasionally causes stiffness of neck or shoulder, fatigue, or psychiatric depression (De Boever and Adriaens 1983). Recent studies suggest that deterioration of the oral environment causes various effects on the central nervous system, such as a decrease of spine numbers in the hippocampal CA1 area (Kubo et al. 2008), an increase of plasma corticosterone (Yoshihara et al. 2001), and transient activation of microglia (Kojo et al. 2010). Furthermore, masticatory disorders reduce input activities in the hippocampus, thereby leading to deficits in learning and memory in aged senescence-accelerated prone 8 mice (Onozuka et al. 1999, 2000, 2002; Kubo et al. 2005). In this report, we investigated the effects of increasing the occlusal vertical dimension on the mouse amygdaloid and hippocampal dynorphinergic nervous system using immunohistochemical, biochemical and behavioral techniques.

Materials and Methods

Increase of molar vertical dimension

Animal treatment protocols were approved by the Ethics Committee of Kanagawa Dental College, employing guidelines established by the committee. DDy male mice were housed in a condition under a 12-h light/12-h dark cycle and temperature at 22 ± 3°C with free access to pelleted chow and water until experiment. As a model of dysfunctional mastication, we increased the vertical dimension of mouse molar dentition, the condition of which was referred as “bite-raise” in the subsequent text. Procedures were performed under sodium pentobarbital anesthesia (35 mg/kg i.p.; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The vertical dimension of the bite was increased approximately 0.1 mm by placing ultraviolet-ray polymerization resin (UniFil®LoFlo, GC corporation, Tokyo, Japan) on the all upper molars (6 maxillary molars) after treatment with a Single Bond Dental Adhesive System (3M Dental Product, Irvine, CA, USA). We confirmed remaining of the resin when these mice were sacrificed. Control animals (sham-operation) underwent the same anesthetic procedure, but no resin was applied. After the procedure, the mice were housed under the above conditions.

Immunohistochemistry

Total 20 mice were used for immunohistochemistry (n = 5 each). At 1, 3, and 5 days after the bite-raise, we examined the distribution of dynorphin A-like immunoreactivity in the amygdala and hippocampus of experimental animals in comparison with sham-operated mice (n = 5; 1 day survival time). The mice were deeply anesthetized by pentobarbital sodium (35 mg/kg, i.p. Wako Pure Chemical Industries, Ltd.), followed by perfusion with 0.9% NaCl and, subsequently, with 4% formaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (PB, pH 6.9). The brain was dissected out from the skull, blocked into three parts by cutting transversely at the levels of anterior commissure and caudal to the mammillary body, and further fixed for 1 or 2 days at 4°C in the same fixative. After washing in PB and immersing in 20% sucrose, the middle blocks were cut into 20-µm-thick transverse sections using a sliding microscope equipped with a frozen stage. Every third section was serially collected and these sections (approximately 55 sections per animal) were immunostained using the free-floating method. Immunohistochemistry was performed according to our routine method (Yamada et al. 2008). Briefly, the sections were washed overnight in 0.1 M PB (pH 7.4) containing 0.9% saline (PBS), and incubated for 4 h at 4°C with rabbit anti-porcine dynorphin A (1-17) serum (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) diluted 1:10,000 in PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich Co., St. Louis, MO, USA) and 0.3% Triton X-100 (PBS-BSAT). This primary antisemur cross-reacts less than 1.0% with porcine dynorphin A (1-13), and shows no cross-reaction with porcine dynorphin A (1-8), porcine dynorphin B (1-13), human µ-endorphin, porcine neo-endorphin and Leu-enkephalin, according to the manufacturer’s description. To test antisemur specificity, preabsorbed anti-serum with human dynorphin A (20 µg/ml; Peptide Institute, Osaka, Japan) was used as a primary antibody. After washing in PBS, the sections were incubated for 1 h at room temperature with a secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in PBS-BSAT. The sections were then washed in PBS and incubated for 30 min at room temperature with avidin-biotin-peroxidase complex (ABC; Vector Laboratories) diluted 1:200 in PBS-BSAT. After a final wash in PBS, the sections were reacted with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer solution (pH 7.4). The sections were counterstained with thionin and coverslipped with Malinol (Muto Pure Chemicals, Tokyo, Japan).

