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Masticatory hypofunction effects induced by BTXA injection of hippocampal neurons in developing rats

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ABSTRACT

Background and objective: In clinical practice, malocclusion is often encountered during the period of growth and development of individuals. In addition to nutritional imbalance, some studies have found that mastication affects learning and memory ability. Tooth loss and masticatory hypofunction have been suggested as risk factors of Alzheimer disease. However, relatively little research has been done in developing animals. The present study evaluated the relationship between masticatory hypofunction and neuropathological changes of the hippocampus in developing rats.

Design: Four-week-old Wistar rats were randomly divided into saline-injected and botulinum toxin type A (BTXA)-injected groups. After an experiment period of 4 weeks, the rats were sacrificed for evaluation of neuropathological changes in the hippocampus through Nissl staining and phosphorylated cyclic AMP (cAMP) response element binding protein (CREB) immunohistochemistry.

Results: Nissl staining revealed a significant reduction in the density of neurons in the BTXA-injected rats. The BTXA-injected rats exhibited a decreased level of CREB phosphorylation. The degree of p-CREB immunoreactivity differed significantly between the two groups.

Conclusion: The BTXA-injected rats exhibited a reduction in neuron density and phosphorylated CREB, indicating that mastication might influence the learning and memory ability during the growth period. Therefore, it is strongly suggested that malocclusion be corrected as soon as possible during growth and development.

1. Introduction

With the growth of aging populations around the world, oral health is a widely discussed topic. Oral health is crucial for general health. For example, periodontitis and dental caries have been found to be related to systemic inflammatory diseases as well as diabetes and Alzheimer disease (Bozdemir, Yilmaz, & Orhan, 2016; Kondo, Niino, & Shido, 1994; Taylor & Borgnakke, 2008). Oral health includes maintaining oral functions, such as mastication and swallowing. Some studies have indicated that mastication can promote and maintain memory functions (Chen, Iinuma, Onozuka, & Kubo, 2015; Fukushima-Nakayama et al., 2017; Okamoto et al., 2015; Ono, Yamamoto, Kubo, & Onozuka, 2010; Teixeira et al., 2014). Elderly patients usually experience tooth loss or develop weak masticatory muscles, which lead to deficiency of masticatory function (Hansson et al., 2013; Okamoto et al., 2015; Weijenberg, Lobbezoo, Visscher, & Scherder, 2015). In addition to elderly populations, poor development of learning ability in children is correlated to decreased frequency of mastication (Rolls, Verhagen, & Kadohisa, 2003). Moreover, mastication stimulation may have a greater influence on children than on the elderly (de Almeida et al., 2012).

Animal studies have indicated that mastication correlates with cognitive function controlled by the hippocampus (Ono et al., 2009). Mastication generates sensory signals, which are transmitted to various regions of the central nervous system (CNS) (Teixeira et al., 2014). The hippocampus is one of the sites that receive signals from the oral cavity. The sensory information from masticatory tissues may also affect hippocampal performance (Ono et al., 2010). The hippocampus is located in the medial temporal lobe and is responsible for learning and memory (Whitlock, Heynen, Shuler, & Bear, 2006). Long-term potentiation (LTP), a form of neural plasticity, was first discovered in the hippocampus. Studies on rats and mice have shown hippocampal neurons to be related to spatial coding (Schiller et al., 2015). Given these findings, we hypothesize that masticatory hypofunction may lead to deficiency of spatial memory and neuropathological changes in the hippocampus of

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adult rats, including developing rats. However, relatively little research has been performed in developing rats (Fukushima-Nakayama et al., 2017; Yamazaki, Wakabayashi, Kobayashi, & Suzuki, 2008). Furthermore, typical interventions used to decrease masticatory function in animal studies are a soft diet (Aoki, Kimoto, Hori, & Toyoda, 2005; Fukushima-Nakayama et al., 2017; Tsutsui et al., 2007), removal of the molars (Kondo et al., 2016; Yamazaki et al., 2008), or occlusal interference (Arakawa et al., 2007; Ichihashi et al., 2007; Mori, Katayama, Miyake, Fujiwara, & Kubo, 2013, 2016). These methods affect mastication indirectly and cannot directly affect associated muscle activity. Because rats are rodents and habitually chew, the aforementioned strategies cannot completely eliminate the activity of masticatory muscles. To address these drawbacks, this study applied botulinum toxin to the masseter muscle of developing rats to directly decrease masticatory function.

Botulinum toxin is a neurotoxin protein derived from *Clostridium botulinum*; it has seven subtypes, type A to type G. Botulinum toxin type A (BTXA) has been applied in the fields of cosmetic medicine and dentistry, such as in the treatment of temporomandibular disorders, bruxism, mandibular spasm, gummy smile, and masseteric hypertrophy (Nayyar, Kumar, Nayyar, & Singh, 2014; Srivastava, Kharbanda, Pal, & Shah, 2015). Botulinum toxin acts on the terminal of motor nerves by inhibiting the release of acetylcholine at the nerve–muscle junction, leading to local muscle weakness and decreased muscle activity (Turton, Chaddock, & Acharya, 2002). In clinical practice, malocclusion is often encountered in developing patients. Patients with malocclusion present with certain features, such as insufficient efficiency in the masticatory muscles and abnormal chewing patterns. Some studies have found that patient with malocclusion demonstrate lower masticatory muscle activation (Nishi et al., 2017; Piancino et al., 2012). In the present study, we induced masticatory hypofunction using BTXA to simulate malocclusion during the growth period in young rats.