ELISA

Total 32 mice were used for enzyme-linked immunosorbent assay (ELISA) analysis. To collect amygdala samples, mouse brains were cut into approximately 1.5 mm-thick slices using a mouse brain slicer (Muromachi Corporation, Tokyo, Japan). Slices containing amygdala tissue were collected and the amygdala region was isolated by removing tissue lateral to the external capsule, medial to the optic
Malocclusion Increases Dynorphin and Affects Behavior

Results

Amygdala

In the control mice, dense dynorphin A immunoreactive fibers were observed in the central amygdala (Fig. 1A, B). However, relatively fewer immunoreactive fibers were seen in the basolateral, basomedial, medial and cortical amygdala. In comparison with the control, the density of immunoreactive fibers in the bite-raised mice seemed to be increased at 1 day after the bite-raise (Fig. 1C, D). Immunoreactive soma-like structures became remarkable at 3 days after increasing the vertical dimension (Fig. 1E, F), while somal staining was very faint in the control animals (Fig. 1A). At 5 days after the bite-raise, dynorphin A immunoreactive profiles were similar to those of control animals (Fig. 1G, H). Preabsorption of the antiserum with dynorphin A abolished these staining profiles (not shown). Consistent with the immunohistochemical data, the levels of dynorphin A in the amygdala were increased at 1 day after the vertical dimension was increased (5.09 ± 0.37 pg/mg; p < 0.01) over that of control mice (2.27 ± 0.30 pg/mg) (Fig. 2). Thereafter, the levels of dynorphin A were decreased at 3 (3.30 ± 0.43 pg/mg) and 5 (1.69 ± 0.40 pg/mg) days after the bite-raise (Fig. 2).

Hippocampus

In the hippocampus, dynorphin A immunoreactive fibers were present in the hilus and the zone of mossy fibers (Fig. 3). The latter is a fiber bundle of projections from the dentate gyrus to the CA3 region of the hippocampus. In comparison with control mice (Fig. 3A), the width of the zone and density of immunoreactive fibers were similar to those at 1, 3, and 5 days after the vertical dimension was increased (Fig. 3B, C, D). Preabsorption of the antiserum with dynorphin A abolished these staining profiles (not shown). ELISA analysis indicated that the levels of dynorphin A in the hippocampus were slightly increased at 1 day (1.22 ± 0.04 pg/mg) after increasing the vertical dimension (Fig. 4). However, the difference in dynorphin A levels was not statistically significant compared with that of control animals (1.06 ± 0.10 pg/mg). At 3 (1.12 ± 0.04 pg/mg) and 5 days (1.07 ± 0.05 pg/mg) after the bite-raise, the levels of dynorphin A were similar to control levels (Fig. 4).
Morris water maze test

During the training period, all of the animals tended to improve their scores day by day (Fig. 5). On the first assessment day, the group of normal vertical dimension with nor-BNI injection showed the shortest escape latency times among the four groups (Fig. 6). The bite-raised animals with nor-BNI injection showed the longest escape latency times among the four groups. However, these differences were not statistically significant. From the 2nd to 5th assessment day, the bite-raised animals with saline injection tended to show longer escape latency times compared to the other three groups. This was particularly apparent on the 3rd day, where values were statistically significant ($p < 0.05$) in comparison with the other three groups (Fig. 6). On the 5th assessment day, all four groups showed similar escape latency times (Fig. 6).

Discussion

In this study, we found that increasing the occlusal vertical dimension caused a transient increase of dynorphin A levels in the amygdala but not in the hippocampus based on the morphological and ELISA analyses, both of which have technical limitations (see later discussion). Furthermore, the transient increase of dynorphin A caused by the increase of vertical dimension seemed to impair memory and learning ability as assessed by the Morris water maze test.

The influence of the bite-raise on the brain has been demonstrated in catecholamine levels (Budts-Jørgensen et al. 1981; Areso et al. 1999; Yoshihara et al. 2001), c-Fos

Fig. 1. Changes of dynorphin A immunoreactivity in the amygdala.

Low (A, C, E, G) and high (B, D, F, H) magnification photographs showing dynorphin A immunoreactive somata and fibers in the central amygdala of control animals (A, B), and at 1 (C, D), 3 (E, F), and 5 (G, H) days after increasing the vertical dimension. Note the denser distribution of dynorphin A immunoreactive fibers at 1 day (C, D) after the bite-raise and that dynorphin A immunoreactive somata (arrows in F) are conspicuous at 3 days (E, F) after the bite-raise. Bars: 100 µm (A, C, E, G), 50 µm (B, D, F, H). Abbreviations: BL, basolateral amygdaloid nucleus; Ce, central amygdaloid nucleus.
Malocclusion Increases Dynorphin and Affects Behavior

and its mRNA (Kobayashi et al. 2004), and glial cells (Kubo et al. 2007; Kojo et al. 2010). All of these effects tend to be transient, similar to the present results. It is well known that various stresses increase brain dynorphin levels. For example, immobilization and forced swim stress increase dynorphin A levels in the hippocampus and nucleus accumbens (Shirayama et al. 2004), and infusion of an antagonist of dynorphin receptor, nor-BNI produces an antidepressant effect (Pliakas et al. 2001; Mague et al. 2003). In general, stress is classified as either acute and clonical. A recent paper classified the κ-opioid receptor-mediated reactions for stresses into three categories: acute and delayed responses, and multiple rounds of adaptation (Knoll et al. 2010). The bite-raise increases plasma corticosterone levels, which is a marker indicating stress (Kubo et al. 2007), suggesting that this condition is a type of stress. The increase of dynorphin A levels in the amygdala may be a neural adaptation. In contrast to results obtained from forced swim, immobilization and learned helplessness (Shirayama et al. 2004), dynorphin changes in the hippocampus in response to the bite-raise were not conspicuous, suggesting the increase of vertical dimension is a less robust stress than other stress tasks.

The amygdala plays an important role in emotional perception and expression, and in fear response (Davis 1992; LeDoux 1996; Adolphs and Tranel 2000). Peak times of elevated dynorphin A levels by morphological inspection differed from those by ELISA analysis. In general, morphological assessment is more inaccurate than biochemical quantity. However, we isolated the whole amygdala although morphological inspection suggested that the increased dynorphin A levels limited only to the central amygdala. Furthermore, there is a technical limitation to equalize sampling for ELISA. In any case, dynorphin A levels are transiently increased by the bite-raise within sev-
eral days. The presence of the dynorphin A-containing nervous system in the amygdala is well documented in the rat (Fallon and Leslie 1986; Ma et al. 2003) and mouse (Jamensky and Gianoulakis 1997; Ploj et al. 2000). Interestingly, dynorphin-containing neurons in the amygdala coexist with corticotroph releasing hormone (CRH) (Marchant et al. 2007), which serves as a neurotransmitter regulating diverse extra-hypophysial systems under stress (Vale et al. 1981). Therefore, dynorphin with CRH may play a fundamental role in the stress response. The lateral amygdaloid nucleus receives projections from sensory cortices (Pitkanen et al. 1997; McDonald 1998), which receive oral cavity sensory information and projects to the central amygdaloid nucleus. This pathway is one route by which the bite-raise increases dynorphin A levels in the amygdala in addition to other direct and indirect routes from the oral cavity to the central amygdala. Furthermore, the amygdaloid complex projects to the hippocampus (Pikkarainen et al. 1999; Pitkanen et al. 2000). Therefore, the amygdala may affect hippocampal functions through the increased dynorphin A in the amygdala. This pathway may be responsible for the phenomena that emotional states affect learning and memory.