Hippocampus is responsible for learning and memory. In neuroscience, LTP was first found in the hippocampus and has been a noteworthy discovery. LTP is considered a crucial mechanism in the hippocampus. CREB plays an essential role in many hippocampus-depended ability, LTP is one of them. To maintain LTP, the activation of some pre-existing protein, such as cyclic AMP (cAMP) response element (CRE) binding protein (CREB), seems necessary. CREB regulates gene transcription by binding to CRE, a cis-acting enhancer element in the regulatory region of various genes. The function of CREB is regulated by its phosphorylation, which results in the activation of gene transcription. CREB has been shown to possess an essential role in long-term memory formation (Kida & Serita, 2014; Mizuno et al., 2002). In our study, to evaluate memory function, we detected the expression of phosphorylated CREB.

The purpose of this study is to investigate the effect of masticatory hypofunction induced by BTXA in neuropathological changes of the hippocampus. Additionally, we evaluated the relationship between malocclusion and cognitive function during the growth period of rat models.

2. Materials and methods

2.1. Experimental animals and protocol

The animals in this study were 4-week-old male Wistar rats (N = 20) purchased from BioLASCO Taiwan Co., Ltd. Two rats were arranged in one cage. All cages were placed in a single room with a constant room temperature (22 °C ± 2 °C) and humidity (40%–70%) and a light–dark cycle (with lights turning on at 6 A M and off at 6 PM). Rats were fed a standard diet of hard pellets and water. The body weight of the rats was measured once per week. All experimental procedures were performed at the Research Animal Center of Taipei Medical University. Full protocol and ethical approval was obtained from the council of the Animal Center at Taipei Medical University, Taiwan (LAC-2017-0113).

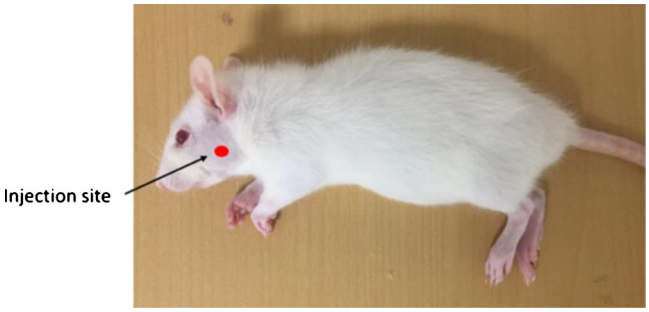

Fig. 1. Site of BTXA and saline injection.
Under general anesthesia, both mandibular regions were shaved, allowing direct visualization of the masseter muscle. BTXA and saline were injected to each side of masseter muscle belly.

A total of 20 animals were randomly assigned to the experimental group (N = 10) and the control group (N = 10). The animals were 4 weeks old at the time of BTXA or normal saline injection. General anesthesia was performed during BTXA or normal saline injection and upon sacrificing the rats. A combination dose of 25 mg/kg tiletamine-zolazepam (Zoletil 50®, Virbac Inc., Carros, France) and 11.66 mg/kg xylazine (Rompun®, Bayer Inc., Kyonggi-do, South Korea) was administered intraperitoneally. The appropriate anesthetic depth monitoring was performed using a toe-pinch. At day 1, after general anesthesia, both mandibular regions were shaved in all animals for direct visualization of the masseter muscle injection sites (Fig. 1). The rats in the experimental group were injected with 0.5 U BTXA (Botox®, Allergan Pharmaceuticals, Dublin, Ireland) at one side of the masseter muscle under general anesthesia, and the total dose was 1 U BTXA in each rat. One vial of BTXA contained 100 U of air-dried toxin, with 1 U equal to the median amount necessary to kill 50% of female Swiss Webster mice weighing 18–20 g each after intraperitoneal injection (LD50) (Jankovic & Brin, 1991). Ten milliliters of sterile, nonpreserved 0.9% normal saline was used to reconstitute BTXA, yielding a preparation of 1 unit per 0.1 mL. In the experiment group, each side of the masseter muscle was injected with 0.05 mL of this solution. In the control group, the same amount of sterile, nonpreserved 0.9% normal saline was injected. After injection, the rats were send back to cages and keep for 4 weeks. The rats were sacrificed at day 28.

2.2. Body weight measurement

Each rat's body weight was measured once per week. During the experiment, changes to body weight were documented and differences between the saline-injected group and the BTXA-injected group were compared.

2.3. Perfusion and tissue preparation

At day 28, the 8-week-old rats were deeply anesthetized and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). After perfusion, the hippocampus was removed and kept in sucrose buffer for cryoprotection at 4 °C overnight. Serial 30-μm-thick sections of the hippocampus were cut with a cryostat (CM3050S, Leica Microsystems, Wetzlar, Germany). Sections were processed for Nissl staining as well as CREB and phosphorylated CREB (p-CREB) immunohistochemistry.

2.4. Immunohistochemical staining for CREB and p-CREB

For CREB and p-CREB immunohistochemistry, sections were rinsed in 0.05 M tris-buffered saline (TBS) and placed in 0.3% hydrogen peroxide for 5 min at room temperature to reduce endogenous peroxidase

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