The hippocampus is often implicated in the neurobiology of stress (Knoll and Carlezon 2010) and is essential for certain forms of learning and memory, such as visuospatial and content-dependent learning and memory (Squire 1992;
Malocclusion Increases Dynorphin and Affects Behavior

Ergorul and Eichenbaum (2004). The present data did not show a statistical significance in the differences in dynorphin levels in the hippocampus by immunohistochemistry and ELISA between control and bite-raised animals. As mentioned above, immobilization and forced swimming increases hippocampal dynorphin A level (Shirayama et al. 2004). However, we could not detect the statistically significant increase of dynorphin A in the hippocampus, suggesting the increase of vertical dimension targets the amygdala rather than the hippocampus. Alternatively, small changes are difficult to detect by morphological assessment. Dynorphin immunoreactive fibers are restricted in mossy fibers and perforant pathway terminals. For ELISA, we used the whole hippocampal formation owing to difficulty of selective dissection of these fiber bundles, which may underestimate changes of dynorphin levels. Therefore, the tendency of dynorphin to increase on the 1st day after the bite-raise may be substantial. Dynorphin is co-localized with glutamate, the primary neurotransmitter in granule cells in the dentate gyrus, and the synaptic release of dynorphin is reported to cause pre-synaptic inhibition of glutamate release from the mossy fibers and perforant pathway terminals (Drake et al. 2007). Opioid receptors are expressed at moderate to high levels in the hippocampus (Clarke et al. 2001; Drake et al. 2007). These lines suggest that the hippocampus is a targeting area for the increased dynorphin in the amygdala and/or hippocampus associated with glutamate-mediated hippocampal functions.

The water maze task has long been regarded as a hippocampal dependent task based on studies of special impairment after selective lesioning of the hippocampus (Jarrard 1978; Morris et al. 1982). The present data from the water maze task indicated that the increase of vertical dimension transiently impaired learning and memory, suggesting that increased dynorphin impairs learning and memory. Supporting this hypothesis, injection of a specific antagonist for κ-opioid receptor improved the impaired learning and memory. At the present time, the detailed mechanism that dynorphin affects learning and memory is not known. However, the increased levels of dynorphin resulting from stress may block glutamate-mediated functional plasticity of the hippocampal formation, and dynorphin antagonism with nor-BNI may restore this response. Spatial memory tasks activate hippocampal subfields, and plasticity of the mossy fiber projections is involved in and required for the storage of spatial representations (McClelland and Goddard 1996; Vann et al. 2000; Ramirez-Amaya et al. 2001). The presence of time lag between the highest dynorphin A levels in the amygdala and the impairment of spatial learning and memory assessed by water maze test suggests that behavioral expression of the amygdala and/or hippocampus is delayed from the neurochemical expression probably owing to establishment of effects of dynorphin A on the glutamate-mediated hippocampal functions. Taken together, results suggest that the induction of dynorphin A by stress may block the plasticity of hippocampal pathways that is required for spatial learning, and this activity may underlie the transient behavioral deficit. Although there is no clinical evidence that occlusal disharmony directly affects the learning and memory, a part of patient’s complaints, such as shoulder stiffness, fatigue and psychiatric depress (De Boever and Adriaens 1983), may associate with the transient increase of dynorphin A levels.

Acknowledgements

A part of this work was supported by JSPS KAKENHI Grant Number 24792119 and MEXT-supported Program for the Strategic Research Foundation at Private Universities, S1203004, 2012-2014.

Conflict of Interest

The authors declare no conflict of interest.

References


Squire, L.R. (1992) Memory and the hippocampus: a synthesis